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Study of lymphocyte autophagy in normal and autoimmune responses

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ABBREVIATIONS:

A

ACR: American College of Rheumatology

AD: Alzheimer's diseases

AIDS: Acquired immunodeficiency syndrome

AIM2: Absent in melanoma 2

ALFY: Autophagy-linked FYVE

ALS: Amyotrophic lateral sclerosis

AMPK: AMP-activated protein kinase

AP-1: Activator protein 1

ASC: Apoptosis-associated Speck-like protein containing a Caspase-recruitment domain

ATM: Ataxia-telangiectasia mutated

ATP: Adenosine tri-phosphate

Aβ: Ameloid β

B

BAG3: BCL2-associated athanogene 3

BAT: Brown adipose tissue

BRCA1: Breast cancer 1

C

CA: Citrullinated antigen

CaM: Calmodulin

CAMK4: Calcium/calmodulin-dependent protein kinase type IV

CDS: Cytosolic DNA sensors

CHOP: C/EBP homologous protein

CIIM: Class II compartment

Class III PI3K: Class III phosphatidylinositol 3-kinase

CLIP: Class-II associated invariant chain peptide

CMA: Chaperone-mediated autophagy

CNS: Central nervous system

CoA: Acetyl coenzyme A

COX IV: Cytochrome c oxidase complex IV

CRAC: Calcium release-activated channel

CREM-α: cAMP response element modulator α

D

DAG: Diacylglycerol

DC: Dendritic cell

DCFP1: Double FYVE containing protein 1

DENV: Dengue virus

DISC: Death-inducing signaling complex

DN: Double negative T cell

DNA: Deoxyribonucleic acid

Dnch1: Dynein heavy chain1

DRAM1: DNA-damage-regulated autophagy modulator 1

Drp1: Dynamin-related protein 1

DSS: Dextran sodium sulphate

E

EAE: Experimental induced autoimmune encephalomyelitis

EBNA 1: Epstein Barr nuclear antigen 1

EBV: Epstein-Barr virus

EGO: Exit from rapamycin-induced growth arrest

ERK: Extracellular signal-regulated protein kinase

ERRα: Estrogen-related receptor α

ES cells: Embryonic stem cells

ESCRT: Endosomal sorting complex required for transport

F

FADD: Fas-associated death domain

FFA: Free fatty acid

FIP200: Focal adhesion kinase [FAK] family-interacting protein of 200 kDa

FYCO1: FYVE and coiled-coil (CC) domain–containing protein 1

G

GABARAP: γ-amino-butyric acid receptor-associated protein

GAP: GTPase-activating protein

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GAS: Group A *Streptococcus*

GATE: Golgi-associated ATPase enhancer

GcAVs: GAS-containing LC3-positive autophagosome-like vacuoles

GFAP: Glial fibrillary acidic protein

GI: Gastro-intestinal

GLUT1: Glucose transporter 1

GOPC: Golgi-associated PDZ and coiled-coil-containing

Grp78: Glucose regulated protein 78

GVHD: Graft vs host disease

GWAS: Genome wide association studies

H

hCMV: Human cytomegalovirus

HCV: Human hepatitis C virus

HD: Huntington's disease

HDAC6: Histone deacetylase-6

HFD: High fat diet

HIF-1α: Hypoxia-inducible factor 1α

HLA: Human leucocyte antigen

HOPS/class C Vps: Homotypic fusion and protein-sorting/class C vacuole protein-sorting

Hsp90: Heat-shock protein of 90 kDa

HSPA8: Heat shock protein family A (Hsp70) member 8

I

IC: Immune complexes

IC-DNA: Immune complexes associated to DNA

IFN: Interferon

iNKT: Invariant natural killer T cells

IP_3: Inositol 1,4,5-triphosphate

IP3R: Inositol triphosphate receptor

IPS-1: Interferon-beta promoter stimulator 1

IRF: Interferon regulatory protein

IRS1: Insulin receptor-substrate 1

ITAM: Tyrosine-based activation motifs

ITK: Inducible tyrosine kinase

J

JNK: c-Jun N-terminal protein kinase 1

K

KSHV: Kaposi's sarcoma associated herpesvirus

L

LAMP2A: Lysosome-associated membrane protein type 2A

Lck: Lymphocyte protein tyrosine kinase

LD: Lipid droplet

LMNB1: Lamin B1

M

MAMP: Microbial-associated molecular pattern

MAP1LC3: Microtubule-associated-protein 1 light chain 3

MAPK: Mitogen-activated protein (MAP) kinase

MCMV: Murine cytomegalovirus

MDA5: Melanoma differentiation associated gene 5

MDP: Muramyldipeptide

MEF: Mouse embryonic fibroblast

MFN: Mitofusins

MHC: Major histocompatibility complex

MOG: Oligodendrocyte glycoprotein

MS: Multiple sclerosis

MTOC: Microtubule organization center

mTOR: Mammalian target of rapamycin

MVB: Multivesicular body

Myd88: Myeloid differentiation primary response 88

N

NBR1: Neighbor of BRAC1 gene 1

NDP52: Nuclear dot protein of 52KD

NET: Neutrophil extracellular trap

NFAT: nuclear factor of activated T cells

NF-kB: Nuclear protein kB

NLR: Oligomerization domain receptors (NOD)-like receptor

NLRP3: NOD-like receptor pyrin domain containing protein 3

NSCLC: Non-small-cell lung cancer

NSM: Non selective microautophagy

O

OPTN: Optineurin

P

PA: Palmitate

PARL: Presenilin associated, rhomboid-like

PAS: Phagophore assembly site

PBD: Peroxisome biogenesis disorders

PD: Parkinson's disease

PDI: Disulfide isomerase

PDK1: 3-phosphoinositide dependent protein kinase-1

PFKFB3: 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3

PI3K:

PINK1: PTEN-induced putative kinase 1

PINK1: PTEN-induced putative kinase 1

PIP_2: Phosphatidylinositol 4,5,-biphosphate

PIP3: Phosphatidylinositol 3,4,5-triphosphate

PKC: Protein kinase C

PLCγ1: Phospholipase Cγ1

PMN: Piecemeal microautophagy of the nucleus

PYHIN: Pyrin and HIN domain family member

R

RA: Rheumatoid arthritis

Raptor: Regulatory-associated protein of mTOR

Rheb: Ras homolog enriched in brain

RIG1: Retinoic acid inducible gene 1

RLIP: Rab-interacting lysosomal protein

RLR: Retinoic acid-induced gene (RIG)-I-like receptor

RNA: Ribonucleic acid

RNase A: Ribonuclease A

ROS: Reactive oxygen species

RTK: Receptor tyrosine kinase

Rubicon: RUN domain and cysteine-rich domain containing, Beclin 1-interacting protein

S

SCV: *Salmonella*-containing vacuole

sdRNA: single stranded RNA

SIN: Sindbis virus

siRNA: small interfering ribonucleic acid

SLAM1: Signaling lymphocytic activation molecule family 1

SLE: Systemic lupus erythematous

SLR: SQTSM1/p62-like receptors

SNARE: Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SNP: Single nucleotide polymorphism

SPI-2 T3SS: Salmonella pathogenicity island 2 type 3 secretion system

SYK: Spleen tyrosine kinase

T

TAP: Transporter associated with antigen processing

TBK1: TANK binding kinase 1

TEC: Thymic epithelial cells

TG: Triglyceride

TIID: type II diabetes

TLR: Toll-like receptor

TOM: translocase outer mitochondrial

TOR: Target of rapamycin

TRA: Tissue-restricted antigen

TREX-1: 3 prime repair exonulease 1

TSC: Tuberous sclerosis complex

U

ULK1/2: Unc-51-like kinases 1 (ULK1) and 2

UPR: Unfolded protein response

UPS: Ubiquitin proteasome degradation system

UV : Ultra-violet

UVRAG: UV resistance associated gene protein

V

VAMP: Vesicle-associated membrane protein

v-ATPase: vacuolar H^+-ATPase (

VDAC1: Voltage-dependent anion channel 1

VMP1: Vacuole membrane protein 1

VSV: Vesicular stomatis virus

W

WASH: Wiskott-Aldrich syndrome protein

WAT: white adipose tissue

WIPI: WD-repeat PI3P effector protein

WT: Wild type mice

Z

ZAP70: Zeta chain of T cell receptor associated protein kinase 70kDa

1 THE AUTOPHAGIC PATHWAYS IN CELLULAR HOMEOSTASIS

1.1 Autophagy: Concept and Cellular Functions

The term “autophagy” (from the Greek for self-eating) was first encountered in the early 1960ties and is tightly linked to Christian de Duve’s discovery of the lysosome ten years before **(de Duve et al., 1955)**. The Belgian scientist was indeed the one who named this process **(de Duve C, Ciba Foundation Symposium: Lysosome; Little, Brown, 1963)** describing the digestion of intracellular content as a contrast to “heterophagy”, which is the lysosomal degradation of material originating from outside the cell. As a matter of fact, the degradation of intracellular structures had been observed first by Sam L. Clark in kidney cells from new born mice and by Alex Novikoff in starved liver cells **(Clark, 1957; Novikoff et al., 1956)**. Later on, Antti Arstila and Benjamin Trump identified by electron microscopy that those structures displayed a double membrane and that they seemed to deliver cytoplasmic content to lysosomes for degradation **(Arstila and Trump, 1968)**. Those were the findings that launched the autophagy era that has been growing and evolving for over five decades.

Years of research on autophagy have allowed to grasp the importance of this process for cellular homeostasis. The first investigations on the subject revealed the role of autophagy in cell metabolism. The fact that treatment by glucagon seemed to increase autophagic bodies as shown in liver cells by Thomas P. Ashfold and Keith R. Porter **(Ashford and Porter, 1962)**, De Duve (Deter and de Duve, 1967) and others gave the first hint in that direction. Glucagon was at that time suspected to be involved in protein catabolism, which was skillfully demonstrated later by Micheal R. Charlton and colleagues **(Charlton et al., 1996)**. Furthermore the studies by Ulrich Pfeifer showed that insulin, also a metabolic hormone, contrary to glucagon was able to inhibit the formation of autophagic vesicles. This and the fact that autophagy seemed to be induced by nutrient deprivation **(Mortimore and Ward, 1976)** and more specifically controlled by the concentration ofcertain amino acids, led to the definite conclusion that autophagy was a catabolic process involved in protein turnover **(Mortimore et al., 1983) (Seglen et al., 1980)**. As already mentioned and often seen by electron microscopy, further studies revealed that autophagy was also responsible for the recycling of organelles like mitochondria, linking this mechanism once more to metabolic functions. Over the years, it became more and more evident that autophagy is an integral cell survival mechanism, conserved among eukaryotes. Autophagy can be induced by starvation, stress or through the activation of certain receptors depending on the tissue and cell type. It provides the cell with new building blocks through the degradation of macromolecules

like long-lived proteins, and allows to maintain cellular homeostasis by eliminating dysfunctional or superfluous organelles.

In addition to the studies on rat liver cells, the use of other models such as yeast (*Saccharomyces cerevisiae*, *Pichia pastoris)* **(reviewed in Ohsumi, 2014)**, *Caenorhabditis elegans* **(Jenzer et al., 2015)** or *Drosophila* **(Nagy et al., 2015)** allowed to shed light on the molecular mechanisms and the functions of this process. Moreover, it became clear throughout the years that the first observations made by De Duve, Clark, Ashfold and others belonged to a phenomenon that we now call macroautophagy and that co-exists in vertebrates with two other forms of autophagy: microautophagy and chaperone-mediated autophagy (CMA) that will be briefly detailed and illustrated below **(Fig 1)**.

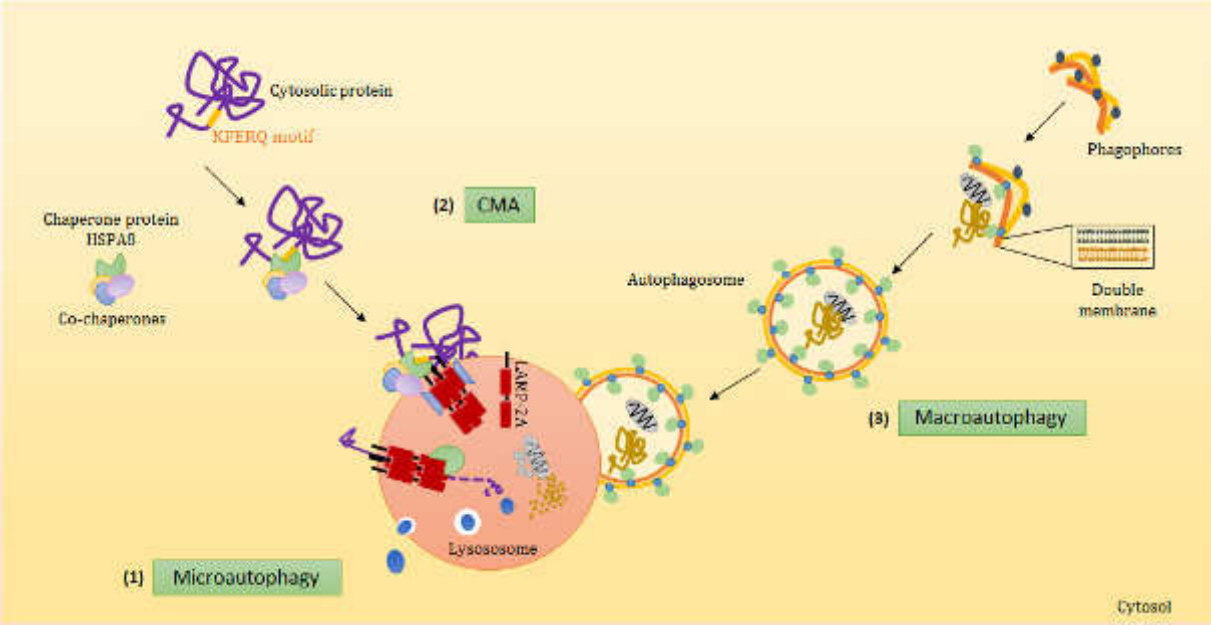

Figure 1: Overview of three types of autophagy.

(1) Microautophagy: Cytosolic content is directly engulfed into the lysosome by lysosomal membrane invagination. **(2)** Chaperone-mediated autophagy (CMA): The cytosolic chaperone protein HSPA8 and its co-chaperones bind to the substrate protein through recognition of the consensus sequence KFERQ. The substrate-chaperone complex is recognized by a lysosomal membrane receptor LAMP-2A. The substrate protein is then unfolded and translocated across the lysosomal membrane and gets degraded in the lysosome. **(3)** Macroautophagy: Cytosolic material is sequestered by expanding membranes (phagophores) forming a double-membrane vesicle, the autophagosome. The autophagosome fuses with the lysosome which leads to degradation of the content by lysosomal hydrolases. **HSPA8:** Heat shock protein family A (Hsp70) member 8**; LAMP-2A:** Lysosome-associated membrane protein type 2A.

1.2 Microautophagy

Microautophagy is a cellular process which was described in 1966 by Christian De Duve as well, while reporting the different functions of the lysosomes **(Duve and Wattiaux, 1966)**. He was indeed referring to this mechanism different from the other autophagic observations because it did not lead to the formation of a specific vesicle but rather induced the engulfment of the cytosolic content for degradation through direct invagination of the lysosome membrane.

In contrast to the other two types of autophagy (macroautophagy and CMA), research on microautophagy remains scarce. Nevertheless studies on yeast (*S. cerevisiae, P. pastoris*), centered on the observation of the vacuoles (the yeast functional equivalent of lysosomes from animal eukaryotes), have allowed to make some advances on the subject and to establish the existence of two types of microautophagy, either nonselective (NSM) or selective (SM) as reviewed by Mijalijca and colleagues **(Dalibor Mijaljica, 2011) (Fig 2)**.

In NSM a degradation of soluble cytoplasmic material without any specificity can be witnessed. Andreas Meyer's team was indeed able to identify four kinetic stages leading to the invagination and the subsequent budding of the vacuolar membrane into its own lumen, also called "inverted budding" **(Kunz et al., 2004)**. Using different pharmacological inhibitors and cooling technics, they were able to dissect those stages *in vitro* after vacuole isolation from yeast **(Fig 2)**. In summary, they established that the first three stages are dependent on vacuolar ATPases and require a constant supply in lipids and membrane to allow the formation and maintenance of a pit-like or tubular structure in the vacuole. The fourth stage is the actual uptake of cytosolic soluble constituents and their release into the lumen. It also appears that the interplay of two protein complexes localized at the vacuolar membrane, TOR (target of rapamycin) and EGO (*e*xit from rapamycin-induced growth arrest), regulate this process **(Dubouloz et al., 2005)**. Thus the mechanisms around non-selective microautophagy-like phenomenon, in yeast, seem to become clearer. It is also triggered by stress factors like starvation or by pharmacological agents such as rapamycin, also known to induce macroautophagy.

In selective microautophagy, it appears that the specific degradation of organelles can be triggered either because the organelles in question are dysfunctional, or to reduce their number. This degradation of mitochondria is called micromitophagy and seems to be induced by the alteration of iron homeostasis **(Nowikovsky et al., 2007)**. The degradation of peroxisomes (micropixophagy) and nucleus (micronucleophagy or piecemeal microautophagy of the nucleus (PMN)) have also been observed in *P. Pastoris* (Ano et al., 2005) and *S. cerevisiae* respectively **(Roberts, 2003)** and are reviewed in Mijaljica *et al* **(Dalibor Mijaljica, 2011)**.

As already mentioned, microautophagy in mammalian cells has not been very well characterized yet and has mostly been observed by electron microscopy.

But a recent study has shown that a microautophagy-like process was involved in late endosome multivesicular bodies (MVBs) biogenesis in a mammalian system. The authors propose that cytosolic proteins are delivered to late endosomes and that some of these proteins are recognized by the chaperone protein HSPA8 (formerly called Hsc70) through the KFERQ peptide motif, suggesting a specific cargo recognition, originally associated to chaperone-mediated autophagy (CMA) that will be discussed below. Even though contrary to CMA it is a LAMP-2A independent mechanism, it requires the endosomal sorting complexes required for transport (ESCRT) I and II for the formation of vesicles in which the proteins get trapped. Whether HSPA8 gets also internalized however remains to be clarified **(Sahu et al., 2011)**.

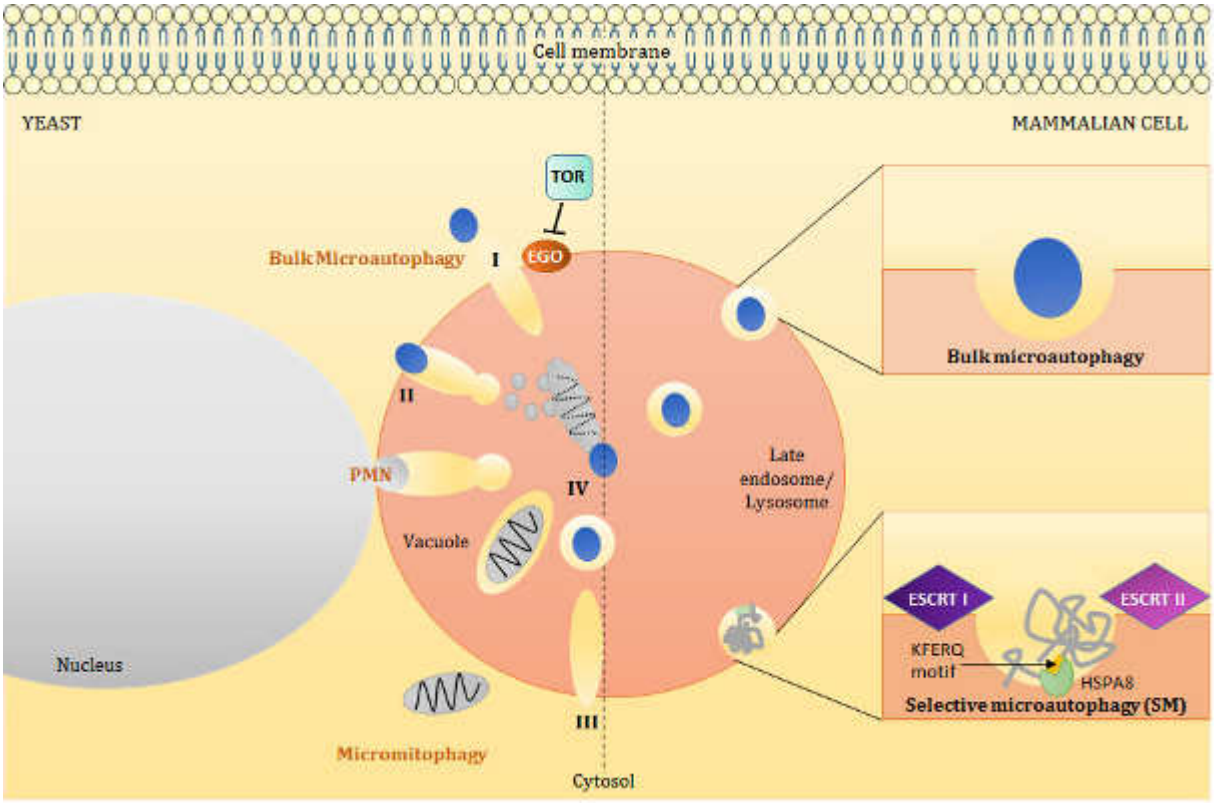

Figure 2: Microautophagy.

In yeast lysosomal degradation via microautophagy is a 4 step process starting with invagination of the vacuole to from a pit-like structure **(I)** leading to engulfment of cytosolic content **(II-III)** and finally degradation **(IV)**. Microautophagy depends on the EGO complex which is negatively regulated by TOR in a nutrient rich environment. EGO regulates bulk microautophagy as well as SM such as piecemeal microautophagy of the nucleus (PMN) or micromitophagy (degradation of mitochondria). Mammalien cells also undergoe bulk microautophagy and SM. SM depends on the recognition of the substrate proteins by HSPA8 via the KFERQ motif and on the presence of ESCRTI/II for the invagination process. **EGO:** Exit from rapamycin-induced growth arrest; **ESCRT:** Endosomal sorting complex required for transport

1.3 Chaperone-Mediated Autophagy

Out of the three types of autophagy, chaperone-mediated autophagy was discovered last. At that time most scientists in that field thought that macroautophagy randomly targeted cytosolic content for degradation. Thus they had overseen CMA as it was the first selective form of autophagy to be described. This discovery can be attributed to James Fred Dice and his team. They observed when following the fate of radiolabeled proteins after microinjection in serum-deprived fibroblasts, that some of those proteins underwent lysosomal degradation while others remained unaffected (Neff et al., 1981). The search for an explanation for this differential degradation led to the identification, after enzymatic fragmentation of ribonuclease A (RNase A), of a pentapeptide motif, the KFERQ motif **(Backer et al., 1983)**. This motif was shown to direct RNase A to lysosomal degradation. The development of an antibody raised against the KFERQ motif allowed them to isolate proteins specifically containing that motif. They observed that by microinjecting these proteins in starved fibroblasts, they were degraded 5 times faster than those without the KFERQ motif. A few years later, Dice's laboratory discovered that this motif was a target sequence for a 70-kilodalton heat shock protein HSPA8 (heat shock protein family A (Hsp70) member 8) and that this chaperone protein was indispensable for the translocation of the proteins into the lysosome **(Chiang et al., 1989**). Around the same period Erwin Knecht, who was also working on lysosomal proteolysis had come to the same conclusion while studying the degradation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), namely that there had to be a selective degradation of proteins in the lysosomes **(Aniento et al., 1993)**. The two laboratories soon noticed that they were probably studying the same protein degradation pathway and started working together to dissect the mechanisms behind this specific proteolysis **(Cuervo et al., 1995)**. The collaboration of these pioneers in selective autophagy turned out to be a major breakthrough for chaperone mediated autophagy (CMA), a term which was actually proposed around the years 2000 **(Cuervo and Dice, 2000)**.

Extensive studies of CMA identified only in mammalian cells so far, have allowed to establish the molecular process step by step, as well as the regulation and functions of this mechanism. Like the other types of autophagy, CMA is induced by nutrient deprivation in order to provide the cell with amino acids for the synthesis of *de novo* proteins or to generate energy. Another function of CMA is the degradation of short-lived proteins like obsolete enzymes or the removal of damaged proteins resulting from oxidative events **(Kiffin et al., 2004)**. Aggregated proteins fall also in the category of macromolecules that can be eliminated through CMA **(Sattler and Mayer, 2000).** CMA has also been described to be involved in the peptide generation for presentation by antigen presenting cells (APCs) **(Uytterhoeven et al., 2015; Zhou et al., 2005)**. This process has been extensively studied in the liver.

Schneider and colleagues generated a mouse model with a conditional knock-out of LAMP-2A in liver cells in order tho study the role of CMA *in vivo*. CMA invalidation in those cells led subsequently to a disturbed lipid metabolism and to a fatty liver suggesting that CMA participates in metabolic liver functions **(Schneider et al., 2014)**. CMA has also been investigated in other cell types such as T lymphocytes for instance. Fernando Macian's team demonstrated in fact that upon TCR engagement CMA is activated and LAMP-2A upregulated. *In vitro* and *in vivo* invalidation of CMA through knock-down and T cell specific deletion of LAMP-2A resulted in reduced activation and immune response against bacterial infection. Moreover they showed that CMA as well as LAMP-2A expression decrease with age in T cells as does their responsiveness to TCR stimulation. Restoring LAMP-2A expression in old T cells led to an improved activation-induced reponse. These results show the importance of CMA in T cell responses and indicate that modulating this process could be beneficial to dodge age-related T cell senescence **(Valdor et al., 2014)**. In many studies CMA has also been shown to be reduced in age-related disorders such as neurodegeneration or metabolic pathologies. Hence using CMA as a therapeutic target could be beneficial in the process of finding new cures against those disorder.

As already mentioned, protein degradation by CMA requires the recognition of a KFERQ or a KFERQ-like motif **(Fig 3)**. In fact it has been established that it is mostly the distribution of the charges that matters for protein recognition by a chaperone complex containing HSPA8. After binding the protein destined for degradation, the complex formed with the chaperone is targeted to the lysosome where it binds to the lysosome-associated membrane protein type 2A (LAMP-2A). This consequently induces the multimerization of LAMP-2A, which at the same time interacts with another chaperone protein, Hsp90, localized at the luminal side of the lysosomal membrane. Hsp90 and the glial fibrillary acidic protein (GFAP) are responsible for the stabilization of multimerized LAMP-2A. In order to cross the lysosomal membrane, the protein targeted for degradation has to be unfolded, a process that is mediated by HSPA8 and the co-chaperones forming the complex **(Cuervo and Wong, 2014)**. Ultimately the substrate protein helped by luminal HSPA8 enters the lysosome where it gets degraded. The degradation of one single protein at a time is actually a specificity of this pathway. Once this purpose is fulfilled HSPA8 contributes to LAMP-2A multimer disassembly. Monomeric LAMP-2A gets then dissociated from the lysosomal membrane by cathepsin A through cleavage of the transmembrane domain **(Kaushik et al., 2011)**.

Even though knowledge about CMA has been growing fast, some questions still remain. It is for example not well understood yet how exactly the internalization of the substrate protein occurs or why LAMP-2A multimerization is relevant for this process. Thus more studies are needed to answer those questions but also to understand the role of CMA relative to each cell type, since it differs depending on the cell studied.

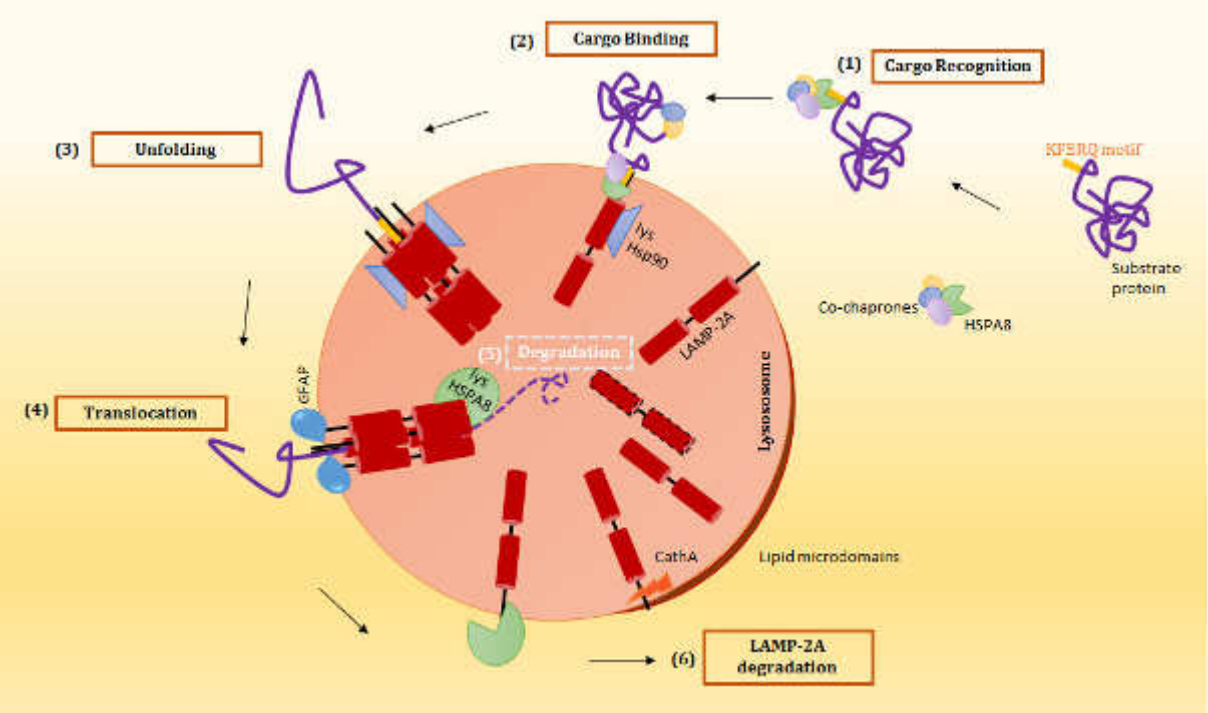

Figure 3: CMA Step by step.

(1) HSPA8/Co-chaperones recognize the KFERQ motif and bind the substrate protein; **(2)** binding of substrate-chaperone complex to LAMP-2A; **(3)** unfolding of the substrate; LAMP-2A multimerization stabilized by Hsp90; **(4)** transmembrane domain of LAMP-2A multimer stabilized by GFAP, substrate translocation mediated by lysosomal HSPA8 and **(5)** degradation of substrate protein in the lysosome; **(6)** disassembly of LAMP-2A multimer mediated by HSPA8, migration to the lipid microdomain where LAMP-2A gets cleaved by CathA and finally degraded by lysosomal proteases. **HSPA8**: Heat shock protein family A (Hsp70) member 8; **LAMP-2A**: Lysosome-associated membrane protein type 2A; **GFAP**: Glial fibrillary acidic protein; **Hsp90**: Heat-shock protein of 90 kDa; **lys**: Lysosome, **CathA**: Cathepsin A

1.4 Macroautophagy

Macroautophagy, most probably the first type of autophagy that De Duve and others observed, is the best characterized type of autophagy so far. As in the rest of this manuscript, it is often simply abbreviated by autophagy. Same as for micro- and chaperone-mediated autophagy, it is a cell starvation-induced mechanism, and is necessary for protein and organelle turnover. Autophagy, has also been shown to be important in cellular development **(Levine and Klionsky, 2004)** and differentiation. Thus it is not surprising that autophagy seems to play a key role in physiopathology, like during neurodegenerative, metabolic and autoimmune diseases or cancer. Even though this process has mostly been associated to survival mechanisms in stress conditions, in some cases it has been shown to induce cell death in a process termed autophagic cell death or type II programmed cell death. This type of non-apoptitic cell death has been shown to take place in conditions where autophagy levels were very high or when the apoptotic pathways were somehow impaired as

demonstrated by Shimizu and colleagues with their Bax/Bak-deficient mouse embryonic fibroblasts (MEFs). These cells were in fact unable to undergo classical apoptosis induced by etoposide, but died anyway through autophagy induction **(Shimizu et al., 2004)**. Indeed, inhibiting autophagy also inhibited cell death which led to the conclusion that this phenomenon was dependent on the autophagic machinery.

In physiological conditions, the autophagic process appears to be mainly a pro-survival mechanism, but plays numerous other roles. In contrast to the two other types of autophagy described above, macroautophagy requires the formation of double membrane vesicles called autophagosomes, that fuse with lysosomes and enable the degradation of the cytosolic content captured inside. Another feature of this mechanism is the multi-step process that involves specific proteins encoded by genes designated as autophagy-related genes (*Atg*). The discovery of those *atg* was made through genetic studies carried out on yeast, and to which Yoshinori Ohsumi and his colleagues were central contributors. The yeast vacuole, identified as the equivalent to lysosomes in mammalian cells **(Jones, 2002)**, was found to endure drastic morphological changes when the yeasts were to be starved (Takeshige et al., 1992). Ohsumi's laboratory was the first to report an autophagic degradation in yeast vacuoles after vesicle formation in the cytosol. Soon after, they identified the first *apg* (for autophagy) gene, *apg1,* using autophagy-defective mutants and utilizing their survival capacities in nutrient deprived conditions as read-out **(Tsukada and Ohsumi, 1993)**. The screen of those mutants and the studies by other research teams led to the characterization of autophagy essential genes **(Ohsumi, 2014)**. *Apg* were then labelled *Atg*, as their products can also be involved in non-autophagic processes. Hence, over thirty *Atgs* have been identified in yeast so far, and for most of them, the homologues in higher eukaryotes are known as well **(Pyo et al., 2012)**.

The discovery of *Atg* and thus of Atg proteins, launched the research aiming at understanding and establishing in details the molecular mechanisms underlying the autophagic process. The knowledge in this field has indeed been growing exponentially these last two decades, leading to the identification of the different stages involved in the autophagosome formation: initiation or nucleation, elongation of the phagophores, sequestration of the cytosolic content, maturation of the autophagosome, fusion with lysosomes and finally degradation of the content. All these stages and their functions/roles, as well as the protein complexes involved, will be taken under scrutiny below, focusing specifically on the events taking place in mammalian cells. Even though it won't be detailed in this manuscript the yeast has been and still is a remarkable tool to study autophagy, since the complexes and the mechanisms involved in this process have firstly been described in this organism before being investigated in higher eukaryotes.

1.4.1 From Initiation to Degradation: Dissection of the autophagic machinery

Initiation (**Fig 6**)

The initiation or nucleation process consists in the formation of a flat organelle called phagophore or isolation membrane. One main interrogation that has not been answered clearly yet is the origin of the membranes forming this structure and leading eventually to the formation of the double membrane of the autophagosome. There is evidence suggesting two sources, the first being that diverse organelles such as the endoplasmic reticulum (ER), mitochondria, the Golgi apparatus, and even the cytoplasmic membrane give birth to the double membrane forming the pre-autophagosomal structures. On the other hand it has been suggested that the membrane could originate from the omegasome, an omega-shaped membrane structure from the phosphatidylinositol-3-phosphate (PtdIns3P)-enriched ER subdomains **(Axe et al., 2008)**. Most evidence points to the omegasome as the main source for the membranes, although it may vary according to the type of autophagy induced and the nature of the triggering signals. The proteins mainly required in the initial steps of the autophagosome formation are called core ATG proteins. They are organized in complexes, starting with the Unc-51-like kinase (ULK) complex composed of ULK1/2, the focal adhesion kinase [FAK] family-interacting protein of 200 kDa (FIP200), ATG13 **(Ganley et al., 2009)** and finally ATG101 **(Hosokawa et al., 2009a, 2009b; Mercer et al., 2009)**. All the proteins forming this complex are associated in a stable conformation independent from the nutritional status of the cell. Furthermore, the ULK1/2 complex is negatively regulated by the activity of the mammalian target of rapamycin (mTOR) in the so-called mTOR-dependent autophagy **(Yang and Klionsky, 2010)**. mTOR activation initiates anabolic events necessary for cell growth and proliferation. Thus mTOR depends on sufficient nutrient and growth factors (GF) to catalize these events. Insulin for instance binds its receptor of tyrosine kinase (RTK) which leads to the activation of the phosphoinositide 3 kinase (PI3K)/AKT (also known as the protein kinase B) signaling pathway. Subsequent phosphorylation of the tuberous sclerosis complex 1 and 2 (TSC1/2) by AKT inbibits its association to Ras homologue enriched in brain (Rheb) GTPase **(Fig 4)**. Free Rheb can then activate mTOR that inhibits the induction of autophagy by binding and phosphorylating ULK1 as well as ATG13 **(Kim and Guan, 2015)**. In contrast AMP-activated protein kinase (AMPK), activated by high AMP/ATP ratios, can initiate autophagy through ULK1 complex direct activation or indirectly via mTOR inhibition. As reported by Kim and colleagues, further sequential phosphorylation events are required in this process. In a nutrient-deprived environment, AMPK gets activated and phosphorylates TSC2 leading to the association of this complex to Rheb. AMPK also phosphorylates a component of the mTORC1 complex, the regulatory-associated protein of mTOR (Raptor) and thus hinders the activation of mTORC1 complex. AMPK also directly inhibits ULK1 complex, by phosphorylating two ULK1 serines **(Kim et al., 2011)**.

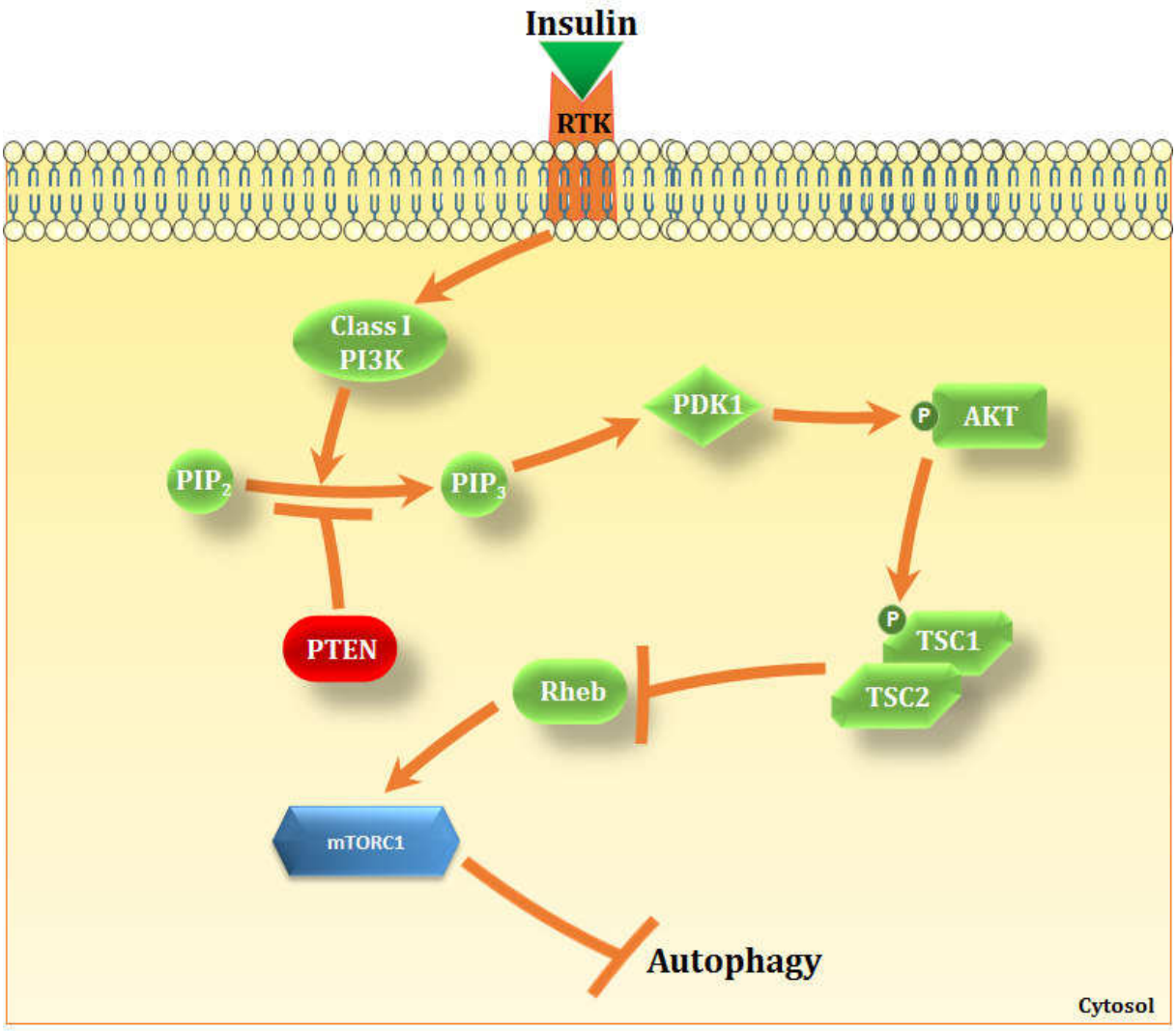

Figure 4: Insulin mediated Class I PI3K/AKT pathway.

Insulin binding to its RTK activates the PI3K. Activated PI3K is then able to phosphorylate PIP_2 to PIP_3 which accumulates and leads to the recruitment of PDK1 and AKT. AKT requires phosphorylation by PDK1 to be activated. AKT can then phosphorylate the complex TSC1/TSC2 thus inhibiting their interaction with Rheb. Free Rheb binds and activates mTOR leading to autophagy inhibition. **RTK:** Receptor tyrosine kinase; **PDK1:** 3-phosphoinositide dependent protein kinase-1; **TSC1/2:** Tuberous sclerosis; **PIP_2:** phosphatidylinositol 4,5,-biphosphate, **PIP_3:** Phosphatidylinositol 3,4,5-triphosphate;

Thus the ULK complex is able to sense nutrient starvation indirectly, through AMPK and mTOR activities. After ULK1 activation, the complex is relocalized to the isolation membrane, where it contributes to the regulation of another major player in the initiation process, the Beclin1-VPS34-VPS15 (vacuolar protein sorting, VPS) complex. One of its members, Beclin 1, has been initially discovered to be a tumor suppressing gene frequently underexpressed in ovarian, breast and prostate cancer **(Aita et al., 1999)**, suggesting a role for autophagy in cancer development, subject that will be discussed in section 2.2. According to many studies this complex displays a kinase activity responsible

for the phosphorylation of phosphatidylinositol (PI) to produce phosphatidylinositol 3 phosphate (PI3P) **(reviewed in Russell et al., 2014)**. Hence this complex is termed class III phosphatidylinositol 3-kinase (class III PI3K) complex. PI3P seems to be involved in the stabilization of ULK1 at the omegasome but also contributes to the binding of other proteins necessary for the formation of the autophagosome, such as the double FYVE containing protein 1 (DFCP1) **(Axe et al., 2008; Karanasios et al., 2013)**. The VPS34 complex can be associated to three other proteins: ATG14L, the UV resistance associated gene protein (UVRAG) and to Rubicon (RUN domain and cysteine-rich domain containing, Beclin 1-interacting protein) **(Fig 7)**. Even though those proteins bind specifically to Beclin1, it is seemingly never at the same time and they are required for different tasks. ATG14L is essential for the relocalization of the complex to the isolation membrane and more specifically to the omegasome **(Fan et al., 2011; Matsunaga et al., 2010)**. When UVRAG is associated to the complex it has been suggested that it enhances the PI3K activity. Rubicon binding on the other hand has an inhibitory effect, since it decreases the PI3 kinase activity and thus prevents autophagosome formation **(Zhong et al., 2009**). When in another conformation, Beclin1 is also able to bind the activating molecule in Beclin1-related autophagy 1 (AMBRA1). The latter protein interacts with the dynein motor complex. It induces autophagy upon release from the complex, by directing the ULK1 complex to the ER **(Di Bartolomeo et al., 2010)**. Beclin1-interacting proteins also comprise Bcl-2 (B cell lymphoma 2), which same as Rubicon negatively regulates autophagy when both proteins are bound **(Pattingre et al., 2005)**. Interestingly, Bcl2 also displays anti-apoptotic properties (Bcl-2 and Bcl-xl) when associated to Bax and Bak on mitochondria. This highlights the tight interplays between autophagy and apoptosis, and the homeostatic role of Beclin1 to that respect.

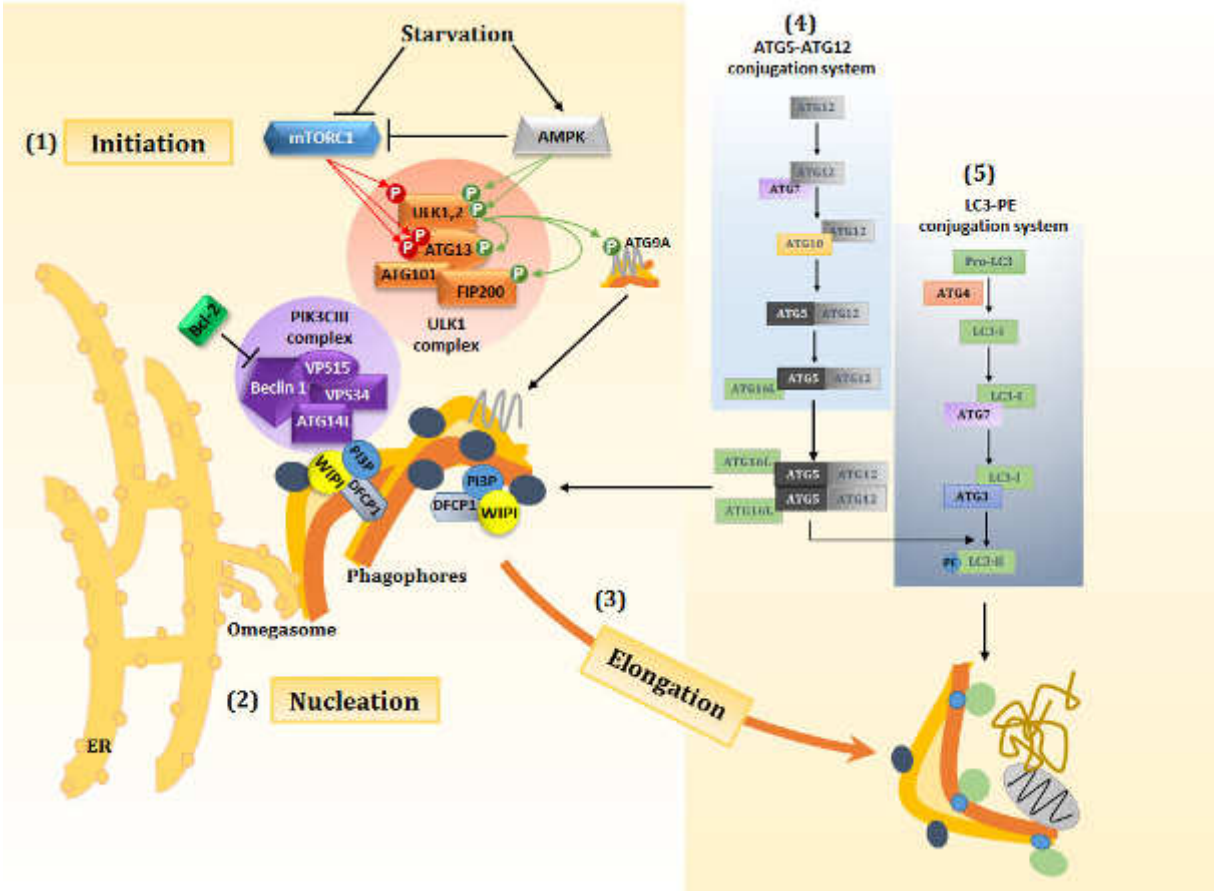

Figure 5: An overview of the autophagic machinery from initiationto elongation.

(1) Detailed here the initiation step controlled by the ULK1 complex (ULK1 ATG13, FIP200 and ATG101). In nutrient rich conditions mTORC1complex interacts with ULK1 and inactivates the complex by phosphorylating ULK1 and ATG13. Under starvation conditions, mTORC1 is inactivated by AMPK leading to the phosphorylation of ULK1 by AMPK and its subsequent activation. **(2)** Then ULK1 phosphorylates ATG13 and FIP200 and consequently induces Beclin 1 dissociation from Bcl-2 which initiates autophagosome nucleation. ATG9A also requires the phosphorylation by ULK1 to be activated and furnishes the membranes for the formation of the phagophores during the nucleation step which takes place at the omegasome, a structure generated from the ER. This process requires the PIK3CIII complex (Beclin1, VPS34, VPS15 and ATG14L). PI3P produced by VPS34 recruits DFCP1 and WIPI to the isolation membrane. **(3**) For the elongation process two ubiquitin-like conjugation systems are required. The ATG5-ATG12 and LC3-PE conjugation systems (blue boxes). **(4)** The ATG12-ATG5 conjugates form a multimeric complex with ATG16L1 through ATG7 and ATG10, E1 and E2-like enzymes respectively. The formed ATG12-ATG5/ATG16L1 conjugate undergoes dimerization and gets recruited to the phagophores by WIPI. **(5)** In the second system LC3-I is generated from pro-LC3 by the ATG4 protease. Then, through ATG7, ATG3 (E2-like enzyme), and the ATG12-ATG5/ATG16L1 complex (acts as an E3-like enzyme), LC3 gets conjugated to the phospholipid phosphatidylethanolamine (PE) to form LC3-II. LC3-II localizes to the growing membranes and the ATG12-ATG5/ATG16L1 conjugates are released from the membranes during the formation of the autophagosome. Cytosolic content is captured while membranes are elongating.
ULK1: Unc-51-like kinase 1; **mTOR:** mammalian target of rapamycin; **AMPK:** AMP-activated protein kinase; **PI3P:** Phosphatidylinositol triphosphate; **DFCP1:** double FYVE containing protein 1, **WIPI:** WD-repeat PI3P effector protein

The generation of the phagophore membrane *per se* is mediated by ATG9A. This ATG is ubiquitously expressed and localized to the Golgi apparatus and late endosomes and cycles from one to the other **(Feng et al., 2014).** Upon autophagy induction by starvation, ATG9A gets phosphorylated by ULK1 which induces its recruitment to the phagophore **(Papinski et al., 2014)** and contributes to phagophore expansion by feeding lipids to the growing isolation membrane **(Yamamoto et al., 2012; Zavodszky et al., 2013)**. One particular characteristic of this protein is that, with the vacuole membrane protein 1 (VMP1), it is the only known transmembrane ATG protein identified so far. VMP1 has mainly been found associated to the ER membrane and has recently been shown to recruit Beclin1 to the phagophore. VMP1 thus regulates PI3K CIII complex activation **(Molejon et al., 2013)**.

Elongation – Sequestration (**Fig 5**)

The elongation step following initiation is mainly mediated by two ubiquitin-like conjugation systems. During this step the phagophore grows and at the same time, cytoplasmic material gets engulfed into the forming autophagosome.

The ATG7 ubiquitin-like conjugation system is composed of three key proteins: ATG5, ATG12 and ATG16L1. In order to build this system, ATG12 has to be conjugated to ATG5 and this is achieved through the combined actions of the E1 ubiquitin-like ligase, ATG7 and the E2 ubiquitin-like ligase, ATG10. First ATG12 gets activated by ATG7 at the C-terminal glycine residue in an ATP dependent manner, subsequently inducing the formation of an ATG12-ATG7 thioester intermediate followed by the transfer of ATG12 to ATG10 to also form a thioester intermediate. Then the conjugation to ATG5 gets established through the covalent attachment of the C-terminal glycine of ATG12 to ATG5's lysine at position 130 **(Mizushima et al., 2002)**. The newly formed ATG12-ATG5 conjugates can then bind the ATG16L1 protein in a non-covalent manner, which in turn induces the homodimerization of this complex resulting in an 800kDa conjugate. The WD-repeat PI3P effector protein (WIPI) recuited by PI3P, interacts with ATG16L1 and facilitates the recruitement of the conjugate to the nucleation site **(Dooley et al., 2014)**. The conjugate ATG12-ATG5/ATG16L1 localizes at the growing autophagosome until it's completely formed and then dissociates from it **(Mizushima et al., 2003)**.

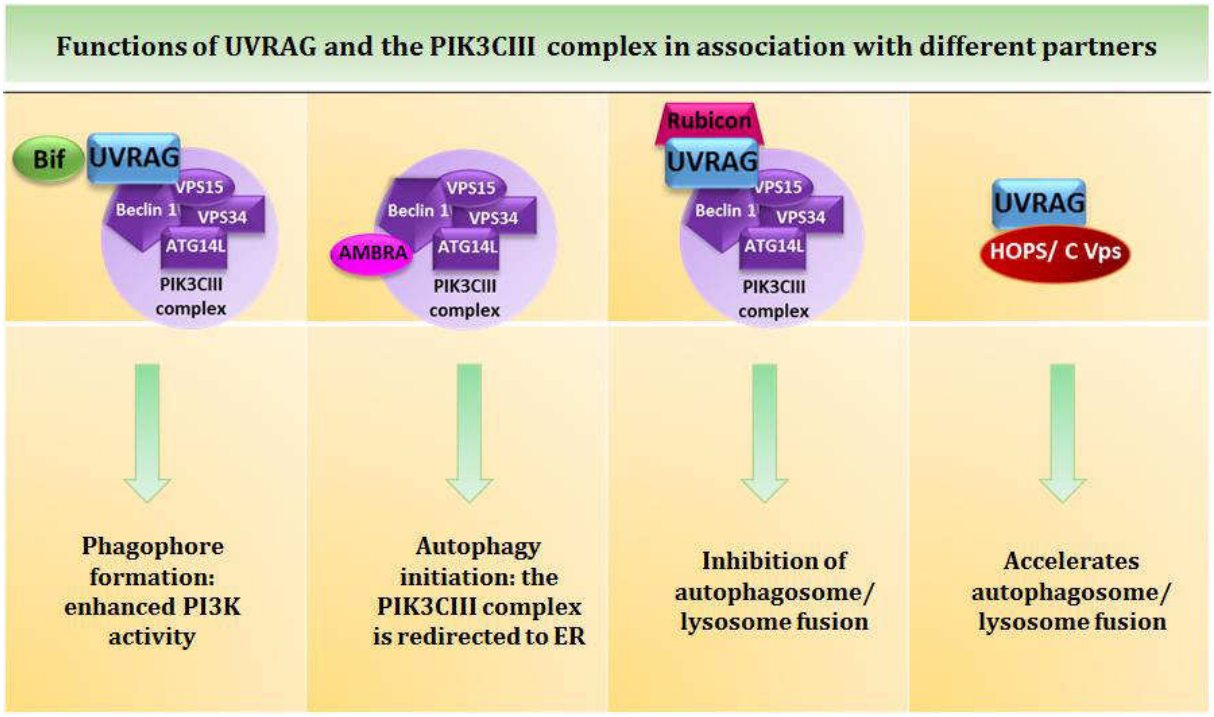

Figure 6: The essential roles of UVRAG and and the PIK3CIII complex in autophagy.

The second ubiquitin-like conjugation system is involved in the elongation of the autophagosomal membrane. This system requires the lipidation of the microtubule-associated-protein 1 light chain 3 (MAP1LC3 or LC3) and its paralogues belonging to the GABARAP family (γ-amino-butyric acid receptor-associated protein), GABARAPL1, and to the GATE family (Golgi-associated ATPase enhancer), GATE-16. Mammalian LC3 family is composed by different isoforms which are LC3A, LC3B and LC3C in human, the most abundant being LC3B. While the LC3 family proteins are important for the elongation step, the GABARAP/GATE family is rather needed for autophagosome maturation **(Weidberg et al., 2010)**. LC3 is generally localized in the cytosol where it undergoes a number of posttranscriptional modifications, the first being its processing from pro-LC3 to LC3-I mediated by ATG4, a cysteine protease also present through different isoforms in mammalian cells (ATG4A-D also called Autophagin 1-4). The most active isoform, ATG4B (Autophagin 1) catalyzes the cleavage at the C-terminal region of pro-LC3 after a glycine residue. It has recently been reported that upon autophagy induction, ATG4B requires phosphorylation for proper function **(Yang et al., 2015)**. In any case, the glycine residue at the C-terminus of LC3-I constitutes the lipidation site where phosphatidylethanolamine (PE) will be conjugated to LC3-I to form LC3-PE, also called LC3-II. This action is mediated as previously for the ATG5-ATG12/ATG16L1 conjugation, by an E1 and E2 ubiquitin-like catalytic system composed of ATG7, and ATG3 an E2-like conjugation enzyme. ATG12-ATG5/ATG16L1 complex in this second conjugation system allows the binding of the PE to LC3-I through its E3 like ligation enzyme activity. As

demonstrated by Fujita and colleagues, ATG16L1 acts like a scaffold protein that transfers LC3 from ATG3 to PE **(Fujita et al., 2008)**. Consequently this lipidated form of LC3 can be integrated both in- and outside of the elongating double membrane. While other proteins attached to the autophagosomal membrane end-up dissociating from it in the initial steps of autophagy, LC3-II is the only protein that remains there from initiation on until content degradation. Hence this protein is commonly used as an autophagic marker. One must however remember that LC3 is partially degraded after autophagosome fusion with the lysosomes. Even though the precise function of LC3-II has not been very well determined yet, it has been suggested, with help of liposomes, to be involved in the hemifusion of the autophagosome **(Nakatogawa et al., 2007)**. Nevertheless LC3-II has been shown to be a major player in cargo recognition in collaboration with sequestosome proteins **(see sections 1.4.3 and 3.1.1)**

Maturation and Degradation **(Fig 7)**

Maturation begins once the autophagosome is fully formed and the cytoplasmic content is sequestered inside. This step leads to the fusion of the autophagosome with the lysosomes and subsequently to the formation of the autolysosome. Many proteins and complexes are involved in the establishment of this process.

UVRAG, involved in phagophore generation through its interaction with the Beclin1-VPS34-VPS15 complex, also plays an important role in the autophagosome maturation process. This complex actually localizes both in endosomes and autophagosomes. UVRAG interacts with a tethering complex (the homotypic fusion and protein-sorting/class C vacuole protein-sorting (HOPS/class C Vps), composed of VPS16, VPS33 and VPS39 among other proteins) independent of Beclin1 **(Fig 6)**. Liang and colleagues show that UVRAG accelerates endosomal trafficking which leads to an increased autophagic flux. Furthermore knock-down of UVRAG by small interfering ribonucleic acid (siRNA) in HeLa cells substantially reduces the colocalization of VPS16 with the GFP-LC3 fusion protein, suggesting an UVRAG-dependent recruitment of the HOPS/class C Vps to the autophagosome **(Liang et al., 2008)**. Tethering complexes are in fact essential in fusion processes since they mediate the coupling of Rab GTPase activation and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARES) assembly. One element of the HOPS/class C Vps complex, humain VPS39 activates such a GTPase, namely Rab7 **(Liang et al., 2008)**. As a matter of fact, Rab7 is one other major key player in this maturation process. This small GTPase usually involved in late endosomal trafficking has been identified as an important mediator of autophagosome fusion with the lysosomes. In fact it has been shown that Rab7 down-regulation in cardiomyocytes resulted in the accumulation of autophagosomes followed by apoptosis, demonstrating the role of this protein in autophagosome maturation rather than in the initiation. It also highlights the requirement of a complete autophagic flux for the

maintenance of cellular integrity in that context. Rab7 actually plays two roles in this process, first mediating the autophagosome transport along the microtubules from the periphery to the microtubule organization center (MTOC), the concentration site of the lysosomes. Secondly Rab7 favors the autophagosome/lysosome fusion. The HOPS complex discussed earlier, seems also to be able to bind Rab7 and to participate both in trafficking and fusion by tethering the lysosome to the autophagosome **(Balderhaar and Ungermann, 2013; Pawelec et al., 2010)**.

UVRAG can also be associated to Rubicon and, as already mentioned, this Beclin1-VPS15-VPS34-UVRAG-Rubicon complex has an inhibitory function towards autophagy **(Fig 6)**. It has been recently proven that Rubicon binds UVRAG and interacts with VPS34 via its RUN-domain and thus inhibits VPS34 lipid kinase activity by blocking UVRAG mediated VPS34 activation. Rubicon is also able to bind Rab7 and to inactivate its capacity to mediate autophagosome/lysosome fusion **(Sun et al., 2011)**.

The endosomal sorting complexes required for transport (ESCRT complexes 0, I, II and III) are important actors in autophagosomes maturation and are able to interact with Rab7 as well. Their primary role is to participate in endocytosis and in multivesicular bodies (MVBs) biogenesis. MVBs are late endosomes containing intraluminal vesicles that can fuse with autophagosomes in certain circumstances (e.g. starvation). The fusion of late endosomes with autophagosomes forms a structure called amphisome, prior to the fusion with lysosomes. Interestingly the ESCRT complexes have been shown to mediate this fusion. Impairments of their function is associated to autophagy dysregulations in neurodegenerative diseases. It has been suggested that the ESCRT complex II could interact with Rab-interacting lysosomal protein (RLIP), inducing the recruitment of Rab7 and thus priming the fusion process **(Wang and Hong, 2006)**. Of note, the HOPS complex also interacts with ESCRT complexes **(Pryor and Luzio, 2009)**.

Other Rab GTPases have been shown to be involved in fusion mechanisms. The Protein Rab11 for example, is required for the fusion of autophagosomes with multi-vesicular bodies (MVBs) as well.

As clearly established, the fusion process is essential for autophagosome maturation and in fact one particular protein family is specialized in catalyzing this process, the SNARE family mentioned previously. Their purpose is to create an opening in the membrane of two adjacent vesicles so that they can fuse. SNARE proteins share a signature sequence of about 60 residues. They often contain a transmembrane domain and tend to be present in opposing membranes destined to fuse **(Gerber and Südhof, 2002)**. The proteins present in each SNARE complex, usually composed of four SNARE proteins forming an alpha helix bundle, depend on the vesicles that need to fuse. The SNAREs Vit1b, VAMP7, VAMP8 and SYNTAXIN17 have been shown to mediate autophagosome/lysosome fusion, in particular in the context of pathogen elimination by xenophagy **(see section 3.1.1) (Furuta et al., 2010)**. It has also been demonstrated that SYNTAXIN17 interacts with tethering complex HOPS (Kim et al., 2001).

Pryor and Luzio proposed a fusion model involving the interaction of SYNTAXIN17 with the tethering complexes HOPS and Rab7. They suggested that HOPS might block SYNTAXIN17 from mediating membrane pairing. After replacement of Rab5 by Rab7 at the endosomal membrane, it would induce the recruitment of VPS39 and VPS41, two members of the HOPS complex. VPS39 would then activate Rab7 through its guanine-exchange-factor (GEF) activity and lead to the dissociation of SYNTAXIN17 from HOPS complex, thus allowing trans-SNARE pairing and fusion of opposed membranes **(Pryor and Luzio, 2009)**.

One other feature of the maturation process is the vesicular microtubule trafficking which has been shown to be a very important component of the autophagic process. The cytoskeleton is composed of four major constituents: actin filaments, microtubules, intermediary filaments and septin. All of them play a role at different autophagic stages with the microtubules being the ones mainly involved in autophagosome maturation. Autophagosome transport along the microtubules towards the MTOC requires the motor protein dynein. In a mouse model for Huntington's disease, dynein mutation through deletion of the dynein heavy chain1 gene (Dnch1) actually blocks protein aggregate degradation and thus mediates the development of neurodegenerative diseases **(Ravikumar et al., 2005)**. Studies have shown that dynein is linked to the autophagosomes through its binding to LC3 family proteins as their name (Microtubule-Associated-Protein) already indicates. Another protein that has emerged as an important player in autophagy maturation is the histone deacetylase-6 (HDAC6). Lee and colleagues report that HDAC6 mediates the fusion of autophagosomes with lysosomes through actin remodeling. This protein is known to regulate microtubule-dependent transport. Interestingly the authors observed that HDAC6 recruits an actin-remodeling factor, cortactin, at the site of protein aggregates (Lee et al., 2010b). HDAC6-defecient MEFs accumulate double-membrane structures containing multivesicular bodies, protein aggregates and have furthermore low levels of cortactin indicative of its role in autophagosome/lysosome fusion in order to mediate protein clearance. However inducing starvation in the absence of HDAC6 did not disturb autophagosome maturation suggesting that this protein is mostly required for quality control basal autophagy **(see section 1.4.3)**.

All in all, many proteins and complexes are required for the maturation process which is differentially regulated depending on vesicles that need to fuse even though the order in which the molecular events take place doesn't seem to have been clearly identified yet. To sum up the events, it can be said that once the autophagosome is formed it needs to be transported along the microtubules to the MTOC where it comes in contact with the lysosomes or endosomes leading subsequently to their fusion and the formation of the autolysosome **(Longatti and Tooze, 2009; Metcalf and Isaacs, 2010)**.

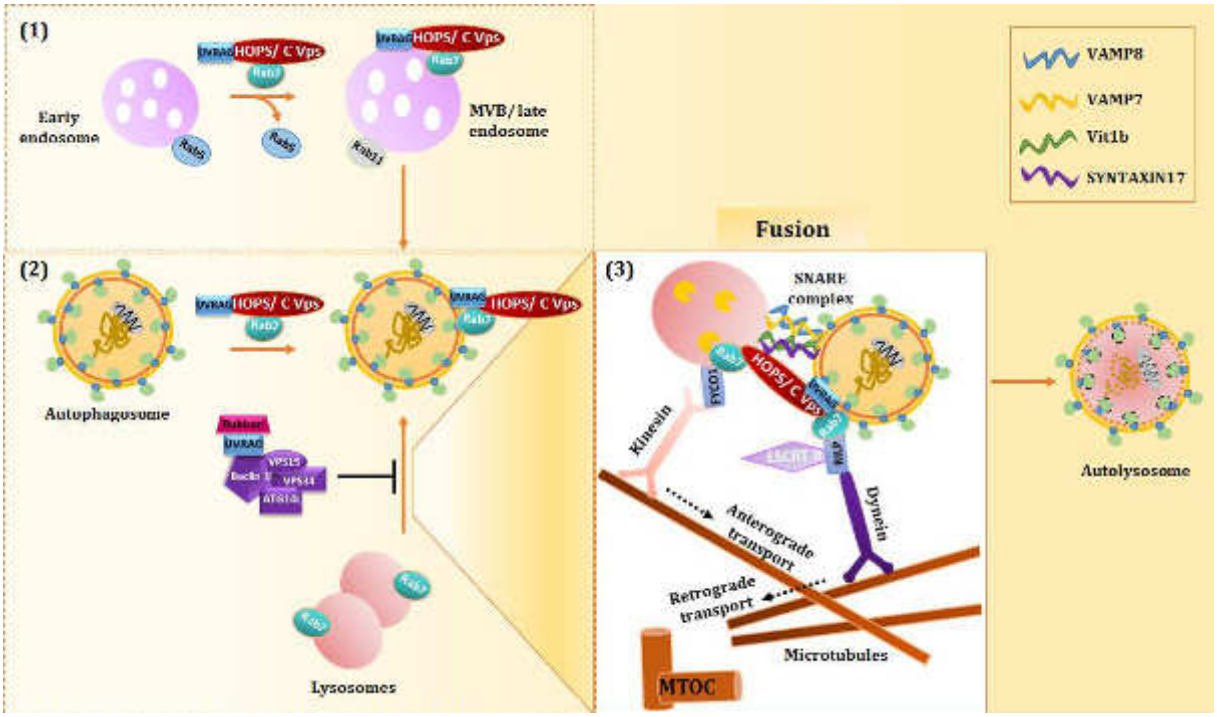

Figure 7: Schematic representation of endosome, autophagsome maturation and fusion with lysosomes.

(1) Rab5 is attached to the surface of an early endosome. UVRAG interacts with the tethering complex HOPS/C-Vps and mediates endosomal maturation by replacing Rab5 with Rab7 and by activating Rab7. **(2)** The same complex integrates Rab7 into the membranes of autophagosomes where Rab7 interacts with LC3-II. **(3)** Starvation promotes the binding of FYCO1 to kinesin and to Rab7 at the membrane of the lysosome while the RILP-dynein complex binds to Rab7 at the autophagosome, a process which requires the intervention of the ESCRT II complex. These interactions provide means to regulate the bidirectional movement of lysosomes and autophagosomes along microtubules towards one another and the MTOC. RILP-Rab7 interaction is further controlled by the UVRAG–HOPS/C-Vps complex which also regulates the fusion process (white square) by tethering the MVB/autophagosome and lysosome/autophagosome to one another. The SNARE complex (VAMP8, VAMP7, Vit1b and SYNTAXIN17) promotes the fusion of the lysosomes with the autophagosome. SYNTAXIN17 (purple) interacts with two proteins of the HOPS complex, Vps39 and Vps41 (not depicted). **UVRAG:** UV-resistance associated gene protein; **FYCO1:** FYVE and coiled-coil (CC) domain–containing protein 1; **ESCRT:** Endosomal sorting complex required for transport; **HOPS/C-Vps:** Homotypic fusion and sorting/Class C vacuole protein-sorting**; MTOC:** Microtubule organization center; **RILP:** Rab-interacting lysosomal protein.

1.4.2 Non-canonical Autophagy

The formation of the autophagosomes requires the activation of various ATG proteins described above. It has however been shown that the autophagosome can be assembled independently of some of the components of the conventional autophagic machinery. This process termed non-canonical autophagy will be discussed here.

ATG5/ATG7-independent autophagy

ATG5 and ATG7 are essential for autophagy because of their requirement for the ubiquitin-like conjugation systems during elongation. Nevertheless some evidence of autophagosome formation and content degradation independent of both proteins has emerged. Shigeomi Shimizu's team has reported the formation of double-membrane structures as well as LAMP-2 postive vesicules in MEFs lacking either ATG5 or ATG7. Those cells had been treated with etoposide, a cytotoxic stressor, or had been starved. Comparing etoposide treated cells, they observed accumulation of double-membrane structures at 12h and less LAMP-2 positive vesicules while at 18h it was the reversed situation suggesting the formation of autophagosomes that fuse with lysosomes to form autolysosomes. Rapamycin treatment however did not induce autophagy in the *Atg5*$^{-/-}$ MEFs, suggesting that non-canonical autophagy induction might not depend on the classical mTOR-related stimulus. Moreover, LC3-II seemed to be missing from these autophagic vesicles, while ULK1, FIP200, Belcin1 and Vps34 were shown to be important for the initiation of this alternative autophagic pathway. Silencing of *Atg12* and *Atg16L1* on the other hand didn't affect autophagosome generation, indicating that the ubiquitin-like conjugation systems might be dispensable. They also observed a colocalization of autolysosome marker LAMP-2 with trans-Golgi apparatus markers as well as Rab9, a GTPase implicated in late endosome to *trans*-Golgi trafficking indicating the involvement of all of these components in this pathway **(Nishida et al., 2009)**.

Beclin1-PI3K-independent

The Beclin1-PI3K complex in association with ATG14L is involved in autophagosome generation and regulates its maturation when bound to UVRAG or UVRAG-Rubicon. Nevertheless, it has been demonstrated that the autolysosome generation does not necessarily rely on this complex, depending on the stimulus used to trigger autophagy.

Using a SH-SY5Y neuronal cell line treated with a neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+, used to elicit mitochondria-targeted injury), Zu and colleagues noticed a strong increase in autophagic vacuole formation. But attempts to inhibit this process in those cells using pharmacological inhibitors of class III PI3K (wortmannin and 3-methyl-adenine) did not exhibit any effect on autophagosome

generation, suggesting the involvement of non-canonical autophagy in their formation. This was confirmed with Beclin1 silencing experiments. Indeed, the authors still observed the presence of autophagic vesicles after treatment with MPP^+**(Zhu et al., 2007)**.

Another case where Beclin1-independent autophagy was demonstrated, stood after treatment of a human breast cancer cell line with pro-apoptotic agents or Resveratrol (Res), in the hope to trigger autophagic cell death. Scarlatti and colleagues observed in Beclin 1 or VPS34 knocked-down cells an accumulation of LC3-II by western blot, upon treatment with Res as well as an increase in GFP-LC3 puncta. This was indicative that autophagy induction in this context was both Beclin1 and VPS34 independent. This process was however ATG7 dependent **(Scarlatti et al., 2008)**. Furthermore the use of another autophagy promoter molecule, C_2-ceramide required the expression of the three proteins demonstrating once more the non-canonical autophagy can be triggered by certain stimulus only.

In a recent and elegant study by Guido Kroemer's team, it was shown that fatty acids (FAs) were able to induce the formation of autophagosomes as well. Depending on the FAs saturation, the authors could either trigger canonical or non-canonical autophagy. They treated a human osteosarcoma cell line with palmitate (saturated) or oleate (unsaturated) and they noticed that while palmitate triggers the usual autophagic pathway, oleate activates an unconventional degradation process. Niso-Santano and colleagues observed that oleate-induced autophagy is Beclin1-independent and requires an intact Golgi-apparatus both *in vitro* and *in vivo* **(Niso-Santano et al., 2015)**. Nevertheless, the physiological relevance of this discovery as well as the detailed molecular mechanisms of this non-canonical autophagy still need to be addressed.

ULK1

The induction of autophagy in an ULK1-independent way has also been investigated. This phenomenon was observed in glucose-starved MEFs. There is indeed converging data demonstrating that autophagosome formation triggered through metabolic stress does not necessarily require AMPK activation nor mTOR inhibition. This suggests that non-canonical autophagy can be independent of the AMPK/mTOR/ULK1 axis **(Cheong et al., 2011)**.

Bcl-2/JNK regulated autophagy

Autophagy can in fact be regulated by elements other than AMPK and mTOR and in that regard can be considered as unconventional. As briefly discussed in **section 1.4.1** Bcl-2 negatively regulates autophagy by binding Beclin1. For a long time the exact mechanisms of this regulation were however poorly understood since most studies were mainly focused on the AMPK/mTOR autophagy regulation axis. Beth Levine's team was among the first to show that in starvation conditions Bcl-2 required

multiple phosphorylation by c-Jun N-terminal protein kinase 1 (JNK1) to dissociate from Beclin1 leading to autophagy activation **(Wei et al., 2008)**. This requirement was further confirmed by the fact that viral Bcl-2 (vBcl-2) from Kaposi's sarcoma associated herpesvirus (KSHV) lacks those phosphorylation sites. Hence vBcl-2 cannot be dissociated from Beclin1 and the virus escapes degradation by inhibiting autophagy **(E et al., 2009)**.

Shimizu and colleagues suggest however that JNK can only be activated to induce autophagic cell death but not in starvation conditions which would promote cell survival **(Shimizu et al., 2010)**.

LC3-associated phagocytosis

LC3-associated phagocytosis (LAP) is a non-canonical type of autophagy that has been shown to take place in macrophages, neutrophils and dendritic cells (DCs) **(Liu et al., 2015)**. This process participates in the elimination of extracellular bacteria, apoptotic bodies or immune complexes through phagocytosis. Same as for canonical autophagy, LAP requires the recruitment of Beclin 1, class III PI3K activity as well as the two ubiquitin-like conjugation systems since deletion of ATG7 and ATG5 haven been associated to reduced LAP. Contrary to canonical autophagy, LC3-II gets integrated into a single membrane vesicle and not in a double membrane. Moreover, LAP induction is independent from ULK1 complex activation.

This process is triggered by some pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs) or Dectin-1 (recognition of β-glucan on fungal walls). The signaling lymphocytic activation molecule family 1 (SLAM), a gram negative bacteria sensor has also been suggested to induce LAP, but the evidence linking this receptor to this process still need to be delivered. Nevertheless LAP is clearly involved in enhanced pathogen and dead cell clearance as well as in antigen presentation linking innate and adaptive immunity.

In fact Douglas Green's team demonstrated that phagocytosis of dead cells induces the recruitment of LC3 to the phagosomes and that the presence of that protein is important to accelerate phagosome maturation as well as the clearance of their cargo **(Martinez et al., 2011)**. LAP has also been shown to promote major histocompatibility complex (MHC) class II antigen presentation triggered by Dectin1 **(Ma et al., 2012) (reviewed by Mehta et al., 2014) (see section 3.2.1)**. Furthermore LAP seems to be important in mechanisms mediating the fusion of phagosomes and lysosomes in response to Fcγ-receptors (FcγRs) signaling. Those receptors are able to sense immune complexes (IC) and lead to a type I IFN response in a TLR9-dependent manner. Henault and colleagues observed indeed that in *Atg7*-deficient pDCs stimulated by DNA-IC, this process was impaired while in *Ulk1*-deficient pDCs, incapable of undergoing conventional autophagy but still competent for LAP, secretion of IFN-α could be induced. Same as for dead cell clearance, continuous DNA sensing has been shown to be one of the

features leading to autoimmunity, thus the studies mentioned above demonstrate that targeting LAP could possibly lead to improvement in autoimmune diseases such as SLE **(see section 4.3.1) (Henault et al., 2012)**.

Membrane re-organisation during bacterial replication

The nature of the proteins forming the autophagic membrane can change during the sequestration of a pathogen as it is the case in xenophagy **(see section 3.1.1)**. This feature was investigated during an infection by *Brucella abortus* a bacteria mediating brucellosis. Upon entry in the cell, the bacteria iniates the formation of a vacuole, the *Brucella*-containing vacuole (BCV), that travels in the early stages of infection to the ER where it starts its replication. Starr and colleagues discovered that after ER replication, the BCV displayed autophagic features such as a double membrane. These autophagic BCVs (aBCVs) were however not positive for LC3 but were revealed to depend on ULK1, Beclin1 and ATG14L, while independent of the two conjugation systems ATG5-ATG12/ATG16L1 and LC3-PE. The authors were able to show that *Brucella* requires a non-conventional autophagic machinery for completion of its replication cycle and cell to cell infection **(Starr et al., 2012)**.

1.4.3 Selective Autophagy

For many years after its discovery, autophagy was thought to be non-specific. It is true that when the autophagic membranes grow they can randomly enclose cytosolic material. It has however become evident that there is a specific targeting of some proteins, cell structures and organelles to the autophagosome. As already mentioned at the beginning of this chapter, studies on yeast have allowed to better understand the autophagic processes on a functional as well as molecular level. Selective autophagy makes no exception to that respect as it was first described in yeast under the form of the cytoplasm to vacuole targeting (Cvt) pathway. Cvt is responsible for the transport of the aggregated hydrolases like aminopeptidase I (ApI) from the cytosol to the yeast vacuole **(Lynch-Day and Klionsky, 2010)**. Thereby using the Cvt pathway as a model has contributed to important advances in the understanding of selective autophagy in mammalian cells.

1.4.3.1 Autophagy receptors and adaptor proteins

Selective autophagy has been shown to be driven by several receptors and adaptor proteins. In the recent years some of these proteins have been identified and studied in order to establish their exact functions. **(Fig 8)**

p62/SQSTM1

The first autophagic receptor to be described was the polyubiquitin-binding protein p62 also called sequestosome 1 (SQSTM1). It was shown to bind huntingtin (a protein involved in Huntington's disease development) aggregates, that can be degraded via autophagy **(Bjørkøy et al., 2005)**. It is now established that p62 possesses a C-terminal ubiquitin binding UBA (ubiquitin associated) domain. Moreover p62/SQSTM1 contains an LC3-interacting region (LIR), necessary to bind LC3 in a non-covalent manner and to mediate cargo degradation by autophagy **(Stolz et al., 2014)**. p62 is constitutively bound to LC3, thus associated to the membrane, and is continuously degraded in the autolysosome. Hence this protein gets used as a marker for autophagic flux. Its decrease is namely correlated to an enhanced autophagic activity while its increase indicates inhibited autophagic flux.

Since p62 recognizes ubiquitin (Ub) motifs, it has been suggested that it could be involved in pexophagy and mitophagy, specific degradation through autophagy of peroxisomes and mitochondria respectively. These mechanisms will be discussed later. As a matter of fact, peroxisomes are dependent on p62 for degradation while in mitochondria, it has been shown that their depolarization induces the ubiquitination of outer mitochondrial membrane (OMM) proteins like mitofusins or VDAC (voltage-dependent anion channel; **Rogov et al., 2014**).

HDAC6

Another protein mentioned before **(see section 1.4.1- Maturation)** that can be considered as an autophagy adaptor protein is HDAC6. HDAC6 preferentially recognizes K63-linked Ub motifs of aggregated proteins and mediates their dynein-transport along the microtubules to a structure called aggresome, that gets degraded by autophagy **(Kawaguchi et al., 2003)**. But contrary to p62 it doesn't possess a LIR domain. Hence it has been suggested that HDAC6 and p62 work in collaboration. HDAC6 delivers the substrates at the degradation site where p62 finishes "the job" by recruiting the autophagic machinery **(reviewed in Lamark et al., 2012)**.

NBR1

Neighbor of BRAC1 gene 1 (NBR1) shares structural and functional similarities with p62. It possesses both a UBA as well as a LIR domain and can also interact with p62 to form an oligomer. Hence NBR1 is

also involved in Ub-positive protein aggregate clearance through the autophagic machinery, in an LC3-dependent manner with or without being associated to p62 **(Kirkin et al., 2009)**. NBR1 has been identified as a receptor for the targeting of peroxisomes to the autophagosomes as well **(Deosaran et al., 2013)**.

Optineurin (OPTN)

OPTN is implicated in various cellular functions. This protein has indeed been identified among other functions, as a major regulator of membrane trafficking, protein secretion and cell division **(Kachaner et al., 2012)**. During autophagy, it has been observed to be able to bind, via its C-terminal domain, aggregated proteins both in an Ub-dependent and -independent manner **(Korac et al., 2013)**. Nevertheless, binding to LC3 via the LIR domain is required for cargo degradation in the autolysosome. Other than aggrephagy, OPTN is able to promote xenophagy, the degradation of microorganisms by autophagy. Philipp Wild and his colleagues showed that when phosphorylated by TANK binding kinase 1 (TBK1) OPTN binding to LC3 was enhanced and that it seemed to promote de degradation of Ub-coated *Salmonella enterica* **(Wild et al., 2011)**.

NDP52

Nuclear dot protein of 52KD (NDP52) is also a xenophagy receptor. One specific feature of this protein is its specific binding to LC3C through a non-canonical LIR motif **(von Muhlinen et al., 2012)**. The interaction of both proteins seems essential for anti-bacterial defense since their absence leads to a significant increase of *Salmonella thyphimurium* intracellular invasion **(Weidberg et al., 2010)**.

ALFY

Autophagy-linked FYVE protein is a scaffold protein binding ATG5, p62 and PI3P. Like several autophagy receptor/adaptor proteins discovered so far, ALFY is involved in aggrephagy through the recognition of Ub motifs of aggregated proteins. Actually it has been suggested that ALFY and p62 may be mediating the degradation of Ub misfolded nuclear proteins via autophagy. How exactly this phenomenon takes place is net fully understood yet **(Isakson et al., 2013)**.

The proteins listed above are only a few of the known autophagy-adaptors but the list has been expanding and others may be discovered in the next years. As pointed out above most of these proteins are involved in aggrephagy but can also mediate specific organelle or even pathogen degradation.

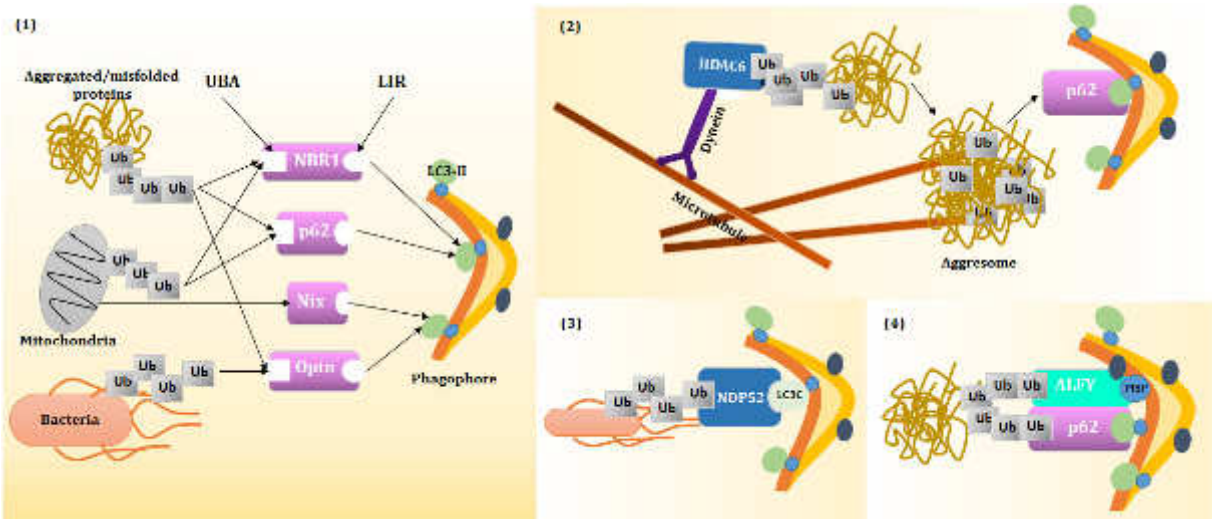

Figure 8: Schematic overview of autophagy receptor/adaptor protein and their targets.

(1) The autophagy receptors NBR1, p62, Nix and Optn with their ubiquitin associated domain (UBA, white square) bind ubiquitinylated targets and the LC3-interacting region (LIR, white circle) to tether the targets to LC3-II at the growing phagophore. **(2)** Sequential interaction of HDAC6 and p62. HDAC6 binds ubiquitinylated aggregates and delivers them at the aggresome and p62 mediates the delivery to the autophagosome for degradation. **(3)** NDP52 recognizes Ub^+ bacteria and binds specifically to LC3C. **(4)** ALFY binds Ub^+ aggregated proteins or misfolded proteins and interacts with the ATG5-ATG12/ATG16L1 conjugate by binding ATG5. ALFY also binds PI3P and p62.

1.4.3.2 Macromolecules and organelle specific degradation

Aggrephagy:

Aggrephagy is the specific degradation of aggregated proteins trough the autophagic pathway. In order for this process to take place, misfolded and/or dysfunctional proteins will associate to form a structure called aggresome. These proteins constituted into an aggresome are ubiquitinated. But so are the proteins destined for proteasome degradation, which is another Ub-degradation system in the cell. So what points a protein's fate to a lysosomal degradation via autophagy rather than to the ubiquitin proteasome degradation system (UPS)? The UPS specifically targets small and soluble polyubiquitinated proteins (linked together through the lysine at position 48 (K48)) while insoluble, unfolded, aggregated proteins will be directed to the autophagosome for degradation. This process can also take place when the UPS or even CMA are deficient, acting as a compensatory mechanism for cellular quality control **(Lamark and Johansen, 2010; Lamark et al., 2012).**

Aside ubiquitinated proteins, the aggresome is composed of intermediate filaments (vimentin or keratin) and has been shown to be localized in proximity of the MTOC. The aggresome formation also

requires a microtubule-dependent transport of protein aggregates which can be mediated either by HDAC6 as previously mentioned **(Johnston et al., 1998)**, by BCL2-associated athanogene 3 (BAG3) or by carboxy-terminus of Hsc70-interacting protein (CHIP) **(Sha et al., 2009)**. Both BAG3 and CHIP bind dynein as well as HSPA8 substrates in order to enable their transport to the aggresome. Contrary to HDAC6 substrates, targeting is ubiquitin-independent **(reviewed in Lamark et al., 2012)**. Nevertheless the type of proteins forming the aggresome seem determinant for its further degradation by autophagic machinery **(Wong et al., 2008)**.

Aggrephagy is, as already mentioned, mediated by adaptor proteins such as p62, NBR1, Optn or ALFY. Dysfunctions or mutations in receptor proteins mediating this process have been shown to lead to accumulation of protein aggregates and have thus been associated to several neurodegenerative diseases such as Huntington's disease, Parkinson's disease or Alzheimer's disease, where misfolded proteins like huntingtin, α-synuclein or ameloid β (Aβ) respectively form neurotoxic aggregates **(Nixon, 2013)**.

To sum up, aggrephagy is an important quality control mechanism allowing the elimination of protein aggregates. These aggregates can result from mutations or errors during translations. Other factors able to induce protein aggregation can be cell senescence, environmental triggers like temperature variations or oxidative stress resulting from reactive oxygen species (ROS) production that alter protein folding **(Drummond and Wilke, 2008)**. Even though it is not aggrephagy *per se,* long-lived protein turnover is also selectively mediated by the autophagic machinery in a Ub-p62 dependent mechanism **(Kim et al., 2008)**.

Mitophagy (**Fig 9**)*:*

Mitochondria produce a substantial part of the cell energy. They indeed participate in the generation of adenosine tri-phosphate (ATP) needed to insure proper cell function. In order to achieve ATP production, mitochondria need to breakdown carbohydrates and fatty acids in a process called oxidative phosphorylation. By-products of this process are ROS which are released into the cytosol. When in excess they can oxidize cellular elements leading to modifications in protein folding or induce DNA damages, resulting in oxidative stress subsequently leading to cell death **(Circu and Aw, 2010)**. Thus in order to maintain cellular homeostasis, dysfunctional or excess mitochondria need be removed from the cell. One way to eliminate those mitochondria is a selective autophagic pathway called mitophagy **(G Ashrafi, 2013)**.

This specific recognition of damaged mitochondria has been shown to be mediated by the PTEN-induced putative kinase 1 (PINK1)/E3 ubiquitin ligase Parkin pathway. Loss of mitochondrial membrane potential induces the recruitment of full-length PINK1 (64 kDa) to the inner mitochondrial membrane,

where it cannot be processed to its smaller form (52 kDa) by presenilin associated rhomboid-like (PARL) protease, as it happens in healthy mitochondria. PINK1 subsequently accumulates on the outer membrane and recruits Parkin from the cytosol to the mitochondrion **(Deas et al., 2011; Vives-Bauza et al., 2010) (Narendra et al., 2010)**. How exactly Parkin is recruited is however not very well understood yet. It was shown that after its relocalization to the mitochondrial membrane, it acquires its active conformation through phosphorylations by PINK1 **(Kane et al., 2014; Kondapalli et al., 2012)**, and induces the ubiquitination of mitochondrial proteins. Depending on the ubiquitination site and the polyubiquitin chains formed the entire mitochondria will be degraded via mitophagy. Mitophagy induction requires sequential steps starting with proteasomal degradation of small GTPases called mitofusins (MFN), necessary for mitochondrial fusion. Studies using Drosophila as a model, demonstrated that loss of MFN allows to single-out damaged mitochondria from the functional ones **(Poole et al., 2010; Ziviani et al., 2010)**. The second step consists in the ubiquitination of mitochondrial proteins by Parkin, preferentially linking the Ub moieties together through the lysines 6, 11 and 63 (K6, K11 and K63) **(Cunningham et al., 2015; Geisler et al., 2010)**. These posttranslational modifications seem particularly important for the translocase outer mitochondrial (TOM) proteins TOM20 and TOM70 as well as for the voltage-dependent anion channel 1 (VDAC1) to allow mitophagy. This was actually demonstrated for TOM20 in cultured cells treated with the respiratory chain uncoupling agent CCCP. In that case, downregulation of a deubiquitinase protein, USP30, conjugated to the overexpression of Parkin, resulted in increased ubiquitination. On the contrary, after overexpression of USP30, the reduction of ubiquitination was correlated with decreased mitophagy **(Bingol et al., 2014)**. Geisler and colleagues reported that Parkin recruits p62 as an adaptor protein for autophagy in a Ub-dependent manner **(Geisler et al., 2010)**. They also demonstrated the requirement of VDAC1 as a substrate for Parkin-mediated mitophagy through siARN knock-down of VDAC in CCCP treated HeLa cells which resulted in defective clearance of mitochondria.

One cell type particularly sensitive to mitochondrial clearance are erythrocytes. Mature erythrocytes are devoid of any kind of organelles, a phenotype they acquire in a 2-3 days maturation process during which the organelles are progressively removed. Mitochondrial clearance seems particularly important because of erythrocyte's function as oxygen carrier. In these conditions presence of mitochondria would lead to excessive oxidative stress and ROS production and finally to cell death. Consequently it has been observed that mitophagy is essential for erythrocyte development. The mitochondrial outer membrane protein Nix has emerged as a key player in that process. Nix deficient mice develop indeed anemia which has been shown to be associated to poor survival of mitochondria-loaded erythrocytes **(Schweers et al., 2007)**. Furthermore inhibiting autophagy in reticulocytes with pharmacological drugs results in mitochondrial accumulation and *Nix*$^{-/-}$ red blood cells form autophagosomes as

demonstrated by LC3 staining while co-staining of the mitochondrial cytochrome c oxidase complex IV (COX IV) does not show any co-localization contrary to what is observed in WT erythrocytes **(Sandoval et al., 2008)**. It appears that Nix interacts with LC3 through a LIR domain and acts as a receptor to target mitochondria to the autophagosome but the upstream events still need to be investigated **(G Ashrafi, 2013)**. Nevertheless it has been suggested that Nix-mediated mitophagy might not be restricted to erythrocytes only but may play a role in other cells as demonstrated by Wen-Xing Ding and colleagues who were able to induce Nix-dependent mitophagy in CCCP treated HeLa cells **(Ding et al., 2010)**.

All in all mitophagy mechanisms in most cells require PINK1/Parkin interaction, parkin-mediated polyubiquitination of mitochondrial proteins and especially of the TOM proteins and VDAC and finally the recruitment of p62 as an adaptor protein **(reviewed in Durcan and Fon, 2015)**. Furthermore it has been shown that the PINK1/Parkin pathway has a neuroprotective function and mutations in both proteins have been linked to Parkinson's disease a neurodegenerative disorder linking once more autophagy to neurodegeneration, subject that will be discussed in the second part of this introduction **(see section 2.1).**

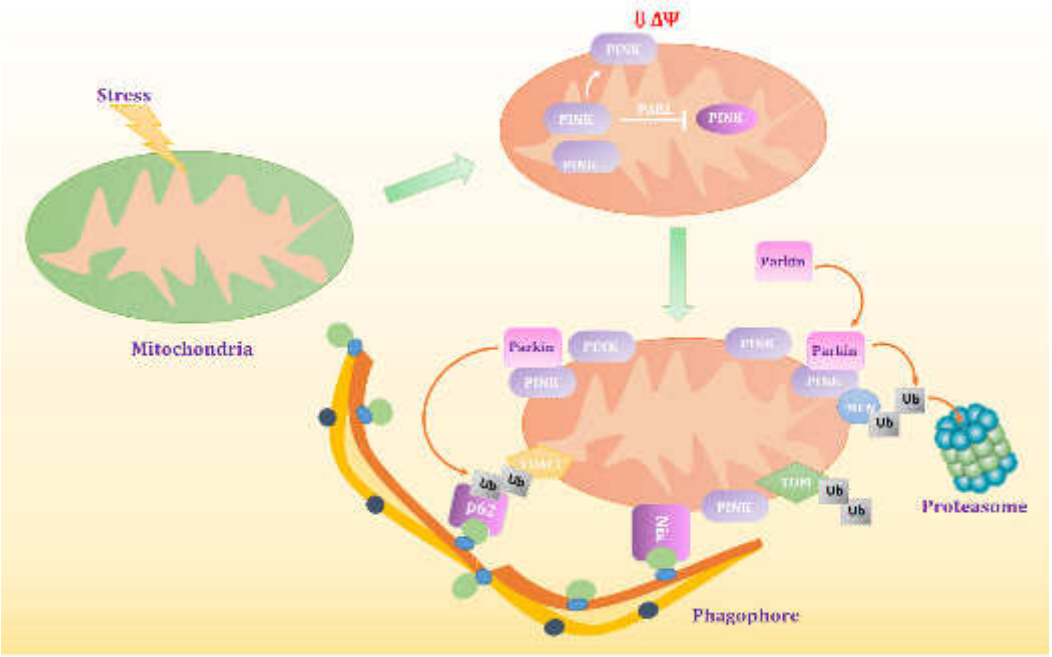

Figure 9: Regulation of mitophagy.

Damaged mitochondria undergo membrane depolarization. In these conditions PARL is not able to process PINK1 and the protein accumulates at the outer mitochondrial membrane. PINK1 recruits Parkin which mediates the ubiquitinilation of various proteins. The first proteins to be ubiquitinilated are the mitofusins. Their subsequent degradation by the proteasome constitutes a signal for mitophagy induction. Parkin further ubiquitinilates VDAC1 and the TOM proteins. Autophagy proteins such as p62 or Nix (in reticulocytes) get recruited to the mitochondria and mediate the interaction with the phagophores via LC3 leading in the end to the degradation of the mitochondria by the autophagic machinery. **PARL:** Presenilin associated rhomboid-like; **PINK1:** PTEN-induced putative kinase 1; **VDAC1:** Voltage-dependent anion channel**; TOM:** Translocase outer mitochondrial

Pexophagy

Peroxisomes share some similarities with mitochondria since they metabolize fatty acids in a β-oxidation reaction ending up in generating acetyl coenzyme A (CoA) and hydrogen peroxide (H_2O_2) respectively. But contrary to mitochondria they do not produce energy and they do not possess their own DNA either. Thus they have to import all the proteins needed for their proper function. They contain up to 50 different enzymes that mediate the oxidative reactions and are considered as detoxifying organelles. For mammals this function is particularly important in podocytes in the kidney where peroxisomes are responsible for clearing toxic elements from the bloodstream. They are also quite abundant in hepatocytes. Moreover peroxisomes are involved in the synthesis of plasmalogens, glycerophospholipids predominantly present in the myelin sheath of neurons **(Agrawal and Subramani, 2016)**.

In general, the number of peroxisomes increases in response to intra- and extracellular environmental changes. Hence it induces, same as for mitochondria, an increase of ROS production potentially toxic for the cell which is why in order to maintain cellular homeostasis, their number has to be regulated as well. As a matter of fact, the lack of peroxisome clearance has been associated to diseases like peroxisome biogenesis disorders (PBDs), cancer and Alzheimer's disease among others **(Fransen et al., 2012)**.

Hence it is now established that specific degradation of peroxisomes can be achieved through the autophagic pathway via a mechanism called pexophagy. In mammalian cells pexophagy has been shown to be regulated through the cellular damage sensor Ataxia-telangiectasia mutated (ATM), a kinase that activates TSC1/2 in response to elevated ROS, leading subsequently to an inhibition of mTOR and thus to the activation of autophagy **(Alexander et al., 2010)**. Moreover ATM and TSC1/2 have recently been identified as peroxisomal proteins **(Zhang et al., 2015)**. Using mostly HEK293 cells, Zhang and colleagues demonstrated indeed that ATM was recruited to the peroxysomal membrane by PEX5, a peroxisome import receptor, through direct binding. This leads to PEX5 ubiquitination by ATM, resulting in recognition by p62 through its UBA domain and thus targeting the peroxisomes to the autophagosome. Another autophagy adaptor protein, NBR1, has also been suggested to promote pexophagy in a p62-independent manner **(Deosaran et al., 2013)**.

Lipophagy

Lipids are for one part primary components of organelles and cell membranes, and secondly they are an essential energy source for the cell. Interestingly, a cell seems to be able to maintain the same lipid amount all throughout its life. Those lipid stores, also called lipid droplets (LDs) contain mainly triglycerides (TGs) and cholesterol. Energy requirements induce the hydrolysis of triglycerides into free

fatty acids (FFAs), a phenomenon further increased in starvation conditions. This conversion from TGs to FFAs was long thought to be uniquely mediated by cytosolic hydrolytic enzymes and lipases. The discovery of autophagy involvement in this process emerged only recently and was first observed in hepatocytes **(Singh et al., 2009)**.

Lipophagy, specific lysosomal degradation of lipids, has been shown to be associated to the maintenance of stable lipid stores **(Liu and Czaja, 2013)**. Both siRNA knock down of *Atg5* in hepatocytes as well as pharmacological inhibition of autophagy, were associated to an important increase in TGs and in the number of LDs. This could also be observed *in vivo,* in mice with a conditional knock-out of *Atg7* in hepatocytes. Interestingly those mice demonstrated also a colocalisation of the LDs and LC3-I in nutrient deprived hepatocytes linking further autophagy to lipid metabolism **(Singh et al., 2009)**. Even though there is clear evidence of lysosomal degradation of LDs, how exactly they are recognized by the autophagic machinery is not very well understood yet. Nevertheless it has been shown that SNAREs, primarily identified as mediators of LDs fusion **(Boström et al., 2007)**, also play a role in autophagosome/lysosome fusion **(see section1.4.1-Maturation) (Moreau et al., 2013)**, suggesting a possible link between both pathways.

Lipophagy seems particularly important in hepatocytes, stellate cells, macrophages, neurons and as such its deregulation could be involved in numerous disorders like human liver diseases (alcoholic liver disease, nonalcoholic fatty liver disease), atherosclerosis or even obesity **(reviewed by Liu and Czaja, 2013).**

Nucleophagy

Some components from the nucleus can also be degraded by autophagy. This process has mainly been described in yeast and is termed nucleophagy. It can be induced in case of shortage in amino acids or by other cell stress signals like DNA damage. Studies have shown that some perinuclear double-membrane vesicular structures observed in cells, are actually autophagosomes containing nuclear material **(Park et al., 2009)**. Moreover a recent study has demonstrated that keratinocytes undergo specific nucleophagy during their terminal differentiation in the epidermis, a phenomenon which was not observed in undifferentiated keratinocytes. The authors observed in fact double membrane structures by electron microscopy as well as colocalization of LC3, LAMP2 and p62 which was correlated with miss-shaped nuclei in the granular layer of human skin. They also noticed that autophagy was decreased in the keratinocytes of patients suffering from psoriasis, an inflammatory skin disease characterized by incomplete terminal skin cell differentiation since organelles including the nuclei aren't properly removed through-out the eperdemis establishment **(Akinduro et al. 2016)**.

Interestingly, a treatment activating autophagy, like Rapamycin (mTOR inhibitor) could have a beneficial effect in this kind of pathologies.

Even though p62 is shown to act as an adaptor protein for nuclear material recognition, the exact molecular mechanisms involved in nucleophagy are not quite clear. There is however some evidence implicating Atg39 as a nuclear envelope receptor capable of inducing nucleophagy in yeast, but no such demonstration was made in mammalian cells **(Mochida et al., 2015)**. Akinduro and colleagues propose that the nuclear material might be recognized because it is damaged, modified or bound to heterochromatin protein 1α, a marker of genetically inactive, tightly packed DNA. Another model that has been suggested, based on observation in fibroblast, is the direct recognition of nuclear membrane protein lamin B1 (LMNB1) by LC3.

Lysophagy

Same as the other organelles, lysosomes can be damaged (e.g. by silica, bacterial toxins or β-ameloid) which as a consequence leads to lysosomal membrane breaks and to the liberation of their content into the cytosol. Lysosomes contain many hydrolytic enzymes and among those the cathepsins. Their release in the cytosol can activate caspases and thus induce apoptosis. Another consequence of cathepsin cytosolic activity can also so be the activation of pro-inflammatory pathways (e.g. NLRP1 inflammasome). Hence the presence of free lysosomal content can be toxic for the cell and the surrounding tissues, which is why those damaged lysosomes need to be quickly removed. Lysophagy, the elimination of lysosomes by autophagy, is a process controlled by ubiquitin and involving p62 and LC3 for specific recognition. How the ubiquitination process occurs and which other ATG proteins are involved still needs to be addressed in future research **(Hasegawa et al., 2015; Maejima et al., 2013)**.

Reticulophagy

When unfolded or misfolded proteins accumulate in the ER, it leads to a stress response designated as the unfolded protein response (UPR). The UPR has been suggested to initiate selective ER degradation through the autophagic machinery in a process called reticulophagy. The link with UPR and autophagy was further strengthened by the fact that the C/EBP homologous protein (CHOP) and the activating transcription factor 4 (ATF4), activated as a consequence to ER stress, have also been shown to regulate *Atgs* **(Yorimitsu and Klionsky, 2007).** CHOP initiates *ATG5* transcription while ATF4 associates with the LC3 promotor region **(Rouschop et al., 2010)**. Moreover CHOP down-regulates Bcl-2 leading to ULK1 activation and subsequently to autophagy induction. While investigating the role of autophagy in T lymphocytes You-Wen He's team uncovered that invalidating *Atg7* specifically in T cells leads to ER expansion and to increased ER stress markers Grp78 and Grp94 leading to defective Ca^{2+} signaling.

Ca^{2+} signaling is indeed critical for these cells thus reticulophagy is essential for T cell homeostasis **(Jia et al., 2011)**.

Even though we will not discuss the degradation process of each organelle, it would appear that organellophagy is primordial for cell homeostasis and that autophagy might be specific for every single organelle. However other than the organellophagies discussed above, ribosome degradation termed ribophagy, is the only other organelle specific turnover event that has been described so far **(Cebollero et al., 2012)**.

One main feature of specific organelle and macromolecule degradation through autophagy that should be kept in mind is the requirement of Ub and adaptor proteins in order to initiate the process. But ATG proteins and molecular mechanisms might differ slightly depending on the autophagic substrate in question. Moreover it appears that any dysregulation in this process tends to lead to disruption of cellular homeostasis and is subsequently likned to the development of various diseases like neurodegenerative, metabolic or inflammatory disorders.

1.4.4 Autophagy-independent roles of *Atgs*

As already mentioned over 30 *Atg* genes have been identified so far and it has been established that most of them are essential for autophagic functions. Some of them are however involved in other pathways and functions. Thereby we will discuss in this part autophagy-independent roles of some autophagy key player proteins. **(Table 1)**

LC3

LC3 plays a lead role in autophagy and has been shown to interact via its LIR domain with over 65 different proteins some of which are not necessarily implicated in the autophagic pathway. This suggests that LC3 is involved in many other cellular functions.

Some of the candidate proteins interacting with LC3 are small GTPases. These enzymes are guanine binding proteins divided into five families (Rho, Ran, Ras, Arf and Rab) that are present in the cell in an active and inactive form (GTP and GDP-bound respectively). They are essential for various cellular functions given that they regulate, among other things, gene expression, cell proliferation or cytoskeleton reorganization. Some of the LC3-interacting GTPases are clearly involved in autophagy regulation (Rab7, Rab11) **(Bento et al., 2013)**. LC3 interacts trough the LIR with the TBC (Tre2, Bud2 and Cdc16) domain-containing protein family which are Rab-GTPase inhibitors involved in membrane trafficking. They possess a Rab-GTPase-activating protein (GAP) activity which inhances GTP hydrolysis

and is thus responsible for the inactivation of Rabs **(Subramani and Malhotra, 2013)**. Popovic and colleagues idenfied through a screen that 14 TBC proteins are able to interact with LC3. One of these proteins TBC1D5 had even two LIR motifs and was shown to interact in a LIR-dependent mechanism with Vps29, an endosomal membrane protein associated to the retromer complex. This interaction is necessary in order to mediate retromer transport of transmembrane receptors from the endosome to the *trans*-Golgi network **(Popovic et al., 2012)**. LC3 binding to TBC1D5 displaces however Vps29 which redirects the membrane trafficking from the endosome to the autophagosome suggesting that LC3 has the ability to reprogram membrane trafficking pathways.

The lipid-free LC3 form has been shown to be involved in viral replication. As a matter of fact, in a study by Reggiori and colleagues, LC3 was found to be associated to the double-membrane vesicles generated by coronaviruses and necessary for their replication. They noticed indeed that downregulation of LC3, but not autophagy inhibition through *Atg7* knock-out, had a negative impact on coronavirus infectious capacities, suggesting an autophagy-independent role of LC3 in promoting viral replication **(Reggiori et al., 2010***)*.

Another autophagy-independent function of LC3 is the regulation of cytoskeleton remodeling through the interaction with a guanine nucleotide exchange factor called AKAP-Lbc (A kinase anchoring protein, AKAP). In this process, AKAP-Lbc is responsible for RhoA activation and thus promotes RhoA-dependent cytoskeleton reorganization. LC3 has been identified as a negative regulator of this mechanism and that through the binding to AKAP-Lbc which keeps this protein in an inactive state **(Baisamy et al., 2009)**.

LC3 as well as other ATG proteins (ATG5, ATG4B, ATG7) are essential for polarized lysosomal secretion in osteoclasts. This mechanism is important for bone resorption since it mediates polarized release of the digestive enzyme cathepsin K (CatK) **(DeSelm et al., 2011)**. In this situation ATG5 and ATG7 are required for the orientation of lysosomal secretion. LC3 on the other hand is localized to the plasma membrane probably to mediate the fusion with the lysosomes. But the exact mechanisms involved in the fusion process and the precise role of LC3 still need to be addressed. It has been hypothesized that SNARE proteins such as the vesicle-associated membrane protein 7 (VAMP7), already known to mediate fusion processes in lysosomal exocytosis, might also be involved **(Moreau et al., 2013)**.

There is some evidence of LC3 and ATG2A involvement in lipid droplets (LD) generation in an autophagy-independent manner. Co-staininig of LC3 and lipids in PC12 cell line revealed a co-localization. Moreover LC3 silencing resulted in defective LDs generation. LC3 localizes at the membrane of the LDs and is dependent on the LC3-PE conjugation system **(Shibata et al., 2010)**. As for ATG2A usually associated in a complex with ATG18 localized to the autophagic membrane, it is also

recruited to LDs and seems required to maintain LD morphology. ATG2A silencing leads in fact to the accumulation of enlarged LDs **(Velikkakath et al., 2012)**.

ATG5-ATG12

The ATG5-ATG12 conjugate has been shown to be implicated in other cellular processes than autophagy. It has indeed been identified as a suppressor of innate antiviral immune signaling. As a matter of fact the ATG5-ATG12 conjugate is able to bind two pattern recognition receptors (PRRs) involved in viral recognition, retinoic acid inducible gene 1 (RIG1), melanoma differentiation associated gene 5 (MDA5) and interferon-beta promoter stimulator 1 (IPS-1). These receptors sense double-stranded or 5'-phosphorylated immunostimulatory RNA (isRNA) from RNA viruses, like vesicular stomatis virus (VSV) or influenza virus. They trigger a signaling cascade through their caspase recruitment domains (CARDs) that leads to a strong type 1 interferon (IFN) response supposed to inhibit viral replication. ATG5-ATG12 conjugate is able to directly interact with the CARD domain and inhibits the type I IFN response. Thus this conjugate favors viral replication which makes it an evasion tool for some viruses. Since ATG5-ATG12 binds RIG1, MDA5 and IPS-1 also in absence of any virus, it has been suggested that it might act as a break that prevents unnecessary IFN production and as such helps to maintain cellular homeostasis **(Jounai et al., 2007; Subramani and Malhotra, 2013; Takeshita et al., 2008).**

ATG5 has been shown to be involved in IFNγ-induced cell death. This phenomenon is actually mediated by ATG5 interaction with the Fas-associated death domain (FADD). ATG5-FADD association triggers the formation of a death-inducing signaling complex (DISC) which initiate the signaling cascade resulting in cell death. HeLa cells transfected with a GFP-LC3 fusion protein displayed LC3 dots when treated with IFNγ which was not the case when ATG5 expression was down-regulated. Furthermore cells transfected with a truncated form of ATG5, unable to interact with ATG12, were resistant to IFNγ-induced cell death as well, suggesting that the autophagic machinery is required. This led to the conclusion that ATG5-FADD interaction actually induces autophagic cell death **(Pyo et al., 2005).**

ATG16L1

ATG16L1 has been shown to regulate an unconventional secretion pathway in neuroendocrine PC12 cell line, commonly used as a hormone secretion model. It appeared that ATG16L was localized at the neurites of these cells and that in interaction with Rab33a, a small GTPase, they allow neuropeptide Y secretion **(Ishibashi et al., 2012).**

ATG16L1 is also able to regulate NOD1 and NOD2 driven cytokine response independent of the autophagic machinery. It has been shown that ATG16L interacts directly with both PRRs and negatively

regulates their response to intracellur pathogens such as *Listeria* or *Shigella* by inhibiting activation of the adaptor protein RIP2 required for mediating NOD signaling. This further inhibits NF-kB signaling and thus inflammatory cytokine production. This mechanism is autophagy-independent as demonstrated by the fact that *Atg16l1* knock-down but not that of *Atg5* resulted in increased pro-inflammatory cytokine production such as CXCL-1 or IL-8 upon bacterial infection of MEFs or epithelial cells. Furthermore truncated form of ATG16L1 lacking the ATG5 –interacting N-terminal region still had the capacity to regulate NOD1-NOD2 signaling axis suggesting that autophagy has no role to play in this specific process. The ATG16L1^{T300A} variant associated to Crohn's disease has been shown to be unable to interact with NOD1 and NOD2 which partly explains the exacerbated inflammatory phenotype **(discussed in section 4.2.1) (Sorbara et al., 2013)**.

Table 1: Autophagy-independent roles of *Atgs*

LC3	ATG5-ATG12	ATG16L1
→ **Cell proliferation, cytoskeleton remodeling** → Interaction with small GTPases → Interaction with AKAP-Lbc **(Baisamy et al., 2009)** → **Trafficking reprogramming** → Interaction withTBC15 **(Popovic et al., 2012)** → **Promoting viral infection** → Coronavirus **(Reggiori et al., 2010)** → **Lysosomal secretion in osteoclasts** → Cathepsin K secretion **(Moreau et al., 2013)**	→ **Regulation of RIG-1, MDA5, IPS-1 mediated type I IFN response** → Interaction with CARD domain **(Jounai et al., 2007)** → **IFNγ-induced cell death** → ATG5 interaction with FADD **(Pyo et al., 2005)** → **Lysosomal secretion in osteoclasts** → Cathepsin K secretion **(Moreau et al., 2013)**	→ **Regulation of neuropeptide secretion** → Interaction with Rab33a **(Ishibashi et al., 2012)** → **Regulation of NOD1/NOD2 mediated cytokine response** → Direct interaction with NOD1 and NOD2 **(Sorbara et al., 2013)**

2 AUTOPHAGY IN HEALTH AND DISEASE

With the growing knowledge about autophagy that has led to identifying the importance of this process in cellular homeostasis, it is not surprising that its dysregulation has been associated to various diseases, which will be discussed in details here.

2.1 Autophagy in Neurodegenerative Diseases

Neurons are long-lived cells subjected to important trafficking activities and thus displaying a high metabolic activity in order to generate the energy intended for this purpose in form of ATP. Neuronal proteins are moreover exposed to extensive stress which is why maintenance of efficient quality control mechanisms is essential. One of these mechanisms is autophagy.

Studies aiming at understanding the various causes of neurodegeneration have indeed shown that life span of neurons is strongly dependent on autophagy. As a matter of fact, the link between autophagy and neurodegenerative diseases was established based on various observations. Cataldo and colleagues noticed lysosomal abnormalities in brain tissues of early onset Alzheimer's disease patients prior to other degenerative signs like brain atrophy for instance **(Cataldo et al., 1994)**. Furthermore neurons of patients suffering from a neurodegerative disease were often shown to display important accumulations of protein aggregates **(Ross and Poirier, 2004)**. Specific deletion of autophagy in neurons under the control of the nestin promoter to drive cre expression, confirmed these observations. Mice affected by the deletion exhibited a neurodegenerative phenotype characterized by defects in movement coordination, reduced reflexes and loss of neuronal cells as well as overall decreased survival of the mice **(Hara et al., 2006; Komatsu et al., 2006)**. Given these discoveries, research in this field has been focused on studying the different neurodegenerative diseases in order to establish clearly the various neuronal defects in regard to autophagy. A better understanding of the role of the autophagic pathway in neurons could indeed help design new therapeutic targets for diseases like amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's diseases (AD) and Huntington's disease (HD). The advances made so far in some of these disorders affecting the nervous system will be discussed below.

2.1.1 Parkinson's disease (PD)

PD is a central nervous system disorder affecting the capacity to control movements as a result of an important loss of dopamine-producing neurons of the sustancia negra. The first therapies were in fact centered on treating patients with dopamine agonists in order to overcome this defect. However, since PD is a chronic disease that worsens with age at some point those therapies were not efficient anymore. Thus the necessity to find other alternatives for treatment became evident **(Savitt et al., 2006)**. Other features of this disease have indeed emerged from intensified investigations on the cellular and molecular level. Some of them led to the identification of Lewy bodies, neuronal cytoplasmic inclusions mainly containing alpha-synuclein aggregates. This protein was found in excess during PD, which seems to strongly contribute to disease development and aggravation. Consequently the accumulation of alpha-synuclein has been associated to autophagy inhibition. Alpha-synuclein inhibits Rab1 resulting in subsequent aberrant localization of ATG9, impairing phagophore formation **(Winslow et al., 2010)**. Hence the aggregation of alpha-synuclein becomes toxic for the neurons. Genetic modifications like mutations, gene duplications leading to changes in functions or misfolding of this protein, are tightly linked to PD development. Loss-of-function mutations in genes coding for PINK1 and Parkin, two proteins crucial for autophagy-mediated mitochondrial turnover, have also been linked to PD, and in particular during juvenile forms of the disease **(Geisler et al., 2010) (Kitada et al., 1998; Valente et al., 2004)**. Moreover Parkin is involved in alpha synuclein clearance through the autophagic machinery.

Other mutations in genes coding for VPS35 and Wiskott-Aldrich syndrome protein (WASP) involved in endosome recruitment also link autophagy and PD development. VPS35 is a large complex responsible for retrograde transport of membranes from the endosomes to the Golgi apparatus, which is why it is also called retromer complex. WASP interacts with VPS35 and thus mediates indirectly endosomal protein trafficking. Hence VPS35 mutations result in the impairment of this cellular activity. One such mutation has been associated to PD and has allowed to uncover a role for WASP in autophagosome formation since PD patients with a mutated allele of *VPS35* displayed deregulated autophagy. Moreover transfection of HeLa cells with the mutated allele and the depletion of WASP in these cell resulted in abnormal trafficking of ATG9 leading to autophagy inhibition **(Zavodszky et al., 2014)**.

The leucine rich repeat kinase 2 (LRRK2) whose mutated forms are very often linked to an autosomal-dominant inheritance of PD **(Singleton et al., 2013),** seems to be involved in autophagy regulation. This protein possesses a GTPase and kinase domain but its cellular function remains hazy. One study suggested an interaction of LRRK2 with Parkin, which seemed to induce an increase in ubiquitinated aggragates in the cytoplasm. They didn't however discuss a possible impact on other forms of

autophagy **(Smith et al., 2005)**. On the other hand, a more recent study showed that LRRK2 interacts with Rab7L1, a homolog of Rab7 suggesting a role of this protein in autophagosome maturation **(Beilina et al., 2014)**.

2.1.2 Alzheimer's disease (AD)

Alzheimer's disease, same as Parkinson's disease is a late-onset chronic and progressive neurodegenerative disorder. AD progression results in cognitive impairments like dementia and massive memory loss. A characteristic feature of AD is the formation of extracellular amyloid plaques in the brain, as a result of both an accumulation of amyloid-β (Aβ) and hyperphosphorylated tau protein deposits, forming tangles due to protein misfolding. Consequently the neurons die and the brain shrinks.

The involvement of autophagy in AD was suspected after transmission electron microscope observations of neurons from AD patients showed that compared to healthy controls their number of autophagosomes was increased. It was first thought that this was due to increased autophagic activity but it proved to actually be the result of impaired autophagic flux. As a matter of fact Ralph Nixon's team showed recently that a defect in lysosomal acidification caused by presenilin 1 (PS1) mutations was behind this accumulation of autophagosomes **(Wolfe et al., 2013)**. PS1 is indeed part of a complex involved in Aβ generation. Its other function, namely the involvement in lysosome acidification, is however the source of the observed autophagosome accumulation. Mutated PS1 loses its capacity to participate in the N-glycosylation of a subunit of the lysosomal vacuolar H^+-ATPase (v-ATPase). As a consequence the lysosomes are poorly acidified and as such are not functional and thus cannot participate in autophagosome cargo degradation **(Cataldo et al., 2004).** Additionally Aβ accumulation has been associated to another gene essential for autophagy, Beclin1. Pickford and colleagues were able to show that Beclin1 expression was decreased in brains of AD patients and that down regulation of Beclin1 in a mouse model for AD resulted in accumulation of Aβ in neuronal cells **(Pickford et al., 2008)**.

It has been recently suggested that autophagy is involved in Aβ secretion. It appears that specific ATG7 knock-out in neurons results in accumulation of Aβ as a consequence of a secretion defect. However, neurons recover their secretory capacity through a rescue of ATG7 expression by lentivirus introduction, confirming a central role for autophagy in Aβ secretion pathway **(Nilsson et al., 2013)**. This decrease in Beclin1 was observed in non-neuronal cells as well in AD patients.

All in all, it appears the autophagy plays a central role in AD pathogenesis. In addition, treatment of AD mice models with rapamycin, a known autophagy activator, resulted in Aβ clearance and in improved cognitive function. Thus modulating autophagy could be an interesting target for treating AD patients.

2.2 Autophagy and Cancer: A double edged sword

Since the discovery of autophagy in the 60ties, this mechanism has mostly been associated to survival mechanism. Cancer research has revealed however that autophagy plays quite a confusing role is this field. While in neurodegenerative diseases decreased autophagy clearly plays a role in their aggravation, it appears that in cancer it can promote both tumor survival and death. The conditions potentially leading to one or the other situation are variable and extremely context dependent.

2.2.1 Autophagy and cancer cell suppression

At steady state, autophagy has been proposed to be involved in tumor suppression by regulating the presence of oncogenic proteins in the cell and by eliminating cellular stress-inducing elements such as dysfunctional organelles or misfolded proteins. Thus by regulating production of ROS and occurrence of inflammatory events, autophagy could prevent the apparition of oncogenic mutations **(Cenci, 2014; Maiuri et al., 2008)**.

The description of autophagy as a tumor suppressor results from findings indicating that in some cancers such as breast, ovarian or even prostate cancer, Beclin1 was under-expressed. In breast cancer for example, deletion of the gene coding for breast cancer 1 (BRCA1) is, as the name of the protein indicates, associated to tumor development. It turns out that *Beclin1* and *BRCA1* are localized in close proximity on the same chromosome. Furthermore, the *Beclin 1* gene locus is localized into a region that has been shown to be frequently monoallelicaly deleted (40-70%) in human breast, ovarian and prostate cancer thus *Beclin1* is considered as a haploinsufficient tumor suppressor gene. As a matter of fact heterozygous deletion of Beclin1 in a mouse model leads to tumorigenesis in some organs like the lungs, the liver or the lymph nodes **(Qu et al., 2003)**. The fact that Bcl2 has been shown to be overexpressed in breast cancer and to confer a resistance to some anticancer drugs is another hint suggesting that loss of Beclin1 mighty be involved in human cancer pathophysiology **(Yip and Reed, 2008)**. Whether loss or downregulation of Beclin1 is related to autophagy has been addressed in some studies and since autophagy was decreased it has been suggested that this might be in favor of

carcinogenesis. Moreover the binding of Bcl-2 to Beclin 1 inhibits autophagy and on the other hand, Bcl-2 knockdown has been associated to autophagic cell death induction in leukemia and breast cancer cell line, suggesting once again a positive regulation of autophagy in tumor suppression **(Akar et al., 2008)**. However in some malignant cancers like colorectal cancer or gastric epithelial cancer, Beclin1 appeared to be mostly upregulated **(Ahn et al., 2007)**. Hence it is difficult to make a definite conclusion on whether Beclin1 is a tumor suppressor or not but as often seen in cancer this might be context dependent. In Takamura and colleagues' study for instance mosaic deletion of Atg5 in mice resulted in development of liver-localized benign tumors even though the deletion affected other organs like the hart, skeletal muscels or the brain **(Takamura et al., 2011).** Since in the same study they made similar observations in Atg7 deficient mice (a mosaic deletion as well) it can be speculated that autophagy plays indeed a tumor suppressor role. Because the tumors were begnin it suggests that autophagy might be required as a protective mechanism against the establishment of newly formed tumors. In regard to the results obtained in Beclin1 deficient mice it can be speculated that tumor formation observed in some organs might be linked to a Beclin 1 deficiency but not necessarly to an autophagy dependent mechanism since in Atg5/Atg7 mosaic deletion the tumours were localized in a specific organ.

There are other elements pinpointing towards an involvement of autophagy in tumor cell suppression. Some of the proteins participating in autophagy induction through inhibition of mTOR signaling like TSC1/2 have been described as tumor suppressor genes. On the other hand, PI3K and AKT which activate mTOR leading to autophagy inhibition, promote cancer development **(reviewed by Levine and Kroemer, 2008)**. Besides p53, the tumor suppressor gene mutated in most cancers also positevely regulates autophagic cell death **(Levine and Abrams, 2008)**. Furthermore some molecules used for cancer treatment have been shown to induce autophagy. One such example is a small molecule (STP-62247) used to treat renal cell carcinoma **(Turcotte et al., 2008)**.

2.2.2 Autophagy as a pro-survival pathway for cancer cells

Cancer cells require high metabolic demands that the micro-environment they evolve in does not necessarily provide. Thus some tumour cells have been shown to be strongly dependent on autophagy for their maintenance and growth. As a matter of fact impairment in autophagy seems to have a negative impact on tumor development **(Degenhardt et al., 2006)**. Guo and colleagues demonstrated that *Atg7* deletion in a mouse model for non-small-cell lung cancer (NSCLC) tumor progression was abrogated. It appears that autophagy deficiency led to the accumulation of defective mitochondria

which led to the activation of p53-induced cell death of the tumor cells **(Guo et al., 2013)**. Combined deletion of p53 and *Atg7* in the same model did not lead to resurgence of tumors in the lungs suggesting that there is another p53-independent pathway that still inhibits tumor formation. Another model of combined deletion of *Atg7* and Nerf2 confirmed the requirement of mitophagy to promote tumorigenesis by regulating oxidative stress. Nerf2 has a cytoprotective role during hypoxia and its deletion combined to *Atg7* deficiency did not show any cumulative effect **(Strohecker et al., 2013; reviewed in White, 2015)**.

Some cancer treatments have been shown to be inefficient either because the tumor cells are not sensitive to the drug or because they develop a resistance. In some cases this resistance has been attributed to an increased autophagy also because some of those drug inhibit mTOR. Another illustrating example is the ATP-competitive inhibitor of mTOR kinase activity AZD8055 used in clinical trial for colorectal cancer treatment. This molecule has been shown to induce autophagy as a cytoprotective mechanisms in a colon cancer cell line **(Huang et al., 2011)**.

Seeing the role of autophagy in tumor maintenance and chemotherapy resistance, inhibiting this process has been considered for therapeutic purposes **(reviewed in Yang et al., 2011)**. As a matter of fact drugs like chloroquine or hydroxychloroquine that have been shown to be potent autophagy inhibitors are currently in clinical trials as anticancer treatments for renal cell carcinoma, pancreatic cancer and breast cancer among many other cancers and preliminary results appear promising **(Sui et al., 2013)**.

Thus how the cancer cell uses autophagy seems to be dependent on the type of tumor in question but seems to be regulated in time and space. The general idea is indeed that triggering autophagy in early stages of tumor establishment would be beneficial to induce tumor suppression while in late stage cancers it could have the reversed effect.

2.3 Autophagy and Aging

Cellular aging is reflected by impaired cellular functions and an increased susceptibility to cell apoptosis. These cellular disfunctions are the result of a number of different parameters such as progressive genomic instability, telomere shortening, increased mitochondrial dysfunction, senescence and cell exhaustion among other factors **(López-Otín et al., 2013)**.

On the level of the entire mammalian organism, aging has been associated to the development of neurodegenerative pathologies, metabolic diseases and cancer. As mentioned previously these

disorders have also been linked to dysfunctions of the autophagic machinery. This process has indeed been shown to decrease with age in some tissues such as the brain or the skeletal muscle **(Wohlgemuth et al., 2010)**. A calorie restricted diet as well as drugs known to induce autophagy like spermidine or rapamycin were able to dial back or delay some symptoms in age-related pathologies and to prolonge life in some model organisms ranging from *C. elegans* to primates **(Cuervo, 2008; Harris and Rubinsztein, 2012)**. Loss of function mutation of *bec-1* (homologue of Beclin1) in *C. elegans* for example led to premature aging. That was also the case when other *Atg* genes such as *atg18, Unc-51 (ULK1/2) or atg9* were mutated suggesting that autophagy is required for longevity **(Tóth et al., 2008)**.

In some tissues however autophagy was found to be increased. Gamerdinger and colleagues found in fact that LC3-II was augemented in a cell model of aging, the I90 cells, as demonstrated by western blot assays. These cells start displaying characteristics of aging after a limited number of divisions. They become senescent and express the aging marker Caveolin. This autophagic increase has been associated to the co-chaperone protein Bcl2-associated athanogene (BAG3). The BAG proteins are involved in protein quality control mediated by the proteasome. In I90 cells BAG3 was increased while the expression of another co-chaperone BAG1 was diminished. Furthermore BAG3 knock-down was correlated to the decrease of LC3-II. The authors discovered that BAG1 is replaced by BAG3 in aging cells and that BAG3 interacts with p62 to mediate the degradation of accumulating poly-ubiquitinilated proteins by the autophagic machinery. It appears that with age, the proteasomal efficacy might be decreasing, leading to the autophagy machinery to take-over protein degradation **(Gamerdinger et al., 2009)**. The Hutchinson-Gilford progeria mouse model for premature aging was also shown to have high basal autophagy **(Carroll et al., 2013)**.

Most evidence so far points towards a protective role of autophagy in aging tissues. Thus autophagy seems to mediate prolonged lifespan by contributing to protein quality control, by eliminating apoptotic bodies and toxins, in short by maintaining cellular homeostasis. The observed decrease of this process during aging seems to be correlated to a reduced sensitivity of metabolic sensors (AMPK) to external and internal stimuli. Furthermore inflammatory events become more frequent with age and lead to NF-kB activation which has been shown to be inversely correlated to AMPK activation. This suggests that finding strategies to maintain autophagy throughout age could contribute to the development of new therapies for the treatment of age-related pathologies such as neurodegeneration or metabolic disorders. This is indeed an interesting area in autophagy research that still requires to be investigated deeper.

3 AUTOPHAGY AND IMMUNITY

Autophagy plays an undeniable and essential role in cellular homeostasis. Studying this process in different tissues and conditions has allowed to see that it has varied functions depending of the system in question. Thereby in innate and adaptive immunity, autophagy has emerged as a key player in various mechanisms such as the regulation of pathogen invasion, inflammation and antigen presentation.

3.1 Involvement of Autophagy in Innate Immune responses

3.1.1 Autophagy in Pathogen clearance (Xenophagy)

The clearance of pathogens (viruses, bacteria, parasites) through autophagy is termed xenophagy and belongs to the selective forms of autophagy. This process has been shown to be involved in the degradation of *Streptococcus pyogenes* also known as group A *Streptococcus* (GAS). Infection of HeLa cells by GAS leads to endocytosis of the bacteria which ends up in the cytosol in LC3 positive compartments. Nakagawa and colleagues named these compartments GcAVs, for GAS-containing LC3-positive autophagosome like vacuoles. They showed that the GcAVs fuse with lysosomes around 4 hours after infection which was correlated with intracellular decrease of GAS suggesting the involvement of autophagy in *Streptococcus* clearance **(Fig 10)**. Furthermore in ATG5 deficient embryonic stem cells (ES cells) and MEFS infected by GAS there was no detection of those GcAVs and inhibiting lysosomal proteases resulted in an increase of intracellular GAS **(Nakagawa et al., 2004)**. The study of *Salmonella Typhimurium* targeting by the autophagic machinery, has also allowed to shed some light on the mechanisms of xenophagy and to identify molecular actors involved. It actually appears that this mechanism is mediated by autophagy receptors such as NDP52, OPTN or ALFY belonging to the SQTSM1/p62-like receptors (SLRs). In case of *S. Typhimurium,* found in a vacuole termed the *Salmonella*-containing vacuole (SCV) after infecting the cell, autophagy has been suggested to target damaged SCVs and to inhibit their growth **(Birmingham et al., 2006)**. The damaged SVCs seem to be able to target signature molecules like ubiquitin that would then attract p62 which subsequently binds LC3, thus directing the SCVs to the autophagosome **(Zheng et al., 2009)**. Other adaptor proteins have been shown to be involved in this process. NDP52 for example is recruited by galectin 8 (Gal8), a β-galactoside binding lectin, which recognizes β-galactosides exposed on the membrane of damaged SCVs, and thus targets the bacterium to the autophagosome **(Thurston et al., 2012) (Fig 10)**. OPTN on

the other hand requires its phosphorylation (on Ser^{177} in human, Ser^{187} in mouse) by TBK1 in order to be recruited to ubiquitinated *Salmonella* **(Wild et al., 2011)**. Consequently ubiquitin as well as carbohydrate signals seem to be important to mediate xenophagy. Nevertheless some questions remain and still need to be addressed. It is for example not well understood yet if the different SLRs get recruited at the same time to the bacteria. Other bacteria like *Mycobacterium tuberculosis* (Mtb) **(Gutierrez et al., 2004)**, *Shigella flexneri* **(Ogawa et al., 2005)** and *Listeria monocytogenes* **(Py et al., 2007)** have been shown to be targeted by autophagy in the early phases of infection. In the case of Mtb the causative agent of tuberculosis, autophagy is required to counterbalance the capacity of this intracellular bacteria to evade degradation in macrophages by inhibiting phagosome/lysosome fusion. Using the zebrafish as a model van der Vaart and collegues discovered that DNA-damage-regulated autophagy modulator 1 (DRAM1) was actually required in this process. DRAM1 is a lysosomal protein that has been shown to interact with p53 to mediate autophagy and cell death **(Crighton et al., 2006)**. They uncovered that upon bacterial infection DRAM1 was upregulated in a myeloid differentiation primary response 88 (Myd88)/NF-kB dependent mechanism leading to the production of pro-inflammatory cytokine IL-1β. The increase of DRAM1 was correlated to an augmented expression of p62. Genetic invalidation using morpholinos of either proteins resulted in increased bacterial load leading to the bursting of infected macrophages while in WT macrophages the authors observed the formation of membranes around the bacteria and reduction of bacterial load. All of this suggesting an interplay of DRAM1 and p62 to mediate selective sequestration of mycobacteria in autophagosomes and their subsequent degradation **(van der Vaart et al., 2014)**. Further investigations showed that Mtb needs to be ubiquitinilated through the STING-pathway in order to be targeted to the autophagosome. Furthermore DRAM1 accelarates the fusion of lysosomes and autophagosomes leading to an increased autophagic flux.
All in all, autophagy is able to trap pathogen either directly in the cytosol or in vacuoles to mediate their degradation or inhibit bacterial growth.

Other pathogens however use this mechanism to their advantage and avert the autophagic machinery to enable their maintenance in the cell. As already mentioned *S. Typhimirium's* growth is inhibited by autophagy just after infection (infection + 1h) but it has been shown that 4 hours post infection, autophagy gets inhibited and thus the bacteria escapes degradation. Even though the mechanism involved in that case is not very well understood, it has been proposed that SPI-2 T3SS (Salmonella pathogenicity island 2 type 3 secretion system), which through its upregulation, targets mTORC1 to the SCVs, and hence inhibits autophagy **(Eriksson et al., 2003)**. Some other bacteria take complete advantage of this process from the beginning on. *Staphylococcus aureus* and *Serratia marcescens* are some of those bacteria that share the capacity to induce the formation of an autophagosome but

inhibit its fusion with the lysosomes which makes it possible for them to use the autophagic membranes and vacuoles for their replication. S. aureus is indeed able to hijack this pathway first by replicating inside the phagosomes and then induce autophagy-dependent cell death as it could be demonstrated in *Atg5*$^{-/-}$ MEFs **(Schnaith et al., 2007) (rieviewed in Huang and Brumell, 2014)**.

Various studies have also linked viral infection to autophagy induction. In that case autophagy has also been reported to play dual roles. It either has an antiviral effect or it favors viral replication **(reviewed in Chiramel et al., 2013)**. In Drosophila for example it appears that autophagy induced through the inhibition of the PI3K/AKT axis is required for the inhibition of viral replication after vesicular stomatis virus (VSV) infection **(Shelly et al., 2009)**. Orvedahl and colleagues demonstrated an antiviral role of autophagy as well. They saw that *Atg5*-deficient neonatal mice were more susceptible to Sindbis (SIN) virus infection. The virus accumulated in the absence of autophagy, which led to cell death of infected neurons even though replication was not affected by *Atg5* deletion suggesting the requirement of autophagy for viral clearance **(Orvedahl et al., 2010)**. As demonstrated by Rabourdin-Combe/Faure's team, infection by measles viruses also induces autophagy after binding the CD46-Cyt1 surface receptor. Using a GFP-LC3 HeLa cell line and by dectecting LC3-II by Western blot experiments, they showed in fact that autophagosome formation was mediated through CD46-Cyt1 engagement. The mRFP-GFP-LC3 fusion protein made it possible for them to distinguish autophagosomes (RFP^+ GFP^+) from autolysosomes (RFP^+ GFP^-) as the GFP signal is quenched in acidic conditions. Engagement of CD46-Cyt1 after measles infection or by an agonist antibody resulted in the formation of punctated dots that where mRFP+ and GFP-. They further established that CD46-Cyt1 interacts with Golgi-associated PDZ and coiled-coil-containing (GOPC) protein which in turn interacts with the Beclin1 complex and activates autophagy leading to the degradation of the virus **(Joubert et al., 2009) (Fig 10).**

The human hepatitis C virus (HCV) however belongs to that category of viruses that exploit the autophagic pathway to increase their pathogenicity. While some studies suggest that autophagy might be necessary for the initiation of HCV RNA translation **(Dreux et al., 2009)**, others indicate rather a role in replication even though the mechanism proposed by one or the other are not quite the same. Sir and colleagues reported that HCV inhibits the fusion of autophagosomes with lysosomes while Ke and Chen's experiments indicate a complete autophagic flux. The two studies agree however that HCV-induced autophagy is dependent on the unfolded protein response (UPR) related to ER stress **(Ke and Chen, 2011; Sir et al., 2008)**. Another interesting example of a virus utilizing autophagy for its replication is the Dengue virus (DENV). It appears that this virus regulates lipid metabolism by stimulating lipophagy thus leading to the generation FFAs that are use in FAO in mitochondria to produce ATP which DENV requires for proper RNA replication **(Heaton and Randall, 2010)**.

The effect of the human immunodeficiency virus (HIV), infectious agent of the acquired immunodeficiency syndrome (AIDS), has been investigated in various immune cells. In DCs autophagy is downregulated upon HIV infection which is specifically due to the viral envelop (Env) protein. Furthermore it has been observed that viral particles accumulate in that case, which facilitates *trans*-infection of CD4 T cells. In macrophages autophagy initiation is not affected by HIV infection but the HIV factor, Nef, binds Beclin1 and inhibits autophagosome maturation. Moreover HIV infection induces IL-10 production which leads to autophagy inhibition of uninfected, bystander macrophages. It appears that the virus utilizes the autophagic machinery for its early replication but then inhibits this process to avoid degradation in the autolysosomes **(reviewed by Dinkins et al., 2015)**. In CD4 T cells Lucile Espert and colleagues reported Env-induced autophagy in uninfected bystander CD4 T cells that would subsequently lead to apoptosis and immunosuppression **(Espert et al., 2006)**.

It appears that xenophagy is a complex process affecting every pathogen in its own way. Some pathogen will directly be degraded through this pathway or they will try to evade the destructive effect of autophagy. Some bacteria/viruses have evolved and developed a third strategy namely to use autophagy to increase their replicative and infectious capacity.

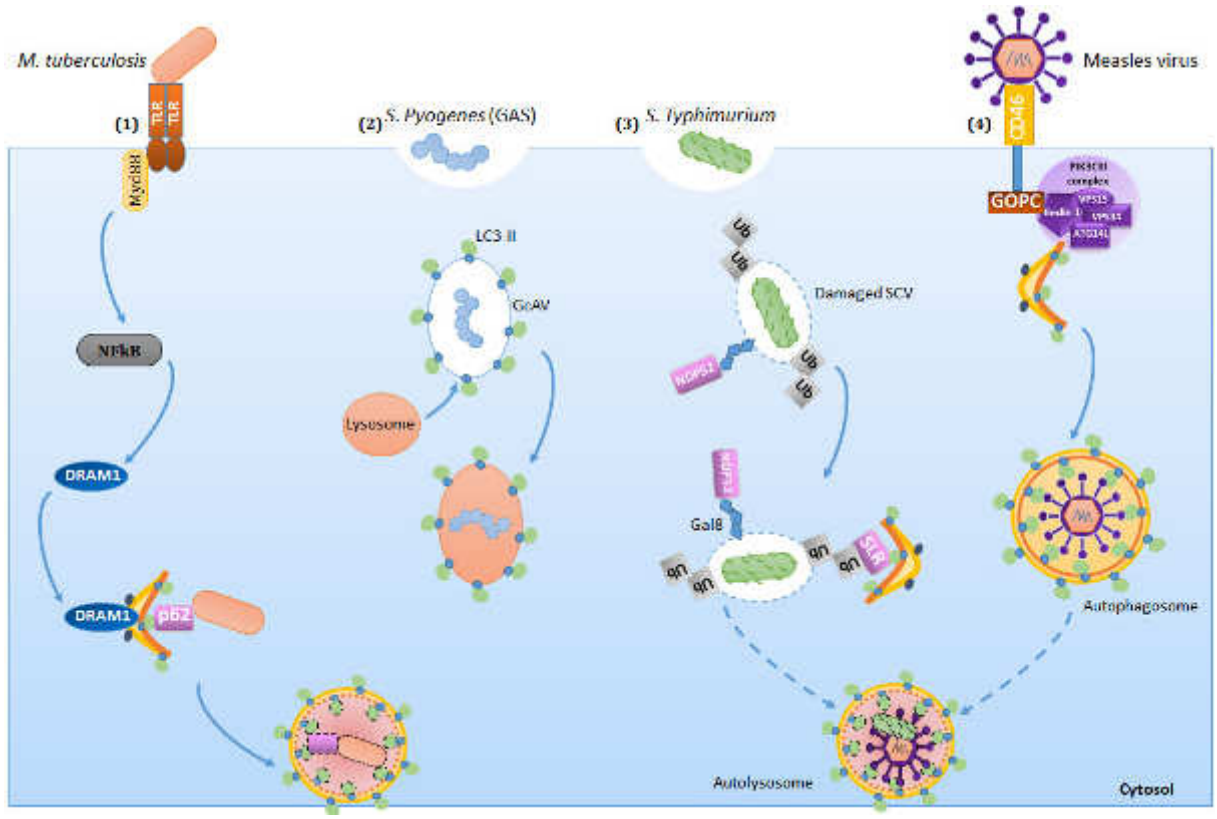

Figure 10: Examples of pathogens degraded via the autophagic machinery.

(1) *Mycobacterium tuberculosis* (Mtb) is recognized by TLRs that activate the Myd88-dependent NFkB pathway. NFkB activates DRAM1 which is able to associate with the phagophore and mediate p62-dependent recognition of Mtb and initiate bacterial degradation. **(2)** *Streptococcus pyogenes* (GAS) enters the cytosol into vesicles that attract LC3-II to form GAS-containing LC3-positive autophagosome-like vacuoles (GcAVs) that fuse with lysosomes leading to *S. pyogenes* degradation. Other bacteria enter the cell in vesicles that undergoe ubiquitinilation when damaged. **(3)** In case if *S. Typhimurium* ubuquitin chains are recognized by SQTSM1/p62-like receptors (SLR) while NDP52 binds galactine 8 chains directing the bacteria to phagophores for degradation. **(4)** Viruses can also be targeted by autophagy. The measles virus binds the CD46 receptor. CD46 interacts with GOPC which in turn is able to attract the PIK3 CIII complex by interacting with Beclin1. This leads to the recruitment of the autophagic machinery, the sequestration of the virus into autophagosomes and finaly to viral degradation in autolysosomes**. TLR:** Toll-like receptor; **Myd88:** Myeloid differentiation primary response 88; **NFkB:** Nuclear factor k B; **DRAM1:** DNA-damage-regulated autophagy modulator 1; **NDP52:** Nuclear dot protein of 52 kDa

3.1.2 Autophagy induction by Pattern Recognition Receptors

The pathogens mentioned in the section above can be sensed by the innate immune system through receptors called pathogen recognition receptors (PRRs). The PRRs are able to bind microbial-associated molecular patterns (MAMPs) and sense danger associated molecular patterns (DAMPs), which leads to the activation of cellular defense mechanisms aimed to clear the pathogens/dangers (Beutler, 2004). The MAMPS can be components of wall or membrane, from bacteria, fungi, parasites but also

nucleic acids. DAMPs are self-encoded molecules resulting from cellular damage like exposure to ROS, or mechanical damage for example. PRRs can be expressed both on immune and non-immune cells. Their activation usually leads to an inflammatory response that can further prime and orientate an adaptive response.

PRRs have been classified into two categories, the first being transmembrane receptors including scavenger receptors, the Toll-like receptors (TLRs) and the C-type lectin receptors **(Takeuchi and Akira, 2010)**. The second category comprises cytosolic proteins such as nucleotide oligomerization domain receptors (NOD)-like receptors (NLRs), retinoic acid-induced gene (RIG)-I-like receptors (RLRs) and the pyrin and HIN domain family members (PYHIN) **(Cerboni et al., 2013)**. Most PRRs activate the evolutionary conserved host defense mechanisms through signal transduction pathways leading to the translocation into the nucleus of mainly three families of transcription factors: nuclear factor kB (NF-kB), activator protein 1 (AP-1) and interferon regulatory factor (IRF).

Some PRRs have been identified as potent autophagy inducers as already mentioned in the context of xenophagy. The TLRs were the first PRRs to be linked to autophagy induction. A report showed that TLR4 stimulation by lipopolysaccharide (LPS) in a macrophage cell line (RAW264.7) leads to autophagy induction **(Shi and Kehrl, 2008; Xu et al., 2007)**. LPS-induced autophagy favors indeed the colocalisation of intracellular mycobacteria with the autophagosomes enhancing their clearance. TLR7 and TLR3 ligands have also been shown to play a role in the activation of the autophagic machinery. Delgado and colleagues showed that stimulation of macrophages infected by mycobacteria with TLR7 agonists, resulted in increased clearance of the pathogen. Even though the bacteria did not express any natural ligand of TLR7, *Tlr7* knock-down reversed the bacterial load decrease and autophagy induction. *Atg5* knock-down in macrophages resulted in the same phenotype. These observations emphasize the importance of TRLs and autophagy interplay in pathogen clearance **(Delgado et al., 2008)**.

Infection by *Listeria monocytogenes* is another interesting case demonstrating the interaction of PRRs and autophagy. This bacteria has been shown to induce autophagy in MEFs, RAW264.7 macrophages as well as in bone marrow-derived macrophages. It appears that this induction is dependent on NOD1 and NOD2 since in *Nod1*, *Nod2* and/or *Tlr2*-deficient cells, autophagy was reduced upon *L. monocytogenes* infection. These cells were however able to exhibit increased autophagic activity after treatment with mTOR inhibitor, rapamycin. Dissection of the mechanisms underlying this process revealed the requirement of the NOD2/RIP2 axis and an ERK/NF-kB dependent signaling pathway in autophagy induction **(Anand et al., 2011).**

Viral infection is also able to induce autophagy through the activation of PRRs. RIG-I and MDA5 are viral sensors belonging to the RLRs. These receptors signal through IPS-1 to induce NF-kB and IRF3 activation and to induce subsequent pro-inflammatory and anti-viral cytokine production. ATG5-deficient cells over-produce type IFN upon vesicular stomatis virus (VSV) infection in *Drosophila*. The reason being that in absence of autophagy nonfunctional mitochondria accumulate leading thus to important ROS production and mitochondrial DNA (mtDNA) release into the cytosol. This stimulus is well-known to induce an inflammatory response through the activation of inflammasomes. Hence in this case autophagy plays a regulatory role in RLRs signaling **(Tal et al., 2009; Orvedahl et al., 2010).**

NLRs are also highly involved in autophagy induction upon bacterial infection. They recognize components of the bacterial wall in the cytosol. One such component is muramyldipeptide (MDP). Cooney and colleagues stimulated indeed human DCs with MDP and observed that after binding this peptidoglycan, NOD2 induces autophagy which is required for antigen presentation. Interestingly this mechanism was defective in DCs from patients suffering from Crohn's disease and bearing the risk allele *Atg16L1*^{T300A} **(Cooney et al., 2010)** suggesting a defective clearance of bacteria a feature that has been linked to disease exacerbation.

3.1.3 Autophagy in the regulation of Type I IFN production

Type I interferons (10 IFN-α subtypes, IFN-β, IFN-ε, IFN-κ and IFN-ω) are key players in anti-viral immune responses. They have also been shown to increase T cell proliferation and display other immunomodulatory functions such as the enhancement of histamine release in mast cells or activation of NK (natural killer) **(Welsh et al., 2012**). The secretion of these cytokines has been associated to autophagy. Based on the results from a study about the infection strategy of human immune deficiency virus (HIV), it seems that upon viral infection, plasmacytoid dendritic cells (pDCs), the main IFN-α producer, failed to secrete the cytokine when autophagy was inhibited either through knock down of ATG7 or pharmacological inhibitors like 3-methyladenine (3-MA) **(Zhou et al., 2012)**. Another virus able to induce IFN-α in an autophagy-dependent manner is the alpha-herpes virus (HSV). HSV is a dsDNA virus that activates the stimulator of IFN gene protein (STING). Viral DNA is however directly sensed by cytosolic proteins such as the GMP-AMP (cGAMP) synthetase (cGAS) which produces a second messenger cGAMP leading to STING activation and the subsequent production of IFN-α. cGAS has been shown to interact with Beclin1 leading to inhibition of cGAMP production. In this case autophagy indirectely negatively regulates IFN production and avoids over-activation of the immune system by mediating viral DNA degradation via the interaction with cGAS **(Liang et al., 2014)**.

Reciprocally, type I IFN are able to induce autophagy. Schmeisser and colleagues demonstrated that IFN-α and IFN-β induce autophagy in some cancer cell lines **(Schmeisser et al., 2013)**. The exact mechanism of autophagy induction in this case is not very well understood yet but it has been proposed that type I IFNs mediate mTOR inhibition leading subsequently to autophagy initiation **(reviewed in Schmeisser et al., 2014)**.

3.1.4 Autophagy in the regulation of inflammatory responses

The inflammatory response as previously discussed can be triggered either by microorganisms such as bacteria, viruses or fungi but also by danger signals as it is the case for ROS or by cytokines. This response requires a tight regulation to avoid over-activation of the immune system which can otherwise lead to important tissue damage.

Autophagy has been shown in some cases to be able to regulate this process. Even though as described in some situations this process contributes to an increased clearance of pathogens (for example infection by *M. tuberculosis*), it can lead to an aberrant and exacerbated immune response as well as to the development of autoimmunity. Nevertheless some studies have reported that autophagy or at least components of the autophagic machinery are able to inhibit such responses.

As ROS producer and possible initiator of apoptosis, mitochondria play a central role in inflammation and their dysfunction has often been associated to chronic inflammatory disorders such as Crohn's disease or neurodegenerative diseases. Thus control of mitochondrial load through mitophagy appears to be necessary in order to limit inflammation. Mitochondria harbour PRRs on their membrane as it is the case for RIG-I and MDA5 that participate in pathogen clearance. Inhibiting autophagy has been linked to hyperactivity of the immune system towards dsRNA as demonstrated by increased type 1 IFN secretion in VSV infected MEFs **(Jounai et al., 2007)**. Secondly autophagy is involved in the recognition of cytosolic DNA and mitochondrial DNA (mtDNA) can also be released into the cytosol by dysfunctional mitochondrial. In autophagic protein deficient macrophages (*Map1lc3b*$^{-/-}$ and *BCN1*$^{+/-}$) cytosolic mtDNA has been shown to stimulate release of pro-inflammatory cytokines IL1-β and IL-18. The mtDNA sensor is not known yet but it has been hypothesized that absent in melanoma 2 (AIM2) might be involved **(Nakahira et al., 2011)**.

ROS also drive production of these proinflammatory cytokines through the activation of the intracellular PRR, the NLRP3 inflammasome **(Fig 11)**. This inflammasome participates in innate immune regulation by mediating the maturation of caspase1 which is responsible for the clavage of inactive

pro-IL1β and pro-IL-18 into their active forms. Autophagy has in fact been identified as a central player in the regulation of the secretion of IL-1β and IL-18. Saitoh and colleagues showed that Atg16L1 deficient macrophages secrete high levels of IL-1β in response to TLR4 stimulation with lipopolysaccharide (LPS). This deregulation was associated to an increased activity of the inflammasome **(Saitoh et al., 2008)**. Harris and colleagues also demonstrated that the combination of LPS stimulation and autophagy inhibition with pharmacological inhibitors (3-MA and wortmanin) lead to secretion of pro-inflammatory cytokines among which IL-1β indicating that inhibiting autophagy improves inflammasome activation. On the other hand activating autophagy with rapamycin after inflammasome activation led to reduced IL-1β secretion by macrophages and in GFP-LC3 transfected macrophages IL-1β was actually found enclosed into LC3 positive vesicles suggesting that this cytokine is targeted by autophosomes for degradation **(Harris et al., 2011)**. It appears that autophagy also directly targets the NLRP3 inflammasome for degradation in order to regulate the inflammatory response. Using GFP-LC3 THP cells (a human monocyte cell line) Kehrl's team gave some evidence in that regard, first by demonstrating that inflammasome induction actually triggers autophagy and secondly that the inflammasome gets ubiquitinated than targeted by p62 in order to be selectively degdraded by the autophagic machinery **(Shi et al., 2012)**.

Nevertheless studies have shown that inflammasome constituents such as NLRC4, NLRP4, NLRP10 and even NLRP3 have the capacity to negatively regulate autophagy by binding Beclin1. For instance in GAS infected cells, NRLP4 dissociates from Beclin1 to bind GAS. Free Beclin 1 can then participate in autophagy induction **(Oh and Lee, 2014)**. Furthermore NLRP4 can bind Class C Vps which leads to inhibition of autophagosome maturation.

All these elements demonstrate that there is a fine tuned inter-regulation between inflammation, inflammasome and autophagy. Thus any disruption at this level can lead to the development of inflammatory and even autoimmune disorders.

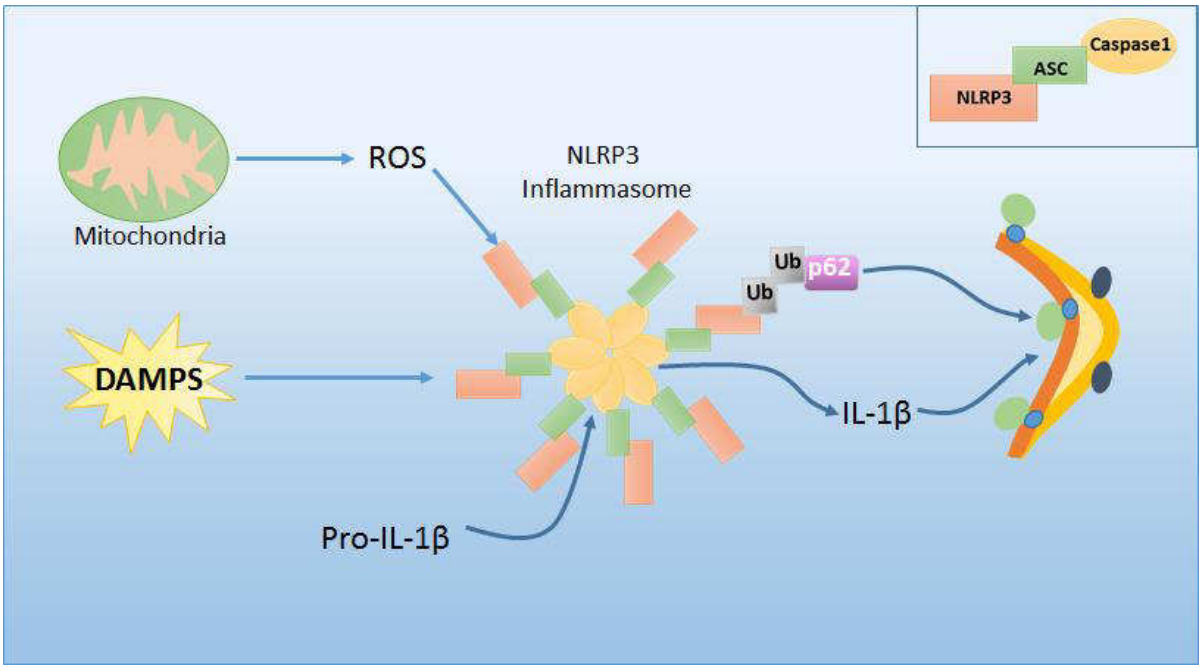

Figure 11: Schematic representation of the regulation of the NLRP3 inflammasome by autophagy.
The inflammasome is composed of the Nod-like receptor protein 3 (NLRP3), the adaptor protein ASC and caspase 1 that assemble together to form a multiprotein oligomer. It can be activated by ROS produced by mitochondria or other danger signals (DAMPS). The NLRP3 inflammasome mediates the maturation of pro-inflammatory cytokines pro-IL-1β into IL-1β but also pro-IL-18 into IL-18 (not depicted). Ubiquitinilation of NLRP3 leads to p62 binding and thus targets the inflammasome to the phagophore for degdradation in the autolysosome. Il-1β can also be degraded by the autophagic machinery. This way autophagy inhibits exacerbation of an inflammasome-induced pro-inflammatory response.

3.2 Involvement of Autophagy in Adaptive Immune responses

In order to mount an appropriate response against external aggressions, higher eukaryotes require the crosstalk between innate and adaptive immunity. While the innate immune system recognizes PAMPs through the PRRs and initiate pathogen control, complete clearance and long-term protection requires adaptive lymphocytes. Those actors, B and T lymphocytes, express variable antigen receptors, B cell receptor (BCR) and a T cell receptor (TCR) respectively which which endow them with the capacity to recognize virtually any antigenic motif. It has been suggested that autophagy is integral to the optimal functioning of adaptive immunity.

Aside its role on antigen presentation that will be discussed further, autophagy was described as essential for lymphocyte activation and survival, as well as in the differentiation and maintenance of their hematopoietic precursors. In fact Katja Simon's team demonstrated that specific deletion of *Atg7* in hematopoietic stem cells (HSCs) in Vav-*Atg7*$^{-/-}$ mice resulted in reduced progenitors of multiple

linages in the bone morrow such as NK, B and T cells, as well as in a myeloproliferative phenotype leading to development of myeloid malignancies resembling human leukemia **(Mortensen et al., 2011)**. They also observed a defective clearance of mitochondria, leading to an increased ROS production subsequently jeopardizing HSC long-term survival. These results suggested that autophagy is essential to maintain HSC functionality **(see section 3.3) (Gurumurthy et al., 2010)**.

3.2.1 Autophagy in antigen presentation

3.2.1.1 MHC class I antigen presentation

MHC molecules, expressed on the cellular surface bind peptide fragments, from various origins, and can present them to cognate T lymphocytes. MHC class I (MHC I) molecules, expressed on all nucleated cells, have been shown to present in most cases endogenous peptides to CD8 T cells.

MHC class I molecules are glycoproteins composed of three alpha domains (α1, α2 and α3) forming the heavy chain and a β_2-microglobulin domain, with a cleft between α1 and α2, designed to bind a specific antigen. These molecules are classically characterized by their capacity to present cytosolic peptides, called endogenous peptides, originating from self or intracellular pathogens. Antigen processing occurs after protein degradation via the proteasome. The resulting peptides enter into the ER, the assembly site of the MHC I molecules, through the transporter associated with antigen processing (TAP), where they can be loaded onto the MHC I **(Hewitt, 2003)**. Loaded MHC I molecules then travel from the ER to the Golgi apparatus before reaching the plasma membrane via the exocytosis pathway and can then present peptides to CD8 T cells.

Since the peptides presented via MHC I are the result of proteasome processing it appears surprising to expect the involvement of autophagy in this process. Indeed, only few studies suggest a direct implication of autophagy in MHC I presentation. Michel Dujardins' team described an autophagy-dependent antigen MHC I presentation of endogenous proteins using herpes simplex virus type 1 (HSV-1) as an infection model in macrophages. They suggest a vacuolar pathway, in contrast to the "classical" proteasomal pathway for endogenous antigen presentation **(English et al., 2009)**. On the other hand Li and colleagues observed an increase of MHC I presentation in autophagy deficient macrophages while MHC I molecules decreased at the cell surface after rapamycin treatment, suggesting a negative regulation of MHC I presentation by the autophagic machinery. Their study of B16 melanoma cells showed in contrast that autophagy is involved in the processing of a part of the tumor antigens in presence of IFN-γ, which also increases antigen presentation and thus degradation

of tumor cells by cytotoxic T lymphocytes (CTLs) **(Li et al., 2009)**. These observations, escpecially the one suggesting a vacuolar pathway for MHC-I presentation by macrophages, could link autophagy and cross presentation.

3.2.1.2 MHC class II antigen presentation

The implication of autophagy in MHC II presentation is on the other hand better documented than for MHC I presentation. Antigen processing in the case of MHC II presentation is in fact dependent on the endosomal/lysosomal pathway making the link with autophagy more obvious. **(Fig 12)**

Contrary to MHC I, only a little group of cells called professional antigen presenting cells (APCs) express MHC II. B cells, DCs and macrophages belong to that category but also thymic epithelial cells responsible for the T cell MHC-restricted selections in the thymus **(Li et al., 2005)**. It has however been shown that stimulation of other cell types such as endothelial cells, stromal cells from secondary lymphoid organs, or fibroblasts can also induce the expression of MHC II molecules. MHC II can also be expressed in human activated T cells.

The canonical pattern for antigen presentation via MHC II involves the endocytosis of exogenous proteins that get delivered to the lysosomal/endosomal pathway and degraded by proteases into small peptides. Those proteins can also enter the cell through pinocytosis or phagocytosis. At the same time, newly synthesized MHC II molecules originating from the ER and associated to a chaperone protein, the invariant chain (Ii) inhibiting premature antigen loading, are transported to late endosomes/lysosomes. Ii gets cleaved in those compartments, leaving only the so called class-II associated invariant chain peptide (CLIP) bound into the groove of the class II molecules. Subsequently the MHC II molecules gain access via the endosomal/lysosomal pathway to the class II compartment (CIIM) characterized by an acidic environment favoring HLA-DM mediated removal of CLIP and the loading of the antigenic peptides onto the MHC II molecules. Finally the peptide-MHC II complexes are transported to the cell surface where they interact with CD4 T cells via the TCR, thus initiating the appropriate effector immune response **(Crotzer and Blum, 2009)**.

Since the exogenous proteins are processed through the lysosomal pathway, it has been suggested that autophagy as well as CMA could also be involved in the generation of antigenic peptides **(Crotzer and Blum, 2009; Schmid et al., 2007)**. MHC II has also been linked to LAP, as already mentioned in a previous section **(3.1.3)**. Ma and colleagues demonstrated that the triggering of the anti-fungal response through Dectin 1 activation is dependent on LC3. Actually it appears that Dectin 1 induces

the recruitment of LC3-II to the phagosomes containing fungi, thus promoting better antigen presentation. As a matter of fact the authors demonstrated that LC3-deficient macrophages stimulated by ovalbumin-expressing yeast, resulted in reduced activation of OT-II cells which are CD4 T cells expressing an OVA specific transgenic TCR. These results and the fact that MCH-II molecules were reduced in phagosomes in absence of LC3-II lead them to consider not only the involvement of LAP in better antigen presentation but also the implication of LC3 in the recruitment of MHC II molecules **(Ma et al., 2012)**. Other studies have shown that LAP induction stabilizes the peptide association with the MHC II molecule by allowing a prolonged interaction **(Romao et al., 2013)**.

Even though MHC II molecules were dedicated first to exogenous antigen presentation, they have also been shown to present peptides of endogenous origin. Same as for MHC I cross-presentation in DCs, autophagy seems to be highly involved in that process. First evidence of endogenous antigen presentation on MHCII to CD4 T cells was given by Jaraquemada and colleagues in the 1990ties with help of their studies on antigen processing of vaccine-infected cells **(Jaraquemada et al., 1990)**. The involvement of autophagy in this process was however first shown only years later by Paludan and colleagues. They demonstrated in an EBV-transformed lymphoblastoid cell line that viral protein Epstein Barr nuclear antigen 1 (EBNA 1), upon acidification inhibition accumulates in lysosomes and localize to autophagosomes. Moreover autophagy inhibition both through pharmacological inhibitors (3-MA) and by autophagy essential gene knock-down (*ATG12*) resulted in reduced CD4 T cell activation by EBV-transformed B cells. In contrast proteasome inhibition had no significant impact on antigen presentation and CD8 T cell activation was not affected. These results lead to the conclusion that endogenous antigen presentation by MHC II is indeed possible and that it requires the autophagic pathway for antigen processing **(Paludan et al., 2005)**. Christian Münz's team hypothesized that the reason why EBNA-1 processing would occur through the autophagic pathway rather than through the proteasome is because this protein has a long half life and autophagy is known to target long-lived proteins for degradation. This explains also the loading on MHC II molecules since they seem to specifically present peptides from long-lived proteins while MHC I presentation rather centers on short lived proteins. Others have since demonstrated the role of autophagy in endogenous peptide MHC II presentation as well **(Dengjel et al., 2005; Schmid et al., 2007).** In mice infected with HSV-1 DCs deficient for *ATG5* were indeed shown to be unable to prime CD4 T cells **(Lee et al., 2010a)**.

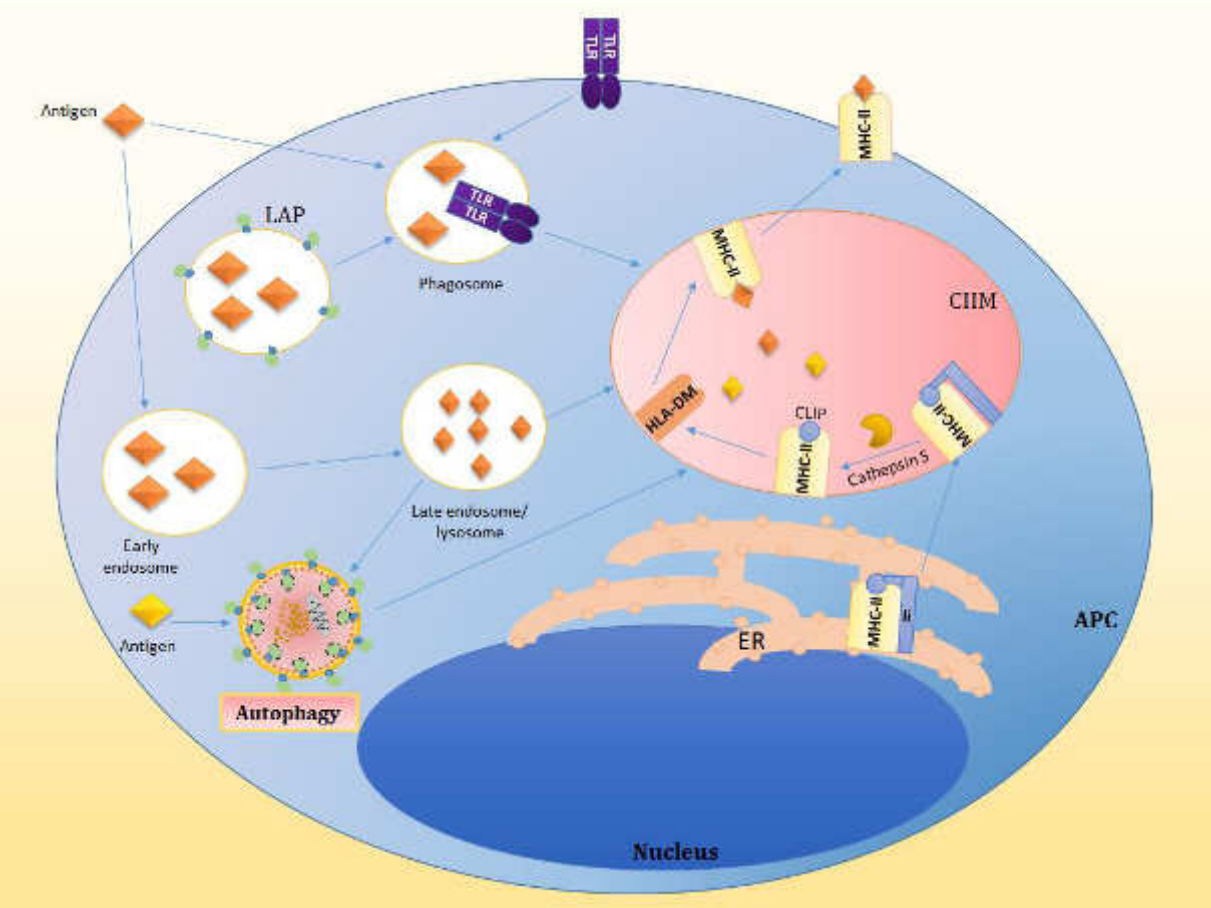

Figure 12: Antigen processing and presentation on MHC II molecules

Antigen presentation mediated by MHC II molecules requires the trafficking of the antigen from early endosomes to late endosomes/lysosomes where it can be degraded into small peptides. The antigen can also be phagocytosed, a process that can be facilitated by activation of TLRs also able to induce LAP. The autophagic machinery contributes to the degradation and the loading of the antigen on MHC II molecules. Autophagy can also capture cytosolic antigen and direct them to MHC II positive compartments where antigen loading occurs. MHC II molecules are generated in the ER and are associated to the invariant chain Ii. They migrate from the ER to CIIM where cathepsin S cleaves the Ii chain leaving CLIP in the MHC II groove. CLIP is removed by HLA-DM and is replaced by the process antigen. The MHC II/peptide complex can then migrate to the cell surfice and present the antigen. **MHC II:** Major histocompatibility complex II; **LAP:** LC3-associated phagocytosis; **TLR:** Toll-like receptor; **ER:** Endoplasmic reticulum; **CIIM:** Class II compartment; **HLA-DM:** Human leucocyte antigen DM; **CLIP:** Class-II associated invariant chain peptide

3.3 Lymphocyte Homeostasis and activation

As mentioned in previous sections, autophagy plays an important role in protein and organelle turnover. As such it is activated at a basal level in most cells including lymphocytes and is highly involved in the maintenance of their homeostasis. As it will be discussed in more details below, lymphocytes depend on autophagy at various levels. T cells are described to depend on autophagy for their development as well as for their survival after activation and their polarization, while B cell lineage was described to need autophagy for development and after for plasma cell differentiation.

The information about the involvement of autophagy in lymphocyte biology were obtained mostly using mouse models with a conditional knock-out of the latter process either in T or B cells, or both, or by using mouse chimeras. As a matter of fact, even though general deletion of autophagy doesn't seem to have an impact on normal embryonic development, it is lethal after birth. Using an *Atg5* total knock-out mouse model, Noboru Mizushima's team observed that the mice died about 1 day after birth. I appears that the neonates are strongly dependent on autophagy in their first hours of life in order to adjust to the drastic environmental changes from the placenta to the "outside world" leading to important requirements in energy but also to a different nutrient intake independent from the placenta. The authors suggested that the neonates face the most important starvation period right after birth and that the only possibility to overcome this problem and to survive is to use autophagy to compensate the lack of essential amino acids and fatty acids **(Kuma et al., 2004)**. Thus the information we have today about the *in vivo* role of autophagy come from tissue or cell type specific autophagy knock-out models.

3.3.1 Autophagy in B lymphocyte homeostasis

First investigations about the in vivo role of autophagy go back to the use of *Atg5*$^{-/-}$ fetal liver hematopoietic chimeric mice. The use of chimaeras as demonstrated by Heather Pua and later by Miller and colleagues showed that impairment of autophagy has an impact on B cell numbers in periphery **(Pua et al., 2007)**. This decrease was demonstrated to be due to defective transition from pro to pre-B cell stage in the bone morrow suggesting the requirement of autophagy in B cell development **(Miller et al., 2008)**. The chimaeras were generated by harvesting fetal liver cells from *Atg5* deficient mice which were then transferred into *Rag1*$^{-/-}$ mice. Thus it cannot be excluded that survival defects observed could be due to an underlying developmental problem of *Atg5* total knock-out from the HSC level before transfer. Indeed autophagy inhibition is detrimental to the generation of hematopoietic progenitors **(Mortensen et al., 2011)**. On the other hand only a few mouse models with a selective autophagy deletion in B cells have been generated so far. Miller and colleagues used a mouse model with a B cell specific *Atg5* knock-out under the control of the CD19 cre promoter expressed from the late pro-B cell stage on. Surprinsingly, in this model, the authors did not report any developmental defect. Moreover, the only B cell population that was impacted in periphery by the lack of *Atg5* was the B1-b B cell population in the peritoneum suggesting that autophagy plays a minor role in B2 B cell development. This contradictory data about the importance of autophagy in B cell development could be due to the reported incomplete deletion of several genes using the CD19

promoter **(Hobeika et al., 2006)**. Hence in these conditions it might be difficult to draw any conclusion on whether or not autophagy has an impact on B cell development.

Nevertheless this partial deletion was actually exploited by Pengo end colleages to demonstrate that autophagy is required for plasma cell (PC) differentiation **(Pengo et al, 2013**). Simone Cenci's team investigated indeed the requirement of autophagy in antibody secreting cells (ASCs) using CD19 cre *Atg5*$^{f/f}$ mice. They agreed on the fact that autophagy inhibition had no major impact on early mature B cell activation and survival. But while quantifying *Atg5* expression by qPCR in LPS-defferentiated PCs from CD19 cre *Atg5*$^{f/f}$ mice they noticed an enrichment in *Atg5* expressing PCs throughout time. This suggests that the leacky promoter confers a selective advantage to autophagy competent PCs. Furthermore it appears that once B cells differentiate into plasma cells (PCs) autophagic activity peaks up and that in transgenic mice expressing GFP-LC3 autophagy is increased in B cells after LPS stimulation. They also observed an increase of LC3 puncta in those cells as well as the upregulation of mRNA of various *Atgs* (*Lc3, Atg9, Atg7, Atg4a*). LC3-GFP positive vesicles were also increased in PCs isolated from the spleen and the bone morrow after immunization with the T-independent (TI) antigen NP-Ficoll, suggesting that autophagy is important for PC differentiation. Furthermore the autophagy-deficient PCs were shown to contain more ER and to secrete a higher amount of immunoglobulins (Ig). These results could be explained by the fact that autophagy regulates ER-induced stress through reticulophagy. PCs are very active protein producing cells and must regulate tightly ER stress. Autophagy indirectly controls in that case Ig secretion, also a source of cellular stress when its production is excessive. Moreover autophagy inhibits premature PCs apoptosis by exercising a cytoprotective role against ER stress. *In vivo* experiments performed by immunizing these mice with either a T-dependent (TD) or a TI antigen resulted in decreased antigen specific humoral response. Restimulation of mice with the same antigen a few months later did not induce a potent response either, indicating a functional defect in autophagy incompetent PCs and a requirement of this process in long-term immune responses **(Pengo et al., 2013)**. Conway and colleagues who were also using the same mouse model to study B cell autophagy came to the same conclusion. They observed a decrease in humoral response after TD and TI immunization as well as after parasite infection. However unlike Pengo and colleagues, upon LPS stimulation the authors identified an early defect in PC differentiation since they detect an impaired upregulation of transcription factors such as *Prdm1* and *Xbp-1* usually upregulated in differentiating PCs **(Conway et al., 2013)**. It is possible that the differences in protocols for B cell activation between the two studies explain the discrepencies on the conclusions about the precise stage where autophagy is needed for PC survival. One could also argue that early defects in *Prdm1* and *Xbp-1* expression contribute to further survival impairments. Two other studies investigated the role of autophagy in long-term humoral responses. They showed that mice with B cell-

specific *Atg7* deletion failed to maintain their memory B cell compartment after immunization with an influenza antigen hemagglutinin (HA). HA-specific memory B cells underwent massive cell death. Germinal center B cells (GC B) however were not affected by this phenomenon confirming previous observations drawn by Cenci's group. Chen and colleagues uncovered indeed that memory B cells could be generated in absence of autophagy. However due to a defective clearance of dysfunctional mitochondria, and thus an accumulation of ROS and peroxidized lipids, apoptosis was observed on the longterm **(Chen et al., 2014, 2015)**. **(Table 2)**

It can appear surprising that autophagy is not required for early B cell activation, considering that autophagy in B cells is suspected to be involved in BCR trafficking. The BCR has indeed been shown to be localized to autophagosome-like structures. It appears that autophagy is required to translocate DNA-containing antigens to TLR9 compartments (Chaturvedi et al., 2008). Ireland and Unanue reported concordant observations by demonstrating that upon BCR stimulation, GFP-LC3 colocalized with internalized IgM in MHC II positive compartments. They suggested as well that autophagy is especially required for the presentation of citrullinated antigens to CD4 T cells **(Ireland and Unanue, 2011)**. This autophagy could be necessary to modulate B cell activation and under certain circumstances, for the recruitment of T cell help via antigen presentation. Moreover presentation of citrullinated self-antigens as well as recognition of self-DNA by TLR9 have been associated to the development of autoimmune diseases such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE), suggesting that autophagy could play a role in autoimmunity.

Table 2: Mouse models of B cell specific deletion of autophagy

Genotype	Phenotype
***Rag1*$^{-/-}$*Atg5*$^{-/-}$ fetal liver hematopoietic chimeric mice (Pua et al. 2007, Miller et al. 2008)**	→ Reduced B cell numbers in periphery → Defective transition from pro- to pre-B cells → Requirement of autophagy in development?
Atg5$^{f/f}$ CD19 cre (Miller et al. 2008, Pengo et al., 2013 ; Conway et al., 2013)	→ Reduced B1-b cell population → No impact on early B cell activation and survival → CD19 = leaky promotor → Defective PC differentiation
Atg7$^{f/f}$ CD19 cre (Chen et al., 2014 ; 2015)	→ Defective maintenance of a memory compartment

3.3.2 Autophagy in T lymphocyte homeostasis

3.3.2.1 Autophagy in T lymphocyte development: From Hematopoietic stem cells to memory T cells

Autophagy in T cell education **(Fig 13)**

Autophagy has been shown to play crucial roles in T lymphocyte biology at various levels. As a matter of fact, starting from the HSC point of view, it has been established that this process is essential for the generation of lymphocyte progenitors. This has been demonstrated thanks to the Atg5$^{f/f}$ Vav-cre mice as previously mentioned **(Mortensen et al., 2011)**. Beyond the role in HSCs, autophagy has been shown to be involved in thymic T cell education. During this process, T cells undergo first positive selection in the cortical part of the thymus. This implies that only T cells with sufficient affinity for recognition of self MHC/peptide complexes survive while the others die by apoptosis. The second phase of T cell education is the counter-selection of T cells with a TCR that strongly binds the MHC associated to a self-peptide from a tissue-restricted antigen (TRA) leading subsequently to their apoptosis. This step, termed negative selection is mediated by the medullary thymic and dendritic cells, and is essential to limit the amount of self-reactive T cells in the periphery. It thus allows the maintenance of tolerance against self-antigens. Thymic epithelial cells (TECs) play a central role in both positive and negative selections since they are responsible for the MHC restricted peptide presentation to the T cells. As previously discussed autophagy has been shown to be involved in MHC II presentation of endogenous antigens. Interestingly, using GFP–LC3 reporter mice Nedjic and colleagues have been able to show that TECs, the main players in this process and especially cortical TECs (cTECs), exhibit a high basal constitutive autophagic activity **(Nedjic et al., 2008).** Furthermore after transplantation of *nude* mice with *Atg5*-deficient thymi, they observed an impact on MHC-II restricted presentation of cTECs to CD4 T cells, and consequently a disturbed positive selection. Accordingly, this model was characterized by the apparition of autoinflammatory events such as colitis and multi organ lymphoid infiltration. The possible implication of autophagy in MHC-II restricted peptide presentation in thymic epithelium was also suggested by Michiyuki Kasai and colleagues who showed that LC3 molecules colocalize with MHC-II compartments both in *in vitro*-established TEC cell lines as well as in thymic cryo-sections **(Kasai et al., 2009)**. The hypothesis of autophagy-dependency of TECs for T cell education, has been argued by another team since. Suskeree and colleagues saw neither a defect in positive or negative selection nor an outbreak of autoimmune events in their mouse model with a conditional knock-out of *Atg7* in the thymic epithelium. They postulated that the previous conclusions might be somehow farfetched as autophagy deficiency impacted all thymic cells and not only TECs **(Sukseree et al., 2012)**. One could however still argue that incomplete deletion in this model impaired any major phenotype occurrence.

Another study still from Ludgers Klein's team, strengthened the idea of direct autophagy implication in thymic selection. They showed indeed that in *Aire*$^{+}$ medullary TECs (mTECs) presentation of a model antigen was autophagy-dependent. They generated therefore a tripartite fusion protein as a model antigen composed of the reporter protein GFP, a human C-reactive protein (hCRP) as a traceable CD4 T cell epitope, and finally LC3 to target the protein to the autophagosome. The resulting fusion protein called GCL (for GFP-hGCL-LC3) was designed to be expressed under the control of the autoimmune regulator *Aire* specific to mTECs. Aichinger and colleagues thus demonstrated quite elegantly that transplantation of autophagy deficient *Aire*-GCL thymi resulted in reduced negative selection **(Aichinger et al., 2013)**. Furthermore a more recent study using a mouse model with a specific deletion of C-type lectin domain 16A (*Clec16A*) in the thymic epithelium, supports the hypothesis of a major role of autophagy in TECs function. *Clec16A* encodes a protein that has been associated to the development of various autoimmune disorders such as type 1 diabetes (TID), SLE, Crohn's disease (CD) and multiple sclerosis (MS) among others and its deletion has been shown to inhibit autophagy. Schuster and colleagues made the following observations namely that in their model TEC autophagy was indeed impaired. Their mice displayed an increase in thymocytes bearing a low expression of the TCR and CD69 suggesting a selection of hyporeactive T lymphocytes. Moreover they saw a decrease in the CD4 single positive (SP) T cell population expressing Helios, a transcription factor which is highly expressed during negative selection and lower during positive selection. These results were indeed indicative of impaired negative selection and of a possible implication of autophagy in this process. To test this hypothesis they transduced a cTEC cell line called MJC1 with an LC3 transgene fused with a DNA fragment encoding for $OVA_{323–339}$ peptide. They could thus follow the autophagy-dependent presentation of this antigen, as the peptide has first to be processed by autophagy in order to gain access to the MHC-II compartment **(Schuster et al., 2015)**. As the reactivity of CD4 T cells bearing an OVA specific TCR (OT-II cells) was reduced in *Atg-5* as well as in *Clec16A*-deficient TECs, they concluded that the phenomenon observed was autophagy dependent. In summary all these works indicate that autophagy in TECs is indeed essential to constitute MHC-II peptide repertoire, and thus is implicated in T cell selection. Hence autophagy impairment in TECs could be an inducer of autoimmune events.

Autophagy in T cell development and peripheral homeostasis **(Table 3)**

The T cell-intrinsic role of autophagy, as for B cell, has been mostly established using conditional knock-out mouse models. But first of all, Pua and colleagues established that T lymphocytes were indeed autophagy competent as they were able to detect both *atg* specific transcripts (*Lc3*, *Atg5*, *Beclin1*) as well as the presence of autophagosomes by transmission electron microscopy. Furthermore they showed that the autophagic machinery could be activated either through starvation or through TCR activation as demonstrated by LC3 conversion assays by western blot **(Pua et al., 2007)**. Starting from

there, autophagy deletion models were generated in order to dissect the role of this process in T cells. Thereby the first deletion models have allowed to demonstrate the importance of autophagy in T cell development. The use of *Atg5* chimaeras as mentioned in the B cell section **(3.3.1)** gave the first indications on a possible relationship between autophagy and T cell development. Pua and colleagues' chimaera model showed indeed a reduction in thymocyte numbers as well as in CD4 and CD8 T cell numbers in the periphery with a more marked decrease for CD8 T cells. This decreased was linked to survival defects in both T cell subsets in the periphery as it could be noted from splenic CD4 and CD8 T cells. T cells also failed to optimally respond and survive to a TCR stimulation suggesting the requirement of the autophagic machinery for proper T cell function **(Pua et al., 2007)**. As already discussed, all these observations could possibly be associated to an autophagy deficiency in early hematopoietic progenitors. Consequently the following models used the cre-lox system to delete *Atg* under the control of a T cell-specific promoter. He's team generated a mouse model with an *Atg7* deficiency in T cells under the control of the proximal Lck promoter ($Atg7^{f/f}$ pLck-cre). They made similar observations in regard to thymocyte numbers, with no change in precursor proportions and same as in the chimaeras, the T cells displayed defects in survival and function. Since the pLck promoter is active from the double negative (DN) stage of T cell development on, they hypothesized that autophagy requirements in thymic T cell development is modest compared to what is found in periphery. Interestingly they noticed that in comparison to wild type mice (WT) peripheral T cells from the $Atg7^{f/f}$ pLck-cre mice contained a significantly higher amount of mitochondria. This is in agreement with the increase in ROS production in the absence of autophagy, and the observed massive apoptosis of the T cells. In contrast mitochondrial content was similar in thymocytes. Interestingly, when comparing WT thymocytes to WT peripheral T cells it appears that the mitochondrial content is much lower in periphery, a phenomenon that was hardly observed in the absence of autophagy. This suggests that this degradation process is involved in developmental regulation of mitochondrial material in T cells **(Pua et al., 2009)**. This was also an indication that metabolic requirements are different from on developmental stage to another. One more important observation from this study was that peripheral CD8 T cell seemed to be able to tolerate only a low mitochondrial content compared to CD4 T cells, as their survival was affected the most in the absence of autophagy. This coincides with the fact that their reduction in mitochondria after thymus egress is higher than it is in CD4 T cells in WT mice. Other studies using slightly different deletion models came to similar conclusions as well while adding new information clarifying a few underlying questions about the relevance of autophagy in T cells. Thereby the works by Stephenson and colleagues confirmed the importance of mitophagy for T cell survival and also identified a set of genes associated to mitochondria that where downregulated in absence of autophagy. They used both an *Atg5* and an *Atg7* deletion model, in order to ascertain that the observations made were surely linked to the autophagic process *per se* and not to an indirect effect of

Atg5 deletion. As a matter of fact *Atg5* has been shown to be involved in other autophagy-independent mechanisms such as in IFNγ-induced cell death due to the capacity of this protein to interact with FADD **(see section 1.4.4)**.

Furthermore He's team demonstrated that the ER-content in T cells is developmentally regulated by autophagy as well **(Jia et al., 2011)**. In normal conditions, DN thymocytes seem to have a high ER content which is rather decreased in DP (double positive) and SP (single positive) thymocytes as well as in peripheral T lymphocytes. The decrease is once again more striking in CD8 T cells. Hence Jia and colleagues detected an expansion of the ER in autophagy-impaired mature T cells, associated to an increase of ER stress markers such as the ER resident molecule protein disulfide isomerase (PDI), ER chaperones glucose regulated protein 78 (Grp78), and Grp94. Other than being involved in protein folding and transport, the ER is a calcium (Ca^{2+}) storage compartment and seeing that the TCR signaling is strongly dependent on Ca^{2+} **(see section 3.3.2.2)**, the authors wanted to investigate the influence of ER expansion on this signaling pathway. They discovered indeed that the Ca^{2+} influx was decreased after stimulation of autophagy deficient T cells and that this was due to an intrinsic defect in ER-content regulation independent of mitochondria as they have also been shown to play a role in Ca^{2+} signaling. This requirement for autophagy-dependent ER and mitochondrial homeostasis in T cells was established in two different mouse models deficient for *Atg7* or *Atg3* respectively **(Jia and He, 2011)**. In both cases invalidating autophagy was dependent on the proximal Lck-promoter expressed early in thymocyte development. Thus the authors speculated that the decreased survival of peripheral T lymphocytes was the result of longterm accumulated defects. This led them to generate a mouse model with an *Atg3* inducible deletion ($Atg3^{ff}$ ER-cre). With a tamoxifen treatment Jia and colleagues menanged to inhibit autophagy in mature T lymphocytes *in vitro*. When cultured in presence of IL-7, a T cell survival factor, the *Atg3* deletion did not have an impact on the T cell survival. But starting from day 10 on these cells started to display an accumulation of ER and mitochondria over time suggesting that autophagy is needed to mediate organelle clearance in order to avoid cell death. These were the pioneer studies that allowed to establish both a role of autophagy in T cell development but also in T cell organelle homeostasis.

Interestingly, it was shown by various studies that autophagy-deficient T cells had an increased expression of the cell surface memory marker CD44 **(Jia and He, 2011; Jia et al., 2011; Stephenson et al., 2009)**. This phenotype has been associated to T cells undergoing spontaneous proliferation (also called homeostatic proliferation) independent of antigen presentation. This phenomenon is usually induced to compensate modifications of the immune system that lead to a lymphopenic phenotype as it can be the case in some chronic diseases, irradiation or thymectomy. The authors suggested that this might be the case in autophagy incompetent T lymphocytes as well **(Stephenson et al., 2009)**.

Kovacs and colleagues, introduced another mouse model. Their mice were deficient for Beclin1 with a deletion controlled by the CD4 promoter (Beclin1$^{f/f}$ CD4-cre). In addition to the phenotype observed in previous models, meaning a reduction of peripheral T cells and massive cell death upon *in vitro* TCR stimulation, the authors noted that these cells displayed an increased expression of cell death related-proteins such as Bim, pro-caspase 3 and 8. Surprisingly they did not see an increase in mitochondrial content contrary to previous descriptions. This firstly suggests that in their model, massive cell death was less linked to defective mitophagy but rather to a decreased degradation of cell death-related proteins. Secondly it indicates that mitophagy in T cells might be largely Beclin1-independent. Furthermore they showed that polarized cells were differentially dependent on autophagy. Interestingly TH17 polarized cells were the least susceptible to cell death in the absence of autophagy, while TH1, TH2 and unpolarized (TH0) T cells underwent massive apoptosis **(Kovacs et al., 2012)**. *In vivo* experiments revealed that their mouse model was resistant to oligodendrocyte glycoprotein (MOG)-induced experimental allergic encephalomyeleitis (EAE). EAE is a mouse model for multiple sclerosis and a Th17-associated disorder. This disease is also partly CD8-dependent and since in Beclin1 deficient T lymphocytes this T cell subset was highly decreased, resistance to EAE might also be linked to that phenotype. As for autophagy in T helper subsets, a previous study by Lu's group had also shown that upon polarization of CD4 T cells transduced with GFP-LC3 retrovirus, Th2 cells displayed a higher autophagic activity compared to Th1 cells, indicative of a polarization-dependent regulation of autophagy in T cells **(Li et al., 2006)**.

In Willinger and Flavell's model, the deletion of Vps34 in T cells led once again to the accumulation of mitochondria observed in previous models. This could appear astonishing as Vps34 is associated to Beclin1 in the PI3KCIII complex and Beclin1 deletion was reported by Kovacs and collegues to have no impact on mitophagy. They suggested that the discrepancies observed concerning mitophagy in the various models could be explained by the fact that there are various levels of autophagy regulation in T cells, meaning that basal autophagy might be needed for organelle clearance while in activated T cells it is required to degrade pro-apoptotic proteins **(Willinger and Flavell, 2012)**. Studying the same model Parek and colleagues also discerned new consequences not yet described at that time namely a Vps34 requirement for the development of invariant natural killer T cells (iNKT) a subpopulation of T cells that recognizes glycolipids in the context of CD1d a non-classical MHC I presentation. They also noticed that old mice developed an inflammatory wasting syndrome that they led back to a reduced number of Treg cells as well as a deficiency in their capacity to regulate effector T cell expansion in the absence of autophagy **(Parekh et al., 2013; Bronietzki et al., 2015).**

To sum up these studies, it clearly appears in all of them that autophagy is essential for T cell development and even more for peripheral T cell homeostasis and function after activation. It must be

said however that all the mouse models had in common the deletion of *Atg* genes early in T cell development which probably exerts an influence on their phenotype in periphery. Moreover since the cell numbers were extremely low and T cells were hardly functional in periphery it made *in vivo* studies difficult in these mice models.

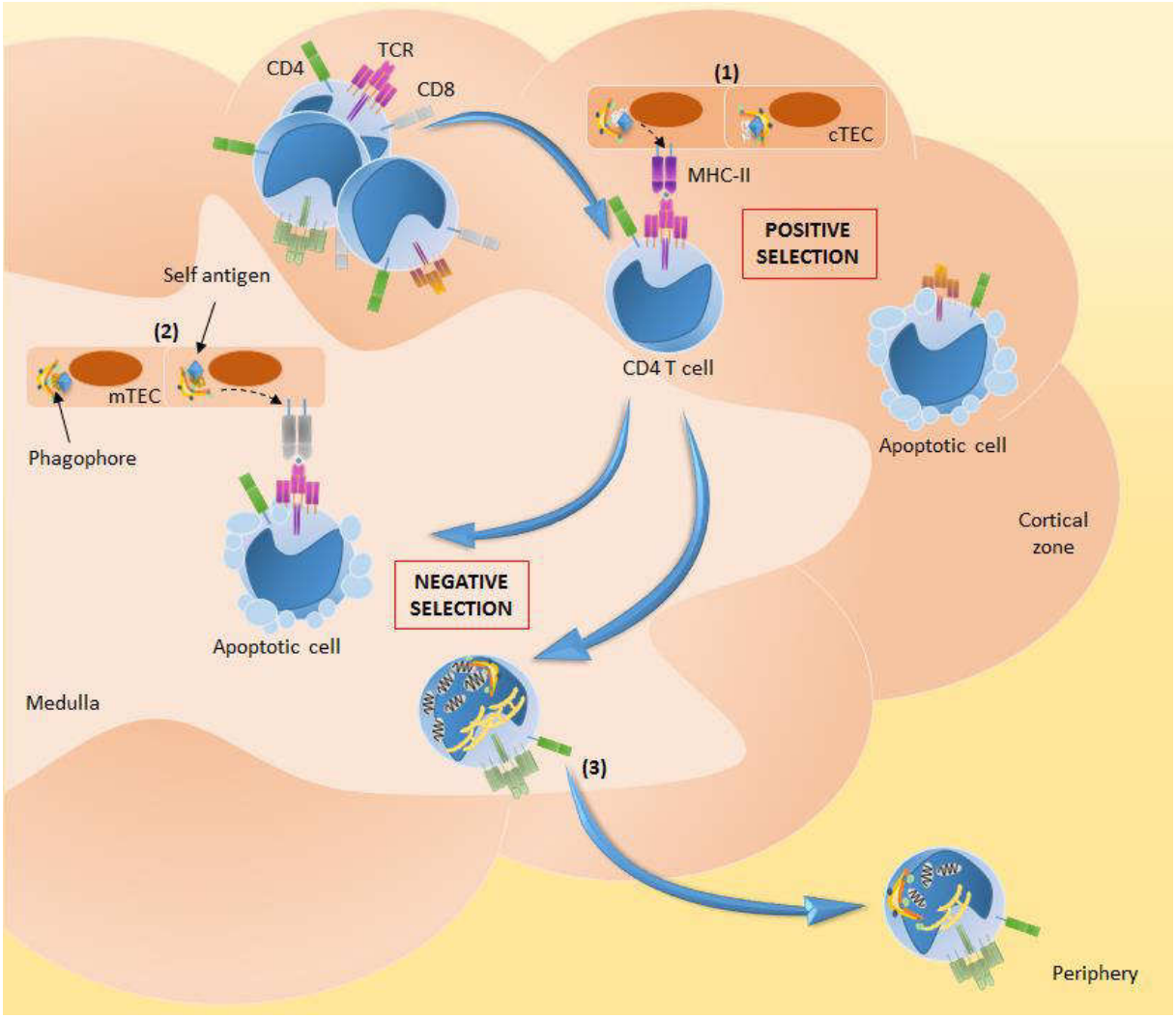

Figure 13: Role of autophagy for thymic T cell development.

(1) cTECs and **(2)** mTECs responsible for positive and negative T cell selection respectively, display high levels of basal autophagy. The autophagic activity is required for presentation of intracellular self-antigens on MHC-II and mediate T cell education. In positive selection T cells with a TCR with no or low affinity for the MHC-peptide comlpex undergoe apoptosis. On the contrary in negative selection the recognition of self peptides results in cell death. **(3)** Thymic T cells contain high levels of mitochondria and an expanded ER which are reduced by the autophagic machinery before exit from the thymus. Peripheral T cells require autophagy in order to maintain their homeostasis.

Autophagy in memory T cells

Two recent studies aimed at overcoming the difficulties linked to existing models in order to draw conclusions about the *in vivo* relevance of T cell autophagy in immune response. Katja Simon's team demonstrated the requirement of the autophagic process in CD8 T cell mediated memory responses using a mouse model with a T cell specific deletion of Atg7 under the control of the CD4 promotor ($Atg7^{f/f}$ CD4-cre). They noticed indeed that even though CD8 T cells were decreased in number and proportions (as were CD4 T cells), the mice were still able to generate an early antigen specific CD8 response after viral infection. But as they followed the response over a longer period of time they started to observe an important decrease of the antigen specific CD8 T cells. Even rechallenging the mice revealed to be unsuccessful, suggesting a defect in the establishment of the CD8 memory compartment. In order to assess if these observations were in fact due to the CD8 T cell intrinsic autophagy deficiency, the authors infected 1:1 ($Atg7^{-/-}$: WT) bone marrow chimeras with the influenza virus. This strategy was meant to exclude factors such as reduced CD4 T cell help, T cell exhaustion or lymphopenia that could also possibly lead to a defective CD8 memory pool. These mixed chimeras also failed to generate antigen specific memory CD8 T cells even though the frequency of CD8 T cell short-lived effector and memory precursors were normal and the exhaustion markers PD-1 and TIM-3 were not further increased. The expression of cytokine receptors (IL-7Rα, IL-15Rα) was also unchanged in murine cytomegalovirus (MCMV) infected chimeras when compaired to controls. Moreover the expression of the transcription factors IRF4 and EOMES, responsible for mediating T cell expansion, differentiation and memory CD8 T cell formation respectively, was normal as well. This was indicative of a defect in memory CD8 T cell maintenance rather than a failure in forming a memory pool. A possible explanation for the defective survival could be that memory CD8 T rely on autophagy to maintain mitochondrial homeostasis and thus supporting a proper mitochondrial respiration crucial for their survival. Furthermore the differentiation of effector CD8 T cells into memory CD8 T cells is accompanied by metabolic changes as the lymphocytes switch from an aerobic glycolysis to mitochondrial respiration also called oxidative phosphorylation **(discussed in section 3.3.2.2)**. It appears in fact that autophagy deficient memory CD8 T cells accumulate dysfunctional mitochondria, display decreased survival and fail to switch to oxidative phosphorylation as demonstrated with the staining of glucose transporter 1 (GLUT1). GLUT1 is usually upregulated in effector CD8 T cells and downregulated in memory CD8 T cells but in the absence of autophagy, it stays higly expressed **(Puleston et al., 2014)**. Puleston and collegues also examined that capacity of their mouse model (*$Atg7^{-/-}$* CD4-cre) to respond to a second challenge with a viral antigen and noted that the recall response was significantly decreased suggesting the requirement of autophagy in this process. Interestingly it has been shown that autophagy decreases with age. This is in agreement with the fact that older

people are more susceptible to infection even when vaccinated. Thus in regard to the results of this study, the authors proposed that enhancing autophagy in elderly people could help induce an efficient memory response to vaccination. They tested this hypothesis by treating old mice with spermidine, a compound naturally expressed in mammals and known to modulate autophagy. They confirmed that this polyamine was able to induce autophagy in T cells in an mTOR-independent manner, and found that its administration to old mice during vaccination restored strong antigen specific recall responses.

Rafi Ahmed's team also came to the same conclusion concerning the requirement of autophagy in CD8 memory responses. As a matter of fact they were the first to generate a mouse model with a specific deletion of either *Atg5* or *Atg7* specifically in effector CD8 T cells, under the control of the granzyme B promoter only expressed when CD8 T cells are differentiating. With this elegant model they were able to go around the lymphopenic phenotype observed in all the previous models. As developmental defects can be ruled out, their models is thus very adapted for *in vivo* functional studies. Xiaojin Xu and colleagues examined autophagy activation status of CD8 T cells after viral infection, using different approaches such as GFP-LC3 expressing CD8 T cells and by detection of *lc3b* and *p62* transcripts. Interestingly, the resulting data indicates that autophagic flux is inhibited during CD8 T cell clonal expansion but gets restored during effector function and memory T cell generation. The experiments on their transgenic mice on the other hand revealed an important loss of effector CD8 T cells in the early stages of infection due to deficient autophagy-dependent metabolic requirements to produce certain growth factors that allow them to maintain their survival. Autophagy seems also to be necessary to maintain metabolic homeostasis for the transition from effector to memory T cells (Xu et al., 2014a).

Thus, the last ten years of T cell autophagy investigations have allowed to shed some light on the role of this process at various stages of T cell development and function. The get-way massage so far is that T cells depend on autophagy for their development, for the maintenance of organelle homeostasis in periphery before and after activation and for the generation of memory CD8 T cells. However, some interrogations still remain because they have not been investigated yet, like the requirement of autophagy in T cell homeostasis in the periphery ruling out any developmental problem during thymic differentiation inherent to descrbed models. Moreover, the question of the need for autophagy in memory CD4 T cells is still unanswered. Finally due to discrepancies from one study to the other, it remains uncertain whether autophagy is induced or blocked right after TCR engagement.

Table 3: Mouse models of T cell specific autophagy deletion

Genotype	Phenotype	Conclusions
***Rag1*$^{-/-}$*Atg5*$^{-/-}$ fetal liver hematopoietic chimeric mice (Pua et al., 2007)**	→ Reduced thymocyte numbers → Reduced CD4 and CD8 T cell numbers in periphery	→ *Atg5* required for lymphocyte development and function → *Atg5* plays a role in the regulation of lymphocyte precursors
Atg7$^{f/f}$ pLck-cre (Pua et al., 2009)	→ Reduced thymocyte numbers → Reduced CD4 and CD8 T cell proportions in periphery → Defective T cell survival and function in periphery upon TCR stimulation *in vitro* → Increased mitochondrial, ROS content in peripheral T cells	→ Mitochondrial content is developmentally controlled by autophagy
Atg5$^{f/f}$ pLck-cre/Atg7$^{f/f}$ pLck-cre (Stephenson et al., 2009)	→ Slightly Reduced thymocyte numbers → Reduced CD4 and CD8 T cell proportions in the thymus and periphery → Defective T cell survival and function in periphery upon TCR stimulation *in vitro* → Increased memory surface marker CD44 expression → Increased mitochondrial, ROS content in peripheral T cells	→ Mitochondrial content in T cell is developmentally controlled by autophagy → Establishment that the observed phenotype is indeed autophagy-dependent → Memory-like phenotype probably due to cell exhaustion and lymphopenic environment
Atg7$^{f/f}$ pLck-cre (Jia et al., 2011)	→ Reduced thymocyte numbers → Reduced CD4 and CD8 T cell proportions in the thymus and in periphery → Defective T cell survival and function in periphery upon TCR stimulation *in vitro* → Increased memory surface marker CD44 expression → Increased mitochondrial, ROS and ER content in peripheral T cells	→ ER content in T cells is developmentally controlled by autophagy → Early autophagy invalidation in T cell development → impact on peripheral T cell functions?
Atg3$^{f/f}$ pLck-cre/ Atg3$^{f/f}$ ER-cre	→ Atg3 deletion *in vitro* → Defective T cell survival and function in periphery upon TCR stimulation *in vitro*	→ Autophagy is dispensable in early T cell activation in presence of a survival factor

(Jia and He 2011)	→ Survival defect ameliorated by IL-7 addition → Accumulation of mitochondria and ER throughout the culture time leading to cell death	→ Autophagy is required for organelle homeostasis in activated cells
Beclin1$^{f/f}$ CD4-cre (Kovacs et al., 2012)	→ Normal thymocyte numbers → Reduced CD4 T cell proportions in periphery → Strong reduction in CD8 T cell proportions in periphery → Survival defects *in vitro* upon TCR activation → T helper polarization → TH0,TH1 and TH2 display strong survival defects → TH17 cells are resistant to apoptosis → No mitochondria accumulation → Increased expression of cell death related proteins → Mice are Resistant to EAE induction	→ Mitophagy → Beclin1 independent? → Autophagy differentially regulated in T helper subsets → Regulation of cell death by *Beclin1* throught the degradation of caspases
Vps34$^{f/f}$ CD4-cre (Willinger et al., 2012)	→ Normal thymocyte numbers → Reduced T cell proportions in periphery → Mitochondria accumulation	→ Mitophagy required for basal T cell homeostasis? → Autophagy required for degradation of pro-apoptic proteins in activated T cells?
Atg7$^{f/f}$ CD4-cre (Puleston et al., 2014)	→ Reduced CD4 and CD8 T cells in proportion and in number in periphery → Intact early antiviral immune response → Generation of antigen specific CD8 T cells → Defective memory immune response → Mitochondria accumulation and increased ROS production in memory CD8 T cells	→ Autophagy is required for the transition from effector to memory CD8 T cells
Atg5 Granzyme-cre (Xu et al., 2014)	→ Deletion autophagy in effector CD8 T cells → No difference in proportion of effector CD8 T cells post activation → No proliferation/survival defects during CD8 T cell expansion phase → Defective survival during transition from effector to memory CD8 T cells	→ Autophagy is dispensable for ealy T cell expansion → Autophagy is required for CD8 T cell effector-memory transition → Dynamic regulation of autophagy in CD8 T cells → Autophagy required for generation of lipids metabolites to fuel FAO

	→ Dysregulated lipid biosynthetic pathways in absence of autophagy	

3.3.2.2 Insights into T lymphocyte activation, their metabolism and the link with autophagy

T cell activation **(Figure 14)**

TCR signal transduction takes place upon presentation of a peptide on MHC to the T cells. It induces activation of different pathways including the protein kinase C (PKC) pathway, mitogen-activated protein (MAP) kinase (MAPK) pathway and the Ca^{2+} pathway, which lead to transduction of transcription factors such as nuclear factor κ B (NFκB), nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1) from the cytosol to the nucleus. These transcription factors promote the expression of genes responsible for T cell survival, differentiation and function as cytokine production. Dissection of the early TCR-induced signals has allowed to establish the numerous events that take place after activation. Activation starts with the conformational change of CD4/CD8 co-receptor that associates with the bound MHC molecule. This allows bringing the lymphocyte protein tyrosine kinase (Lck) associated to CD4 or CD8 cytoplasmic tail, in proximity with the TCR/CD3 complex. This induces the phosphorylation of the tyrosine-based activation motifs (ITAMs) localized on the cytosolic zeta chain of the TCR/CD3 complex by Lck. Phosphorylated ITAMs become docking sites for the zeta chain of T cell receptor associated protein kinase 70kDa (ZAP70), that is further phosphorylated by Lck. Activated ZAP70 is now able to phosphorylate the linker of activation of T cells (LAT) making this protein a new docking site for adaptor proteins and enzymes such as the inducible tyrosine kinase (ITK) and phospholipase Cγ1 (PLCγ1). As a result of this proximity ITK phosphorylates PLCγ1 which activates its capacity to hydrolyze phosphatidylinositol 4,5,-biphosphate (PIP_2), a membrane-bound phospholipid, into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). While DAG stays encored in the cytoplasmic membrane, IP_3 travels to the ER and induces Ca^{2+} release into the cytosol by binding to its receptor on the ER membrane the inositol triphosphate receptor (IP3R). Cytosolic Ca^{2+} triggers the activation of the calcium release-activated channel (CRAC) at the cytoplasmic membrane and the subsequent influx of extracellular Ca^{2+}. Accumulating Ca^{2+} thus binds to calmodulin (CaM) inducing a conformational change which makes it possible for CaM to interact with protein phosphatase calcineurin. Activated calcineurin can then dysphosphorylate NFAT leading to its translocation into the nucleus (Fracchia et al., 2013). NFκB translocation on the other hand is dependent on PKC activation by DAG that induces indirectly phosphorylation of inhibitor of NF-κB (IκB), which is otherwise bound

to NFκB and prevents its access to the nucleus. PKC also mediates the activation of the extracellular signal-regulated protein kinase (ERK)/MAPK pathway via the small GTP binding protein Ras. Ras induces a phosphorylatyion cascade that starts with Raf, a MAP kinase kinase kinase (MAPKKK), then MEK (MAPKK) leading finally to ERK activation. Activated ERK can phosphorylate the AP-1 components Jun and c-Fos in the nucleus leading to the expression of genes involved in survival and differentiation. To sum up this simplified overview of the TCR signaling, there is an interconnection of various players that allow T cell activation. It must be noted however that TCR signaling alone is not sufficient to induce complete activation. As a matter of fact a second messenger is required, namely the activation of co-stimulation receptor CD28 which activates the PI3K/AKT pathway **(Jones et al., 2002)**. This pathway promotes signal transduction that leads to the expression of survival factors such as IL-2 but also by regulating a major actor in metabolic pathways, mTORC1 that will be discussed further below. In any case mTORC1 gets activated indirectly by AKT since this protein actually inhibits the TSC1/TSC2 complex leading to liberation of a small GTPase Rheb which is the direct activator of mTORC1. The interplay of all these pathways including metabolic pathways, are critical for T cell activation, survival, growth, proliferation and differentiation **(Abraham and Weiss, 2004).**

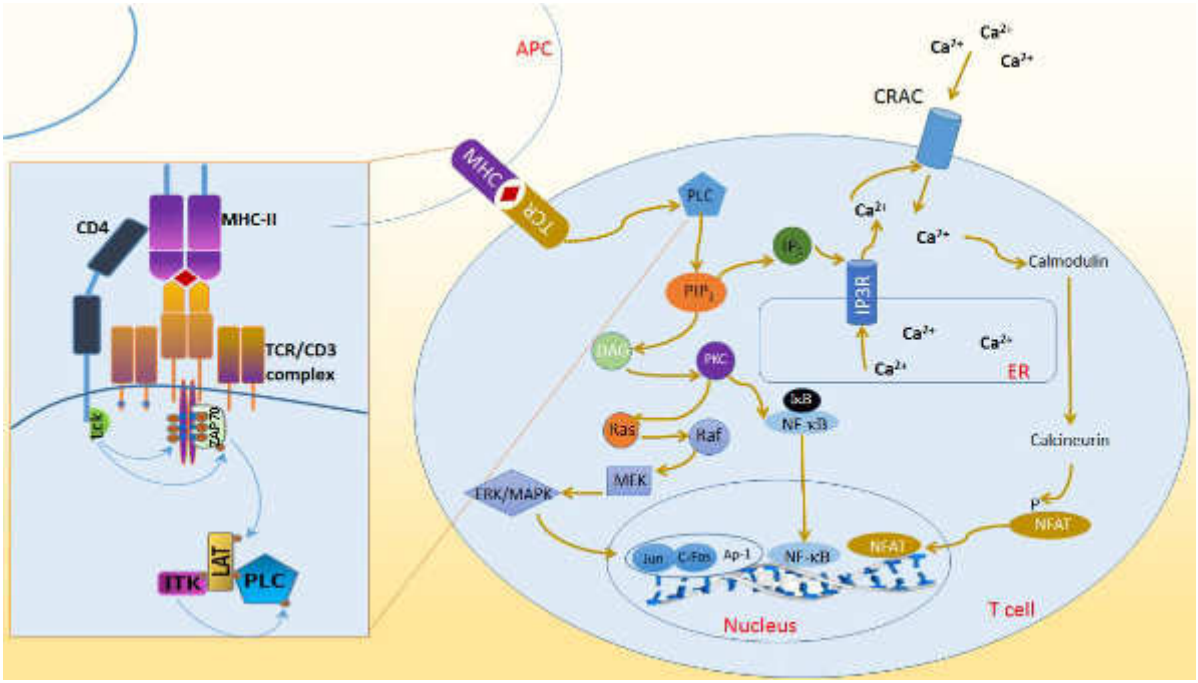

Figure 14: Signaling pathways induced after TCR activation.

Early events induced after TCR stimulation by a peptide bound MHC complex lead to conformational changes of co-receptor CD4 (or CD8) which induces an interaction with the MHC complex as well as the activation of Lck bound to the cytoplasmic tails of CD4 (CD8). Lck phosphorylates ITAM motifs (in blue) in the CD3 zeta chain inducing the recruitment of ZAP-70. ZAP70 undergoes a conformational change as well, which allows the phosphorylation by Lck. Activated ZAP-70 phosphorylates LAT inducing the recruitment of PLC and ITK. PLC is activated through phosphorylation by ITK. Activation of PLC causes the cleavage of PIP2 and the release of DAG and IP3. IP3 binds the IP3R receptor at the ER and induces the release of Ca^{2+} into the cytosol leading to an increase in intracellular Ca^{2+} levels, which activates the CRAC channel at the cytoplasmic membrane and subsequently allows the entry of extracellular Ca^{2+} into the cell. This supply in Ca^{2+} activates CaM which in turn activates calcineurin. Calcineurin is directly involved in the dephosphorylation and nuclear translocation of NFAT. DAG on the other hand activates PKC which phosphorylates IkB and thus orchestrates the liberation of NF-kB and the transclocation of this transcription factor into the nucleus. PKC is also involved in initiating the cascade of other serine-threonine kinases, including Ras and Raf and MAP kinase kinase (MEK) leading to ERK/MAPK activation. This phosphorylation cascade leads to activation of AP1 (composed of Jun and c-Fos).

T cell metabolism a dynamic process **(Fig 15)**

T cell metabolism has been shown to vary depending on the differentiation status of the cell. This characteristic is essential in order to provide the cell with the proper energetic demands required for differentiation, clonal expansion, survival and cytokine production. Thus the activation of a T cell has been shown to induce complete metabolic reprograming dedicated to enhance their proliferative capacity, a feature that they actually share with cancer cells (reviewed in Pearce and Pearce, 2013). As a matter of fact, while metabolic requirements of naïve T lymphocytes depend on oxidative phosphorylation (OXPH), effector T cells rely on aerobic glycolysis also called the Warburg effect. Hence the T cells undergo a switch from a catabolic to an anabolic metabolism.

Naïve T cell metabolism

Naïve T cells depend mainly on glucose, glutamine and fatty acid availability for OXPH, which is a slow process but leads to the generation of 36 ATPs from the glycolysis of one mole of glucose as a starting product. OXPH takes place in mitochondria and involves the degradation of those macromolecules in a catabolic process into intermediate metabolites such as pyruvate, amino acids and fatty acids in order to generate acetylCoA which enters the tricarboxylic-acid cycle (TCA cycle). The TCA cycle produces nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) that fuel OXPH with protons and electrons required for the phosphorylation process of ADP, aiming to generate the end product ATP in an oxygen-dependent reaction **(Verbist et al., 2012)**. Thus OXPH is required for the maintenance of cellular homeostasis in resting cells to insure that basic cell functions such as protein turnover, membrane integrity or ion transport are not compromised. Glucose uptake is thus an important component of OXPH and requires functional glucose transportor 1 (GLUT1) localized at the cell membrane and which has been shown to be upregulated upon T cell activation **(Macintyre et al., 2014)**.

Effector T cell metabolism

The Warburg effect on the other hand only allows the generation of 4 ATPs per mole of glucose but is much faster due to the fact that the glycolysis process is incomplete and leads to the generation of lactate as the major end-product. This metabolic process first described in cancer cells by Otto Warburg, is indeed very particular because it does not use oxygen even though oxygen is readily available which is why this process is also termed aerobic glycolysis. Furthermore it has been suggested that OXPH generates a high amount of ROS susceptible to induce premature senescence or to damage DNA that could then lead to apoptosis. This could explain why highly proliferating cells choose aerobic glycolysis rather than OXPH **(Vander Heiden et al., 2009; Verbist et al., 2012)**. Nevertheless contrary to anaerobic glycolysis, the mitochondria is still functional and a small amount of pyruvate still gets used in the TCA cycle.

Memory T cell metabolism

After encounter with an MHC/peptide complex a small part of the activated T cells differentiate into memory cells. This cells are characterized by the fact that they are in a resting state but can be quickly reactivated upon encounter with the antigen. As such they are phenotypically close to naïve quiescent cells and have therefore a similar metabolic profile meaning that they switch back from aerobic glycolysis to OXPH. Nevertheless they rely mainly on mitochondrial FAO to meet their energetical demands **(Verbist et al., 2012)**.

Metabolic checkpoints

As already mentioned upon activation, T cells require a lot of energy to proliferate rapidly. This goes hand in hand with precise transcriptional changes such as the up-regulation of the transcription factors c-Myc and the estrogen related receptor α (ERRα) that enhance glycolysis and glutaminolysis for *de novo* synthesis of nucleotides while inhibiting fatty acid oxidation (FAO). Hypoxia-inducible factor 1α (HIF-1α) is another transcription factor that intervenes in so-called "metabolic checkpoints". As indicated by its name HIF-1α acts under hypoxic stress and regulates genes that promote cell survival and enhances glucose uptake as well. These are transcriptional check-points but posttranslational metabolic check-points have been identified as well. Thus T cell metabolic events depend on cellular energy sensors such as the adenosine monophosphate (AMP)-activated protein kinase (AMPK) or mTORC1. AMPK gets activated by a high AMP/ATP ratio associated to low energy in the cell as it is the case in starvation or hypoxic conditions and favors OXPH, glycolysis and FAO. Activation if this enzyme enhances glucose uptake and at the same time inhibits mTORC1 by phosphorylating Raptor one of the 4 subunits of this protein complex. mTORC1 has been shown to be activated through the PI3K/Akt pathway that is induced by growth factors and in case of T cell stimulation by CD28 binding. mTORC1 activation promotes protein synthesis but also induces up-regulation of HIF-α and c-Myc expression. Therefore this complex has generally been shown to be involved in anabolic processes, promoting cell growth and proliferation **(reviewed in Fernández-Ramos et al., 2016)**. Thus in T cells mTORC1 rather favors aerobic glycolysis and is a very important factor for T cell survival, proliferation and differentiation after activation. As a matter of fact, mTORC1 regulates T helper cell polarization (Th1, Th2, Th17). AMPK on the other hand promotes naïve and CD8 memory T cell survival through activation of FAO **(Pearce et al., 2009)**.

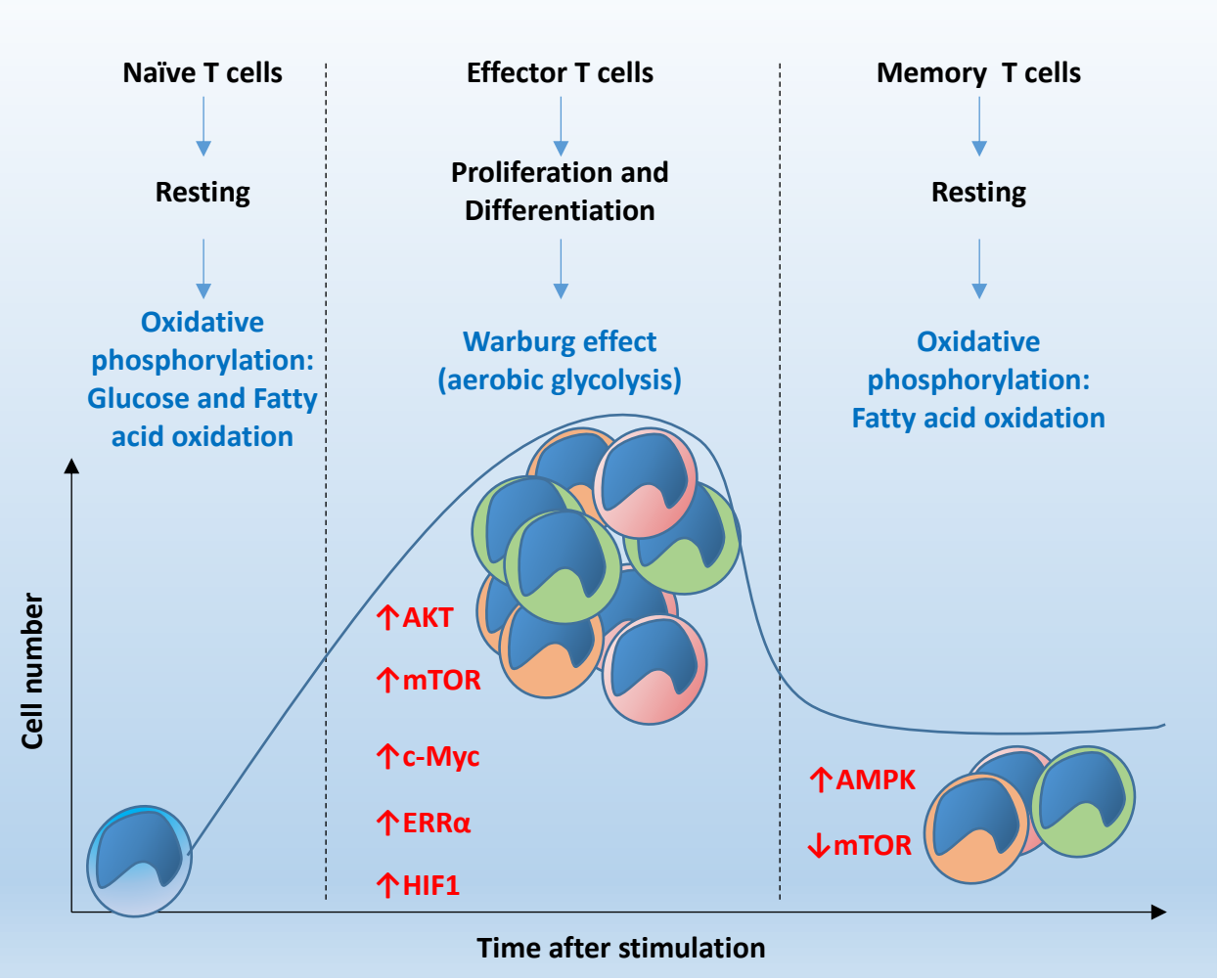

Figure 15: Schematic representation of metabolic T cell regulation during an immune response.

Naïve T cells are resting cells and only require oxidative phophorylation (OXPH) to maintain them in a stady healthy state. After activation they acquire an effector phenotype and thus a high amount of energy to proliferate and differentiate. To respond to these metabolic demands effector T cells undergo metabolic reprogramming reflected by the upregulation of AKT, mTOR and transcription factors such as c-Myc, ERRα and HIF1 resulting in aerobic glycolysis. Memory T cells on the other hand fall back into a resting state and use fatty acid oxidation for their maintenance. They upregulate AMPK which induces mTOR downregulation. **(modified from Verbist et al., 2012). mTOR:** mammalian target of rapamycin; **HIF1:** Hypoxia-inducible factor 1α; **ERRα:** Estrogen-related receptor α, **AMPK:** AMP-activated protein kinase

T cell metabolism and autophagy

The hereafter mentioned energy sensors are also important regulators of the autophagic machinery. As AMPK inhibits mTORC1 it also activates autophagy since mTORC1 inhibits autophagy by phosphorylating ULK1 **(Kim et al., 2011)**. In a low glucose environment, AMPK actually activates the tuberous sclerosis complex 1 and 2 (TSC1/2) a negative regulator of mTOR. Since mTOR is not able to interact with ULK1, this allows AMPK to gain access to this protein and to phosphorylate two of its serines. Activated ULK1 is then able to induce the initiation of the autophagic process. In regard to the

convetional regulation of autophagy by mTORC1 and knowing that TCR activation also induces autophagy, this pathway should actually be inhibited. Yet mTORC1 activation is indeed essential for T cell survival but also differentiation into effector helper T cells. Actually it has been shown that when mTOR activity is decreased and AMPK is highly expressed, it directs the T cell fate towards the differentiation into regulatory T cells which are dependent on FAO, rather than aerobic glycolysis **(Michalek et al., 2011).** This consequently suggests that autophagy induction in effector T cells might be mTOR-independent. There is indeed some evidence supporting this hypothesis as it is the case for Thomas O'Brien and colleagues' study. To summarize in a few words, the authors generated a T cell specific TSC1 knock-out mouse model which leads to a constitutive mTOR activation. Nevertheless autophagy could still be detected in a steady state in T cells and could even be induced in starvation conditions **(O'Brien et al., 2011)**. However a contrasting and surprising discovery by Xiaojin Xu and colleagues was that they saw a blockade of the autophagic flux after CD8 T cell activation via the TCR which is in total contradiction with the data from previous studies. This would in fact be in aggrement with the fact that mTOR is activated in effector T cells and which is usually associated to autophagy inhibition. The authors suggest that this specific aspect could have been missed in previous studies due to differences that can occur between *in vitro* and *in vivo* experiments. It could also be because the previous investigators only used one parameter to evaluate autophagic activity, either determination of the flux by detection of LC3-II or detection autophagosome accumulation. The observed accumulation of LC3-II and the autophagosomes might be the result of a blockade in the autophagic flux **(Xu et al., 2014)**. Another interesting study by Araki and colleagues has shown that treatment of LCMV-infected mice with rapamycin had a beneficial effect on memory CD8 T cell survival **(Araki et al., 2009)**. This is indeed in contradiction with the properties of this molecule mainly used as an immunosuppressive drug but goes hand in hand with recent findings cited in above sections **(3.3.2.1)**, indicating that autophagy plays indeed a role in memory CD8 T cell formation and survival (Puleston et al., 2014; Xu et al., 2014a). Autophagy induction in memory CD8 T cells appears indeed to depend on mTOR inhibition. It is possible that in early stages of T cell activation might be mTOR-independent according to most studies cited above. But the established idea of autophagy induction after TCR stimulation has been recently challenged by Xu and collegues. Thus it seems that further investigations are required in order to clearify wether or not autophagy is induced and required for T cell activation.

4 AUTOPHAGY IN IMMUNE DYSFUNCTIONS

The fact that autophagy plays such an important role in immunity it is to be expected that it might be involved in some immune dysregulations. This process has indeed been suggested to be implicated in the pathogenesis of various diseases such as inflammatory or metabolic disorders as well as in autoimmunity. The link with autophagy and inflammation-associated diseases will only be briefly discussed hereafter. This subject has indeed been addressed in more details by our team in a recent review which has been added to this manuscript **(see Publication 3)**.

4.1 Autophagy in Inflammation-induced Metabolic Disorders

Disorders such as obesity, type II diabetes (TIID) or heart diseases like atherosclerosis often result from a nutritional lifestyle that leads to the development of a so-called metabolic syndrome. A hereditary component is however also involved in the disease pathogenesis. People prompt to develop such a syndrome accumulate metabolic dysfunctions leading to high blood pressure, high insulin level and high cholesterol and triglyceride levels. The resulting disorders mentioned above are also linked to inflammatory events that initiate and aggravate the disease **(Hotamisligil, 2006)**. Autophagy has been shown to be implicated in both in the initiation of inflammation as well as in cellular metabolic functions. Thus this process has been suspected to be involved in the development of these disorders. Furthermore autophagy has been shown to decrease with age which is correlated to an increased susceptibility to TIID and hart diseases **(Cuervo, 2008)**.

4.1.1 Obesity

Autophagy has been shown to regulate lipid droplets turn-over in a process termed lipophagy **(see section 1.4.3) (Singh et al., 2009, 2012)**. On the other hand obesity is clearly characterized by an abnormal accumulation of fat stores. Moreover results from a study by Kovsan and colleagues showed that autophagy was upregulated in adipose tissue from obese people as demonstrated by increased expression of *Atg5*, *Lc3α* and *Lc3b* as well as increased autophagic flux. It was however evidenced that autophagic activity depends on the type of adipose tissue in question. As a matter of fact autophagy was most increased in omental than in subcutaneous adipose **tissue (Kovsan et al., 2010)**. It must be noted that expansion of the omental adipose tissue is associated to an increased risk to develop an insulin resistance leading to TIID **(Bjorndal et al., 2011)**.

Specific invalidation of *Atg7* in a mouse model led to resistance to high fat diet (HFD)-induced obesity. The mice also lost weight, had a better sensitivity to insulin and increased β-oxidation. In the same study the authors were able to show that the observed phenotype was actually due to a change in the balance between brown (BAT) and white adipose tissue (WAT) in favor of BAT **(Zhang et al., 2009)**. WAT adipocytes are responsible for triglyceride storage of and brake-down by mitochondria to generate energy in form of ATP while the BAT is mostly dedicated to generate energy in form of heat. Thus WAT adipocytes seem to be more dependent on autophagy for their maintenance. All of these parameters suggest that autophagy might fuel maintenance of obesity as well as insulin resistance.

4.1.2 Diabetes

Two types of diabetes can be distinguished. Type I diabetes (TID) results from autoimmune events that lead to pancreatic β islets destruction and thus to an impairment in insulin production a hormone required to maintain appropriate sugar levels in the blood. As already mentioned obesity and TIID are often linked to one another. Type II diabetes is associated to an insulin resistance that leads to high blood sugar since glucose cannot be processed properly to generate energy. It also characterized by a β cell dysfunction and increased apoptosis.

Autophagy has been suggested to be implicated in both TID and TIID. However this degradation process seems to be differentially regulated in insulin metabolism. First of all insulin has been shown to negatively regulate autophagy by activating mTOR needed for protein synthesis. Additionally insulin also activates protein kinase B which inhibits FoxO3 a transcription factor involved in the regulation of *Atg* gene expression **(Mammucari et al., 2007)**. In contrast it appears that autophagy might be useful for β-cell homeostasis in order to maintain structure, mass and function of these cells. Specific knock-out of *Atg7* in β-cells resulted indeed in development of hyperglycemia and in reduced insulin production. This could be explained by the fact that β-cell death is increased and insulin granules and β cell mass are reduced in these transgenic mice. Furthermore the β cells exhibited mitochondria swelling and increased colocalization of p62 and polyubiquitinated proteins **(Ebato et al., 2008)**. It appears that ER stress is an important component in diabetes development and that inducing autophagy could eventually contribute to the reduction of this feature. This was indeed observed in a mouse model for diabetes treated with rapamycin **(Bachar-Wikstrom et al., 2013)**. Interestingly autophagy invalidation in β cells from a mouse model for obesity (*ob/ob* mice) resulted in a TIID-like phenotype caused by increased UPR-induced ER stress that led eventually to β cell death **(Quan et al., 2011)**. It was demonstrated that in TIID β cell death could also be induced through an accumulation of intracellular islet amyloid polypeptides (IAPPs) which are co-expressed with insulin. Rivera and

colleagues showed that autophagy was required for p62-mediated IAPPs clearance in β cells **(Rivera et al., 2014)**.

IAPPs as well as high glucose have also been proposed to be implicated in IL-1β mediated inflammation. This pro-inflammatory cytokine depends on the activation of the NLR pyrin domain containing 3 (NRLP3) inflammasome and has been shown to contribute to β cell apoptosis thus to aggravate TIID pathogenesis **(Masters et al., 2010)**. IL-1β seems also to promote TNFα production by adipocytes. Together these cytokines favor resistance to insulin and their interplay is one explanation of the link between TIID and obesity. A study published by Wen and colleagues demonstrated that IL-1β and TNF induce insulin resistance by mediating phosphorylation of the insulin receptor-substrate 1 (IRPS1) at position 307. This has indeed been shown to inhibit the PI3K/Akt signaling pathway. Since insulin resistance has been linked to increased fatty acids availability in the blood with palmitate (PA) being the most abundant, they investigated its effect on the inflammasome. The found out that PA could indeed activate the inflammasome but that it also led the inhibition of AMPK and thus to decreased autophagic activity. These events were also associated to enhanced ROS production by mitochondria probably due to defective mitophagy. ROS being also activators of the NLRP3 inflammasome it seems that an inflammatory loop maintains the TIID phenotype **(Wen et al., 2011)**. In a more recent study the authors used heterozygous mice deficient for *Atg7* (*Atg7*$^{+/-}$) and noticed that they develop an inflammation associated to TIID when crossed with *ob/ob* mice **(Lim et al., 2014)**. Their findings were a break-through in the metabolic field as they demonstrated for the first time the role of autophagy on the whole organism rather than in a specific tissue or cell type. Evidence gathered so far from the different studies clearly indicate that dysregulated autophagy contributes to development of TIID in obesity which makes it an interesting target for the development of drugs to treat metabolic disorders in general and TIID in particular. Furthermore autophagy appears to decrease with age which could thus explain the resurgence of those disorders in that regard.

4.2 Autophagy in Autoinflammatory Diseases

4.2.1 Special focus on Crohn's Disease

CD is charaterized by chronic inflammation that can affect any part of the gastro-intestinal (GI) tract but mostly the end of the small intestine. This inflammation is provoked by a dysregulated immune system which starts reacting against pathogen invading the GI tract but gets overactivated. The exacerbated immune system start recognizing commensal bacteria and the immune response is further fueled by inflammatory cytokines leading to injury of the bowel wall.

Same as in autoimmune disease,s CD development is favored by an interplay of different factors which are environmental (smoking, infections, antibiotics) and genetic. Variants of *NOD2* genes have for instance been strongly linked to CD **(Hugot et al., 2001)**. Different studies have identified loss of function mutations in *NOD2* in most CD patients **(Vignal et al., 2007)**. Genome wide association studies (GWAS) also revealed a link between some variants of *ATG* genes and CD development **(Rioux et al., 2007)**. Single nucleotide polymorphisms were indentified in the *ATG16L1* gene region with the replacement of a threonine by an alanine at position 300 (T300A) being the most recurant variant **(Hampe et al., 2007)**. Further studies lead to the discovery of other variants in other genes essential for autophagy function as it was the case for immune-related GTPase M (IRGM) mainly implicated in xenophagy in response to mycobacteria and viruses **(McCarroll et al., 2008; Parkes et al., 2007)**. Functional studies reveald in fact an interplay of ATG16L1 and NOD2 in association with NOD1 in response to invasive bacteria and more precisely to muramyldipeptide (MDP). Another interesting discovery was that the risk allele *ATG16L1*^{T300A} as well as the most prevalent polymorphism for NOD2 in CD lead to impaired clearance of MDP expressing bacteria through xenophagy. Thus bacteria-induced autophagy seems to be essential to maintain gut homeostasis and to protect against inflammaroty bowel disease **(Travassos et al., 2010)**. McCarroll and colleagues investigated the role of IRGM in the autophagic process and how it is involved in CD. They showed that down-regulating IRGM in intestinal cells led to reduced clearance of pathogenic adherent-invasive *Escherichia coli* (AIEC) known to colonize the ileum of CD patients. In this case mutations in the *IRGM* gene region pontentially alter the elimination of pathogenic bacteria in CD. In contradiction with these results the analysis of one particular IRGM risk allele showed that the mutation induced dedreased binding of IRGM mRNA to its target microRNA 196 (miR-196). It appears however that in healthy subjects miR-196 tightly regulates IRGM induced xenophagy. In CD, miR-196 is overexpressed which generates a strong downregulation of the protective variant of IRGM and thus reduced xenophagy. On the other hand, the risk variant increases but is inefficient in pathogen clearance. Hence in CD, IRGM regulation is disturbed and pathogenic bacteria accumulate in gut epithelial cells **(Brest et al., 2011)**.

The pro-inflammatory status in CD was the first human disorder linking dysregulated autophagy to inflammation. Further investigations showed that *Atg16L1* deficiency resulted in impairment of granule secretion by Paneth cells which are specialized epithelial cells that contain antimicrobial peptides. Specific invalidation of *Atg5* and *Atg7* in the intestinal epithelium using the cre-lox system under the control of the Villin promoter resulted in an indentical phenotype as for the *Atg16L1* deficiency suggesting that autophagy plays a major role in the maintenance of functional Paneth cells. It appears however that these observations were dependent on outside environmental factors. As a matter of fact the same transgenic mice did not display any Paneth cell defect in a pathogen free animal

facility. Cadwell and colleagues finally identified the CR6 strains of norovirus as the mediators of this phenotype. Dispite the viral infection and the genetic invalidation of autophagy the mice still did'nt spontaneously develop colitis indicative of the requirement of additional environmental triggers to develop CD like symptoms. Dextran sodium sulphate (DSS) or ER-stress for instance were shown to be such triggers favoring intestinal inflammation in mice **(Cadwell et al., 2008, 2009)**. However some studies aiming to uncover the role of autophagy in CD patients surprisingly showed that autophagic activity was increased in Paneth cells. Further investigations revealed that the observed autophagic activity was the result of increased autolysosomal degradation of secretory granules resulting in disturbed antimicrobial functions of the Paneth cells **(Thachil et al., 2012)**. On the other hand it has been suggested that autophagy might regulate IL-23 secretion a cytokine known to drive chronic inflammation **(Ciccia et al., 2014)**. Thus it appears that deregulated rather than reduced autophagy is in part responsible for the CD phenotype.

As indicated previously **(see section 3.1.4)** *Atg16L* deficiency leads to increased IL-1β secretion which was linked to the requirement of autophagy for negative regulation of the inflammasome. Both ATG16L1^{T300A} and another *Atg16L1* variant (ATG16L1^{T316A}) strongly linked to CD pathogenesis have in fact been shown to be responsible for enhanced IL-1β response to bacterial infection in macrophages **(Murthy et al., 2014)**. Thus autophagy serves as a break to inhibit exacerbation of the inflammatory response characteristic for Crohn's disease. However ATG16L1^{T300A} appears to be unable to interact with NOD1 and NOD2 which favors inflammation in an autophagy independent mechanism **(see section 1.4.4).** As previously mentioned antigen presentation could also be impacted since Nod induced autophagy has been shown to enhance antigen presentation **(Sorbara et al., 2013**).

4.3 Autophagy and Autoimmunity

The interplay of innate and adaptive immune systems is very efficient in recognizing and eliminating infectious agents as well as cancer cells. The efficiency of the adaptive immune system to recognize an infinity of antigenic structures, imply the potential recognition of self-antigens. This recognition leads to the activation of the immune system after the onset of autoimmune disorders. Autophagy has been shown to be tightly linked to both innate and adaptive immunity regulation. Furthermore autophagy is regulated by environmental factors and single nucleotide polymorphisms highlighted some *Atg* genes as candidates in autoimmune disorders. Thus it appears quite relevant to think that this process is possibly involved in the establishment of autoimmunity.

Autophagy in the maintenance and break-down of immune tolerance **(Fig 16)**

Autophagy has been shown to be required for T cell negative and positive selections in the thymus. A few studies (discussed in section 3.3.2 of this manuscript) have indeed demonstrated autoimmune and autoinflammatory events occurring due to an autophagy deficiency in the thymus **(Aichinger et al., 2013; Kasai et al., 2009; Nedjic et al., 2008; Schuster et al., 2015)**. The results of these studies suggest indeed a requirement of autophagy in TEC for appropriate T cell education and thus to maintain immune tolerance. The relevance of these observations to human pathologies, if any, remains to be determined.

Given that autophagy seems to play a crucial role in antigen presentation, this process has also been considered to be involved in auto-antigen presentation in the periphery, especially in case of citrullinated antigens (CAs) as proposed by Ireland and Unanue **(Ireland and Unanue, 2011**). Interestingly CAs are major targets for autoantibodies in rheumatoid arthritis (RA) but have been associated to SLE as well. Furthermore neutrophil extracellular traps (NETs), which are chromatin structures dedicated to catch and neutralize pathogens, are an important source of CAs when undergoing NETosis, a form of danger signal-induced cell death that has been linked to the autophagy machinery as well **(Khandpur et al., 2013; Pruchniak et al., 2015; Remijsen et al., 2011).** Thus autophagy could be involved in the release of auto-antigens into the extracellular space which favors the induction of systemic auto-immune events

Considering that autophagy plays an important role in the regulation of the type I IFN response towards DNA recognition by PRRs **(discussed in section 3.1.4)**, it is possible to think that autophagy could also play role in the breakdown of tolerance at this level. This makes actually sense, since IC-DNA have been shown to activate autophagy in a TLR9-dependent manner. These complexes rendered immunogenic components from self which leads to autoimmunity when those are recognized. A source of self IC-DNA can be apoptotic bodies or NETs. Moreover, a defective clearance of those apoptotic bodies has been linked to an imparied LAP, non-conventional autophagy described in **section 1.4.2 (Henault et al., 2012)**. This suggests that inhibition of autophagy in human SLE leads to the well documented deficient efferocytosis favoring autoimmunity.

Autophagy in pathophysiology of autoimmune diseases:

Regarding the role of autophagy in the maintenance of cellular homeostasis, in particular for lymphocytes, it is plausible that this process might play a role in peripheral tolerance at this level. As a matter of fact autophagy deregulation has been associated in some extent to various autoimmune disorders.

In multiple sclerosis (MS) an inflammatory autoimmune disease of the central nervous system (CNS) characterized by dymyelination of axons, T lymphocytes infiltrating lesions have been shown to overexpress *ATG5* **(Alirezaei et al., 2009)**. Furthermore autophagy deletion in T cells renders mice resistant to experimental induced autoimmune encephalomyelitis (EAE), a mouse model for MS **(Kovacs et al., 2012)**. Thus in this case autophagy seems to favor the establishment of an autoimmune disease. It must be noted however that Kovacs and colleagues' model was highly lymphopenic, hence one must be careful before drawing conclusions in these conditions as the effect observed could also be due to limited T cell availability.

In rheumatoid arthritis (RA) another inflammatory autoimmune disease, autophagy has also been proposed to participate in the maintenance of the inflammatory phenotype. RA results in the destruction of the cartilage and the joints due to the accumulation of pro-inflammatory cytokine producing immune cells as well as fibroblasts, the latter secreting matrix degrading proteases. In this context autophagy has been shown to be highly increased in fibroblasts in the presence of TNFα, thus protecting these cells from ER stress and subsequently prolonging their survival **(Connor et al., 2012)**. In addition Lin and colleagues have reported increased autophagy in osteoclasts from RA patients related to an increased expression of *Beclin 1* and *Atg7*. Moreover they noticed a reduced bone erosion in mice with an *Atg7* conditional knock-out in osteoclasts even in presence of TNFα **(Lin et al., 2013)**, suggesting that autophagy might participate in the overactivation of osteoclasts observed during RA. In T cells however, autophagy appears to be downregulated in RA patients. These cells also display an increased sensitivity to apoptosis as demonstrated by Zhen Yang and colleagues. They were able to link these observations to defective upregulation of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3 (PFKFB3) in T cells of RA patients. This protein is involved in the maintenance of the Warburg effect in T cells in situations where a strong metabolic activity is required in order to generate sufficient energy. RA T cells are constantly activated and require a great amount of energy. The defective upregulation of PFKFB3 seems to be correlated to a decreased autophagic activity as well. Through LC3-II detection by Western blotting the authors show that autophagy is downregulated in RA T cells. They confirmed the link with PFKFB3 deficiency by knocking down this protein in TCR stimulated normal T cells. This lead to a decreased detection of LC3 while the overexpression of PFKBFP3 induced the reversed phenotype. The absence of autophagy in RA T cells contributes to their senescence leading to premature aging of the immune system a feature characteristic of this disease. Thus in RA autophagy decrease or increase seems cell dependent, but in one case or the other it contributes to disease pathogenesis.

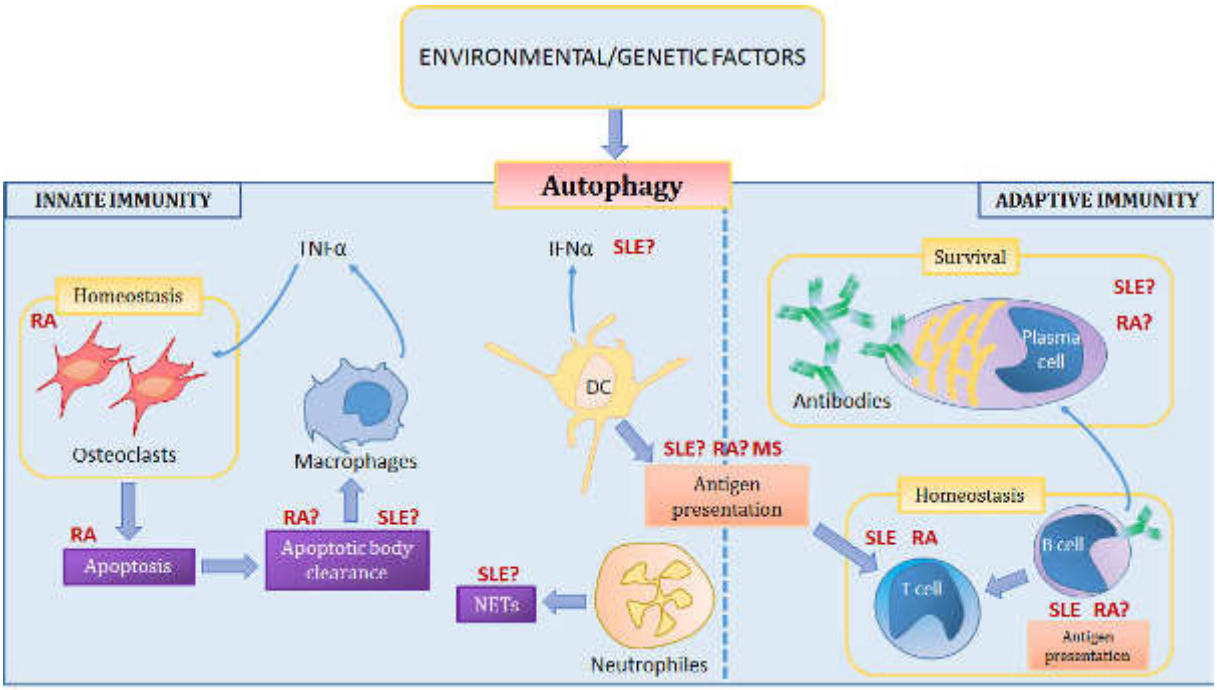

Figure 16: Prouved and suspected implication of autophagy in autoimmune disease development.

Environmental and genetic factors are involved in autoimmune disease pathogenesis and can be regulated the autophagic machinery. Autophagy has been shown to modulate both innate and adaptive immunity. This process regulates cytokine secretion, osteaoclast homeostasis, apoptotic body clearance by macrophages as well as DC-mediated antigen presentation. In adaptive immune responses autophagy has been linked to the maintenance of T and B cell homeostasis, to B cell antigen presentation and to plasma cell survival. The question mark represents the possible involvment of the indicated mechanism in autoimmune disease development. **RA:** Rhumatoïd arthritis, **SLE:** Systemic lupus erythematousis; **MS:** Multiple sclerosis; **NETs:** Netrophil extracellular traps, **TNFα:** Tumor necrosis factor; **IFNα:** Interferon gamma.

4.3.1 Role of autophagy in Systemic Lupus Erythematosus

Autophagy has also been shown to be involved in other autoimmune disorders such as SLE. As a matter of fact various studies including ours, have reported a dysregulation of this process both in T (Alessandri et al., 2012a; Gros et al., 2012a) and B cells of SLE patients as well as in mouse models for SLE **(Clarke et al., 2015)**. In addition, susceptibility loci for *ATG5* and *DRAM1* have emerged from single nucleotide polymorphisms (SNPs) in genome wide association assays (GWAS) **(Zhang et al., 2015b; Zhou et al., 2011a)**.

4.3.1.1 SLE an immunological conundrum

SLE pathophysiology

SLE is a complex systemic autoimmune syndrome. It can indeed affect several organs. Patients suffering from this disease harbor a very polymorphic set of symptoms which vary from one individual to another. It mostly affects women (9 women/1 man) in child-bearing age (15-45 years old) and occurs in flares, meaning that patients experience periods of remission with no apparent symptoms and periods of relapse where the disease is re-activated.

The most common manifestation of SLE is a skin malar rash which forms a "butterfly-like" or a "wolf-like" shape in the face. It is actually thought to be the source of the name given to this disease. Other clinical manifestations are arthritis, uveitis, kidney dysfunction, neurologic disorder among other 11 criteria that have been established by the American College of Rheumatology (ACR) aiming to facilitate lupus diagnosis. Four of them are required to ascertain that a patient is actually suffering from SLE **(reviewed in Tsokos, 2011)**.

SLE pathogenesis **(Fig 17)**

The symptoms underlying the pathophysiology of SLE result from inflammatory events that take place in different organs such as the skin, the kidney, the heart, the lungs and the brain. They can be caused by the deposition of immune complexes (ICs) in the organs due to a defect in clearance of apoptotic bodies. Apoptotic bodies are indeed an important source of auto-antigens such as double stranded DNA (dbDNA), histones as well as constituents of the spliceosome. Defective clearance of apoptotic bodies can indeed lead to chronic activation of the innate immune system through constant stimulation of PRRs via DAMPs but also increases presentation of autoantigens via the MHC thus leading to the activation of autoreactive T cells. Consequently this facilitates the subsequent activation of autoreactive B cells that start producing autoantibodies that form ICs responsible for fueling inflammation in tissue lesions.

The lupus-associated inflammation is correlated to an increased production of type I IFN-induced genes. Interestingly, IFN-α is produced in response to viral infection mainly by pDCs as a consequence of the stimulation of the TLR-7 and 9 by single stranded RNA (sdRNA) and dsDNA respectively. It can also be produced after ICs containing TLR-7 and -9 ligands, like self nucleic acids, thus contributing to the exacerbation of the disease. Moreover, by inducing DCs maturation, type I IFNs in general favor the activation and differentiation of CD4 T cells **(reviewed in Meyer, 2009)**. Mutations in the genes encoding for 3'repair exonulease 1 (TREX-1) have been associated to monoallelic SLE development. TREX negatively regulates the cytosolic IFN-stimulatory DNA response by degrading dsDNA and ssDNA.

TREX deficiency has been shown to be correlated to increased type 1 IFN responses **(Crampton et al., 2014)**. Supplementary evidence demonstrating the pathogenecity of type 1 IFN was given by the lupus mouse model New Zealand Black/White F1 ((NZB/W)F1). These mice are the first generation of the crossing between NZB and NZW mice and spontaneously develop autoimmune symptoms similar to human SLE around 6 months of age. Treating 9-18 weeks old (NZB/W)F1 mice with IFN-α induced an early autoimmune phenotype characterized by detection of anti-dsDNA autoantibodies, proteinuria and development of glomerulonephritis with the first symptoms occurring 10 days post-treatment **(Mathian et al., 2005)**.

B Lymphocytes in SLE pathogenesis

As B cells differentiate into plasma cells that produce antibodies and autoantibodies in an SLE context, they have been extensively studied in order to establish their role in peripheral tolerance breakdown. In short it appears that lupus B cells are hyperactivated in response to BCR stimulation which leads to the increase in calcium flux. Furthermore the stimulation of TLR-7 and 9 by internalized DNA or RNA containing complexes, acts in synergy with the BCR signal, amplifying B cell activation. As B cells are antigen presenting cells, they could also contribute to T cell activation and thus to subsequent inflammation.

T Lymphocytes in SLE pathogenesis

T cells are indeed central players in SLE pathogenesis mostly because they are required to activate autoreactive B cells. Indeed, the treatment of a lupus-prone mouse model (NZB/W)F1 with T cell-depleting antibodies resulted in reduced disease progression and increased survival **(Wofsy, 1990)**. Lupus T cells harbor in many cases a special phenotype. Analysis of T cells from SLE patients has shown that they are in fact hyperactivated when their TCR gets stimulated. This is apparently due to a modification of the TCR/CD3 complex. Contrary to a normal TCR/CD3 complex **(see section 3.3.2)** the ζ chain is replaced by the γ chain of Fc receptor (FcγR) which recruits the spleen tyrosine kinase (SYK) instead of ZAP70. A consequence of this signalosome modification is an increased Ca^{2+} flux. Downstream of this signal calcium/calmodulin-dependent protein kinase type IV (CAMK4) phosphorylates transcription repressor cAMP response element modulator α (CREM-α) that represses IL-2 expression by mediating epigenetic modifications on its promoter while stimulating IL-17 expression **(Hedrich et al., 2011)**. Thus this partly explains one other characteristic of the lupus disease namely a decrease in IL-2 production that has been associated to an impairment in Treg cell survival, and a higher susceptibility of effector T cells for apoptosis. Furthermore CREM is suspected to also regulate SYK expression in lupus T cells **(reviewed in Xu et al., 2012)**. Lupus T cells display other abnormalities such as the increase in mitochondria content as well as augmented mitochondrial

membrane potential, increased ROS production and ATP depletion **(Nagy et al., 2004; PERL et al., 2004)**. These features are also linked to the fact that T cells from lupus patients undergo spontaneous apoptosis. This leads to lymphopenia and even necrosis which is a form of death favoring inflammation **(Emlen et al., 1994)**. Unlike normal T lymphocytes however they are resistant to activation-induced cell death. Additionally an overexpression of the co-stimulation molecule CD40L and the adhesion molecule CD44 could be detected. Thereby, an increased expression of CD40L has been associated to a better costimulation of autoreactive B cell which express CD40 leading to an enhanced autoimmune response. CD44 on the other hand is required for T cell migration to inflammed tissues. In SLE patients it could thus favor T cell infiltration into the kidneys subsequently causing the formation of inflammatory foci **(reviewed in Moulton and Tsokos, 2011)**. As mentioned earlier, lupus T cells overexpress IL-17, a cytokine known to mediate pro-inflammatory responses by favoring the expression of cytokines such as IL-6, TNF-α or CXCL18. Furthermore T helper subset proportions are modified in lupus patients as IL-17 secretion leads to an increase of the Th17 CD4 T cell subpopulation **(Sha et al., 2009)**. Their role in this disease is however not overall accepted **(reviewed in Martin et al., 2014).**

All the T cell alterations have not been discussed hereafter but all in all it appears that the T cell biology is deeply modified during SLE. If these modifications are the consequences or the results of the breakdown of peripheral tolerance is however not very clear. Nevertheless since these cells contribute to the maintenance of an autoimmune phenotype by continuously delivering help to autoreactive B cells, they appear to be good candidates for potential therapeutic targeting.

SLE etiology

SLE is a multifactorial disease which requires both environmental and genetic components to be triggered. The environmental risk factors are diverse and have been identified throughout the years as triggering, maintaining or exacerbating the disease. One such environmental factor is the exposure to excessive ultra-violet (UV) radiations. They can indeed cause DNA damage, induce apoptosis but also lead to the secretion of pro-inflammatory cytokines thus favoring an autoimmune reaction. Furthermore a possible implication of pathogens mainly viruses in lupus pathogenesis has been proposed. Infection by Epstein-Barr virus (EBV) or human cytomegalovirus (hCMV) for instance have been associated to lupus development. Some chemical agents such as cigarette smoke, pesticides or mercury as well as female hormones (estrogens) have been shown to be important risk factors.

The genetic component in lupus was mainly suspected because the disease predominantly affects women in African and Asian populations. Furthermore twin studies have revealed a 24-35% SLE concordance rate in monozygotic twins. Familial aggregation studies have allowed to establish that SLE

is indeed a multigenic disorder but also helped identify a number of susceptibility loci. It is however the genome wide association assays that have led to the identification of a great number of single nucleotide polymorphisms (SNPs) linked to SLE development with about 30 genes clearly associated to SLE disease pathogenesis **(Ceccarelli et al., 2015; Ramos et al., 2010)**. Modern genetics as well as the use of mouse models have greatly contributed to point out genes that could eventually be involved in lupus pathogenesis. The first genes identified to harbor lupus susceptibility alleles were located within the human leucocyte antigen (HLA) region. Among those genes mutations in HLA-DR regions coding in humans for MHC-II were often described in SLE patients. Mutations in the genes implicated in the complement cascade also strongly predispose to SLE development. As a matter of fact complete deficiency in C1q or C4 implicated in the classical pathway are the only mutations associated to a monogenic lupus. These mutations are however quite rare but seeing the crucial role of these molecules in apoptotic body and IC elimination, it demonstrates the importance of clearing the cells from such components in order to maintain tolerance against self-antigens **(Belot and Cimaz, 2012)**. Other SLE susceptibility loci in genes associated to phagocytosis have also been identified. Some of these genes belong the Fcγ receptor family devoted to recognition of the constant region of antibodies when associated into ICs and thus leading to their elimination **(Takai, 2002).** Mutations in genes involved T and B cell development, function or signaling have also been associated to lupus. This is for instance the case for *IKZF1, STAT4, PTPN22, BLK* or *LYN.* The consequence of these mutations are often related to hyperactivation of the lymphocytes which leads to breack-down of tolerance **(Crampton et al., 2014)**.

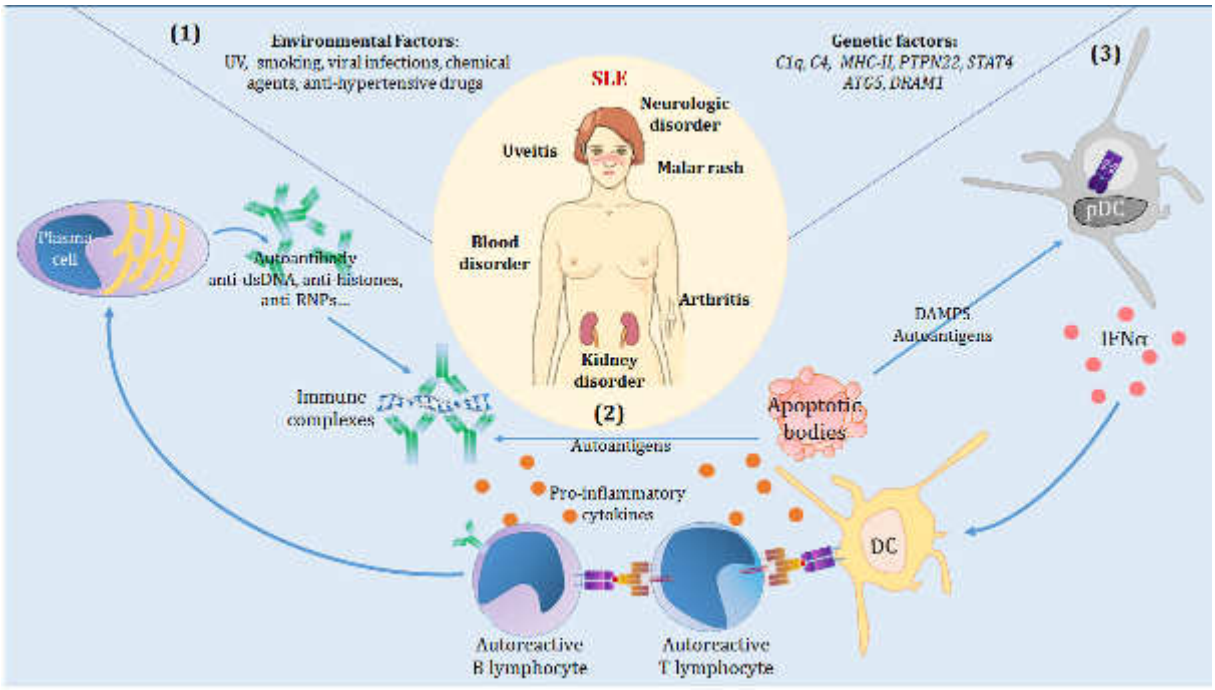

Figure 17: Modelization of factors leading to SLE pathogenesis.

(1) The interplay of environmental and genetic factors appears to favor disease development. **(2)** A set of symptoms that can be found in patients suffering from SLE a disorder affecting predomonantly women. **(3)** Overview of immunological events leading to tolerance breakdown. An excess of apoptotic bodies can be the source of autoantigens and DAMPs. DAMPs can activate cells from the innate immune system and pDCs in particular which leads to IFNα secretion and subsequently activate DCs. The adaptive immune system is activated by the presentation of autoantigen to autoreative T lymphocytes leading to autoreative B lymphocyte activation and differentiation into plasma cell secreting autoantibodies mostly directed againt nuclear antigens.The autoantibodies assemble into immune complexes that can deposit in various organs, provoking tissue lesions that induce inflammation leading to the symptoms in **(2)**. Tissue lesions represent a new source of autoantigen that can stimulate TLRs exacerbating further the autoimmune response leanding to disease progression. **DAMPs**: Danger-associated molecular patterns**; IFNα:** Interferon alpha

4.3.1.2 SLE and autophagy

Genetic links between SLE and autophagy

As previously discussed autophagy has been shown to be involved in a number of autoimmune diseases including SLE **(section 4.3)**. One of the first studies indicating that autophagy might play a role in lupus development demonstrated that sera from SLE patients could induce autophagy in a human neuroblastoma cell line **(Towns et al., 2005)**. Later on, a GWAS conducted in a Chinese population identified a SNPs in the intergenic region between *PRDM1* and *ATG5* (Gateva et al., 2009; Zhou et al., 2011a) as associated to SLE development. Common *ATG5* variants have been identified by other studies within or near the coding region as well as in other *ATG* such as *ATG7* **(Pickford et al., 2008; Zhang et al., 2015b)**. An independent study carried out on a northern European population however could not demonstrate a genetic link between *ATG5* polymorphism and SLE **(Järvinen et al., 2012)**. This

could be explained by the diversities that can be found in the different ethnic populations that could drive susceptibility to develop SLE or not. Interestingly a study once again in an Asian population described a susceptibility genetic variant to SLE near *DRAM1 (*damage-regulated autophagy modulator A) encoding a lysosomal membrane protein able to induce autophagy when activated by p53 **(Yang et al., 2013; Yen Mah et al., 2012)** under genotoxic stress. These studies indicate that autophagy could constitute a pawn in SLE pathogenesis.

Autophagy in SLE cellular functions

On the functional level several cellular dysregulations in SLE have been associated to autophagy. For instance ICs recognition leading to the IFN signature by pDCs, one characteristic in SLE, has been shown to be autophagy-dependent **(see section 1.4.2).** Furthermore autophagy has been shown to mediate clearance of apoptotic bodies. Thus deficient autophagy could be associated to persisting apoptotic bodies susceptible to induce inflammatory responses if various organs but which are alos an important source of auto-antigens. All of this elements can lead to tolerance breakdown. Some studies strongly suggest the involvement of this process in SLE but wether autophagy is implicated in the development or maintenance of the disease still needs to be investigated. Our team and others showed that autophagy markers were increased in T cells from lupus patients as well as in mouse models for lupus ((NZB/W)/F1 and MRL^{lpr+} mice) at a basal level and even more after activation (Clarke et al., 2015; Gros et al., 2012a). Our team demonstrated an upregulation of the autophagic activity in T cells specific to SLE patients and lupus-prone mice, that seemed correlated to disease aggravation in mice. It must be noted however that our study included only a few patients. These results were confirmed by Clarke and colleagues who showed further increased autophagy in lupus B cells a feature that had not been observed in previous studies. In addition, Pierdominici's team detected differences in naïve vs memory T cells with autophagic activity being higher in memory T cells both in healthy controls as well as in SLE patients **(Alessandri et al., 2012b)** which is in agreement with recent studies suggesting that autophagy might be important for the maintenance of memory CD8 T cells **(Puleston et al., 2014; Xu et al., 2014a**). Contrary to the studies by Gros, Arnold et al. and Clarke et al., however, the only difference they saw when comparing healthy donors to SLE patients was an increase of autophagy in naïve cells from SLE patients. Furthermore they observed that sera from SLE patients could induce autophagy in normal T cells but that surprisingly lupus T cells were resistant to that stimulus. According to the authors, this might be due to a chronic exposure to autoantibodies that could lead to selection of autophagy-resistant T cells in SLE but it can be speculated that due to an already high level of autophagy in these cells it is possible that it might not be possible to generate more autophagosomes.

Autophagy, mTOR and SLE

The role of autophagy in lupus is further emphasized by the fact that rapamycin, the mTOR inhibitor, has been shown to restore normal T cell activation by normalizing calcium signaling thus reducing disease activity **(Fernandez et al., 2006)**. This seems surprising as inhibition of mTOR is supposed to lead to autophagy induction. Autophagy however seems to favor SLE pathogenesis as indicated by some studies cited above. But it can be argued that the observed effect of rapamycin might be due to its immunosuppressive effects *per se* rather than the activation of autophagy. It could also be another indication that autophagy induction in T cells is indeed mTOR independent. As a matter of fact while autophagy markers are increased in SLE, so is mTOR activation, especially in T cells. It is documented that mTOR inhibition leads to an increase in Tregs, which participate in the reduction of exacerbated immune responses in SLE but also favors differentiation of CD8 memory T cells **(Fernandez and Perl, 2010)**. Furthermore in other contexts, rapamycin reduces the pDCs induced pro-inflammatory response, inhibits co-stimulation signal as well as graft vs host disease (GVHD). With this in mind it appears that the influence of rapamycin on the immune system is widespread. But it has been suggested that this might also depend on the dose administrated to the patients **(Zhang et al., 2012)**. Nevertheless other drugs used to treat lupus such as chloroquine and hydroxychloroquine have been shown to modulate autophagy by blocking the flux. This suggests that inhibiting autophagy could be interesting for the design of new lupus treatments. Furthermore there is some evidence of autophagy deregulation B cells as well. Clarke and collegues demonstrated using an LC3-specific dye that B cell from lupus patients and lupus prone mice displayed a higher autphagic activity and that this process was especially required for plasmablast differentiation **(Clarke et al., 2015**). Since these cells produce autoantibodies that are the main feature of this disease autophagy could be specifically targeted to eliminate these cells. Recent studies on B cell autophagy have in fact proven a role of this process in the maintenance of long-lived plasma cells strengthening further the possible contribution of this process in the maintenance of an autoimmune phenotype.

The aims of my thesis project:

Prior to the start of my PhD work, our team was able to show that autophagy was upregulated in T cells from both systemic lupus erythematosus (SLE) patients and lupus prone mice ($MRL^{lp/lpr}$, (NZB/W)F1) (Gros, Arnold *et al.*, 2012). My PhD research project aimed then at establishing the implication of autophagy in T and B lymphocyte function in normal and pathological humoral immune responses. For that purpose we generated mice models with a specific invalidation of Atg5 either in B or T cells.

As discussed in the introduction of this manuscript several animal models with specific deletion of essential *Atg* genes in lymphocytes have been generated by other teams. They have allowed to answer a number of questions on the role of this process in lymphocyte biology. But until very recently in most models autophagy invalidation occurred early during development. Autophagy has been shown to play a central role in the generation of hematopoietic progenitor cells. In these conditions it can be speculated that the observations made so far on impaired lymphocyte survival upon activation could be related to an early developmental defect. Thus we were particularly interested in studying the role of autophagy in mature lymphocytes *in vivo*. As a matter of fact only few investigations have taken an interest in *in vivo* immune responses in absence of autophagy in those cells.

The first investigations led to the generation of two mice models with a specific deletion in immature and mature B cells ($Atg5^{f/-}$ Mb1-cre and $Atg5^{f/-}$ CD21-cre respectively). We were able to show that autophagy is dispensable in early B cell development. We further observed that mice with an autophagy deficiency in peripheral B cells ($Atg5^{f/-}$ CD21-cre) were still able to generate an antigen specific short-rem humoral response. When studying the long-term humoral responses in C57BL/$6^{lpr/lpr}$ $Atg5^{f/-}$ CD21 cre autoimmune-prone mice however we noticed that the anti-nuclear IgG response was impaired. Thus it appears that in absence of autophagy long-term plasmocyte survival is compromised and leads to impaired antibody secretion especially in an autoimmune context.

My PhD project was especially focused on studying the role of autophagy in T cells with two main objectives in mind. First we wanted to establish the role of autophagy in T cell-dependent humoral responses, in order to better understand how, when and why this process is important for these cells. Secondly we wanted to delineate through which signaling pathway(s) autophagy is induced in T cells both in a normal and a lupus context

To address the first point, we generated transgenic mice deficient for autophagy only in mature T cells. We assed T cell function of these mice *ex vivo* and observed normal CD4 T cell survival and proliferation after activation. We immunized our mouse models with a T dependent antigen, Ovalbumin, and we

noticed that while the primary and even the secondary immune response was normal in amplitude, their long-term humoral response was impaired. This led us to the conclusion that the memory response was impacted by the absence of autophagy in T cells. In order to asses if the need of autophagy was intrinsic to CD4 memory T cells, we transferred autophagy incompetent CD4 T cells from immunized mice into recipient mice which were then immunized with the same antigen. Compared to the controls, their memory humoral response was indeed weaker. We moreover showed that the absence of autophagy had an impact on memory CD4 T cell survival by inducing a memory phenotype in CD4 T cells *in vitro.* We found that T cells deficient for autophagy had a survival defect under long term IL-7 treatment. Hence it seems that autophagy is essential for normal function of memory T cells.

My second concern was to better understand the signaling pathways leading to autophagy induction after T cell receptor (TCR) stimulation. We either activated or inhibited the main pathways induced after TCR activation, in order to assess their impact on autophagic activity. For the time being we have been able to show that the calcium pathway is mainly involved in the induction of this process. This led us to think that downstream of the Ca^{2+} pathway, nuclear factor of activated T cells (NFAT) transcription factor might be involved in the regulation of some autophagy-related genes (*ATGs*). However quantitative PCR experiments did not confirm this hypothesis. Thereby we started exploring a possible post-transcriptional or post-translational regulation of ATG proteins by the activation of the calcium pathway. We focused more specifically on light chain 3 (LC3) levels regulation, since the expression of this ATG protein varies the most in our conditions when the Ca^{2+} pathway is modified. Preliminary results obtained using transcriptional and translational pharmacological inhibitors, indicate that the regulation might occur at the translational level. We intend to explore further this path and would like to identify which actors are involved in this regulation.

All in all, the studies we have done so far on T and B cells have given new insights into the role of autophagy in lymphocytes biology, at different levels of their development and function. Targeting this process could be an interesting strategy in chronic inflammatory diseases involving lymphocytes.

1 Publication 1

1.1 Forword

Macroautophagy is dispensable for B cell development but required for homeostasis and long-term humoral autoimmune responses

Johan Arnold, **Diane Murera**, Florant Arbogast, Jean-Daniel Fauny, Sylviane Muller and Frédéric Gros

The aim of this study was to delineate the impact of autophagy in B cell development but also to evaluate the importance of this process in B cell mediated humoral responses in an autoimmune context. Previously described models proposed that autophagy is involved in early B cell development **(Miller et al., 2014).** Their observations were however based on a chimera mouse model. In this model B cells are deficient for autophagy already at the progenitor level which makes it tricky to differentiate if the observed survival defects result from autophagy requirements for B cell development or if they result from defects accumulated throughout hematopoiesis. Thus the generation of a mouse model with a specific deletion early in B cell development ($Atg5^{f/-}$ Mb1-cre) showed that contrary to available data autophagy is not required at that stage. Evaluating the role of autophagy in B cell peripheral functions was also of great interest especially since we showed that at the developmental level there was no specific difference. Thus we generated transgenic mice with a B cell specific autophagy invalidation at the mature stage using the Cre-Lox system under the control of the CD21 promotor which resulted in $Atg5^{f/-}$ CD21-cre mice. Immunization experiments on these mice revealed that early responses are not impaired. Using the same autophagy deletion strategy on mice with an autoimmune genetic background ($C57B/L6^{lpr/lpr}$ $Atg5^{f/-}$ CD21-cre) resulted in reduced autoimmune features.

When we started this study only few data on B cell autophagy were available and the role of autophagy in B cell development was not very well established. As for the role in autoimmunity no study had taken real interest in the subject in an *in vivo* context at that time. Hence this works is an important contribution in this erea of research.

1.2 Autophagy is dispensable for B-cell development but essential for humoral autoimmune responses

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Autophagy is dispensable for B-cell development but essential for humoral autoimmune responses

J Arnold[1], D Murera[1], F Arbogast[1], J-D Faury[1], S Muller[1,2] and F Gros*[,1,3]

To gain new insight into the role of B-cell autophagy, we generated two novel mouse models deficient for the autophagy-related gene (*Atg*)*5*, one from the outset pro-B cell stage (*Atg5*$^{f/-}$ Mb1 cre) and the other in mature B cells only (*Atg5*$^{f/-}$ CD21 cre). We show that autophagy is dispensable for pro- to pre-B cell transition, but necessary at a basal level to maintain normal numbers of peripheral B cells. It appears non-essential for B-cell activation under B-cell receptor stimulation but required for their survival after lipopolysaccharide stimulation that drives plasmablast differentiation and for specific IgM production after immunization. Results obtained using Atg5$^{f/-}$ CD21 cre x C57BL/6$^{lpr/lpr}$ autoimmune-prone mice show that B-cell autophagy is involved in the maintenance of anti-nuclear antibody secretion, elevated number of long-lived plasma cells, and sustains IgG deposits in the kidneys. Thus, treatments specifically targeting autophagy might be beneficial in systemic autoimmune diseases.
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Macroautophagy is a catabolic process allowing the degradation of cytoplasmic material in double membrane vesicles, ultimately fusing with lysosomes. Macroautophagy, initially implicated in the generation of nutrients under metabolic stress, is known to have multiple roles, in different physiologic compartments, such as in vacuole trafficking, cell signalling, and cell death. Macroautophagy is deeply involved in the regulation of immunity.[1] It has been shown that autophagy can regulate inflammation related to inflammasome activation and to type I interferon secretion. Moreover, it contributes to antigen presentation by both major histocompatibility complex (MHC) class I and class II molecules.[2]

Macroautophagy is also tightly linked to lymphocyte activation and survival. It has central roles in T-cell basal homeostasis, survival, and polarization.[3] It is also involved in the regulation of T-cell signalling by downregulating the NF-κB pathway[4] and apoptosis processes through the procaspases 3 and 8 degradation.[5]

Macroautophagy has additionally been described to regulate B-cell lineage, in particular during B-cell development. Thus, it has been shown that B cells generated from fetal liver chimaeras, with a complete deletion of the essential autophagy-related gene (*Atg*)*5*, exhibited a block at the pro- to pre-B stage transition.[6,7] However, as the genetic deletion is systemic and occurs very early during development, the question remains over whether the developmental blockade could be due to defects resulting from early haematopoietic development. Indeed, macroautophagy has been shown to be fundamental to haematopoietic stem cell survival and renewal.[8] Moreover, conditional deletion of *Atg5* under the control of CD19 promoter expressed from the pre-B stage does not lead to major developmental breaks, except a decrease in B-1a B-cell population.[8] The contrast with results obtained with chimaeric mice could be due to the partial deletion induced by cre expression under the control of CD19 promoter.[9] At this stage, however, it remains unclear whether macroautophagy is really needed in the first steps of B-cell development.

Studies based on mouse models deleted for autophagy genes at an early stage of development led to fundamental findings regarding the importance of macroautophagy in B cell-related immune responses. Two independent studies, based on T cell-dependent and -independent model antigens or on infectious agents,[10,11] concluded that macroautophagy is necessary *in vivo* for humoral immune responses by regulating plasma cell differentiation and survival. The former study[11] showed that macroautophagy is involved in regulating endoplasmic reticulum (ER) load along with plasma cell differentiation, thus limiting ER stress and contributes to long-lived plasma cell survival. A role for macroautophagy in B-cell early activation was excluded, especially in germinal centre (GC) formation. Two more recent studies, one based on a mouse model of influenza infection and the other on model antigens used as immunogens, demonstrated that in addition to its effect on plasma cell survival, macroautophagy is necessary for the survival of B-cell memory compartment.[12,13] These findings therefore also implicate macroautophagy in humoral autoimmunity.

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Abbreviations: Abs, antibodies; ACTB, β-actin; Atg, autophagy-related genes; BCR, B-cell receptor; CFA/IFA, complete/incomplete Freund adjuvant; CFSE, carboxyfluorescein succinimidyl ester; CMA, chaperone-mediated autophagy; dsDNA, double-stranded DNA; ER, endoplasmic reticulum; FO, follicular; GC, germinal centre; IRF4, interferon-responding factor 4; LC3, light-chain 3 standing for MAP1LC3B, microtubule-associated protein light-chain 3B; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MZ, marginal zone; OVA, ovalbumin; PNA, peanut agglutinin; SLE, systemic lupus erythematosus; T1 and T2, transitional 1 and 2; TCR, T-cell receptor
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Studying the involvement of autophagy in autoimmunity is justified by its central regulatory impact in inflammation and its role on antigen presentation and on lymphocyte activation and survival.[14,15] Our team and others described a deregulation of macroautophagy in T cells from both lupus-prone mice and patients suffering from systemic lupus erythematosus (SLE).[16–18] This deregulation could contribute to autoreactive T-cell survival and can be put in line with the deregulation of both macroautophagy and chaperone-mediated autophagy (CMA) in B cells that has been recently described to occur in lupus.[19,20] To date, however, these data remain correlative and no study has been published describing an *in vivo* model, prone to systemic autoimmunity, with specific autophagy deletion in B cells.

Here, we describe the generation of two new mouse models of conditional ATG5 deletion, one under the control of a promoter active early during B-cell development (Mb1 cre) and the other active in mature B cells only (CD21 cre). They were designed and constructed to clarify the role of macroautophagy in B-cell development and homeostasis. Comparison of the two models should allow us to define whether identified defects are linked to developmental issues or deregulation of homeostasis. In addition, $Atg5^{f/-}$ CD21 cre autophagy-deficient mice were crossed with autoimmune-prone animals to further examine the role of macroautophagy *in vivo* on long-term humoral autoimmunity and study some lupus-related pathophysiological features.

Results

Basal levels of autophagy are necessary for B-cell maintenance. We generated two new mouse models deficient for ATG5 specifically in B cells. The first one, with cre recombinase expression under the control of Mb1 promoter, was deleted early during B-cell development while the second one, under the control of CD21 promoter, was designed to be deleted in mature B cells only. Both mouse models were born at the predicted Mendelian ratio and showed no particular morphologic phenotype.

$Atg5^{f/-}$ CD21 cre (CD21 cre) mice exhibited no deletion of ATG5 in splenic T cells and in these cells, conversion of LC3-I to LC3-II occurred normally (Figure 1a). However, as expected, B cells from CD21 cre mice showed a specific deletion of ATG5 compared with B6 mice (wild type, WT) and littermate $Atg5^{f/+}$ CD21 cre mice at both transcriptional and protein levels (Figures 1b and d). A poor conversion of LC3 was observed in $Atg5^{f/-}$ CD21 cre mice validating the impeding of the autophagy process, even under stimulation by anti-IgM. As previously reported by others,[10] no increase in autophagic activity was found after BCR stimulation in WT and littermate mice. We also checked whether autophagy was decreased in B cells from $Atg5^{f/-}$ Mb1 cre (Mb1 cre) mice. We saw a very efficient deletion of ATG5 in splenic B cells from $Atg5^{f/-}$ Mb1 cre mice, correlating with a total impairment of LC3-II generation (Figures 1c and d). *Atg5* mRNA expression was reproducibly lower than for CD21 cre B cells. Although B cells from CD21 cre mice exhibited a reproducible LC3-II knockdown (Figure 1d), LC3-II was no longer observed in Mb1 cre B cells, arguing for a specific deletion of autophagy that is stronger in this model than in CD21 cre mice. Although ATG5 deletion is observed in littermates relative to both CD21 cre and Mb1 cre mice compared with WT C57BL/6 mice, autophagy levels (deduced from LC3-II staining) were comparable and no significant difference was observed in terms of autophagic flux intensity (Supplementary Figure S1).

To more specifically evaluate the potential autophagy-independent effects of decreased ATG5 expression on phenotypes of both littermates, we then conducted a series of experiments using CD21 cre and Mb1 cre mice separately compared with WT mice. First, we quantified the different subpopulations of B cells in the spleen of these two defective models. Although both types of littermate mice exhibited levels of B cells comparable to wild-type mice, both Mb1 cre and CD21 cre mice showed a diminished number of B cells in the spleen (Figures 2a and b). A decrease in follicular B-cell number and T1 B cells was also observed in both CD21 cre and Mb1 cre mice, as compared with WT mice. However, the homeostatic defect of follicular (FO) B cells in CD21 cre was less severe than in Mb1 cre mice, arguing for a more efficient autophagy impairment in the latter deletion strategy, as described above in Figure 1. Mb1 cre T2 B cells were also diminished compared with the corresponding littermates. However, marginal zone B cells were not seen to be affected by autophagy deletion in both deletion models. These results led us to propose that *Atg5* deletion in B cells induces a defect in peripheral homeostasis, the latter being more pronounced in the context of Mb1 cre-mediated deletion.

Next, we assessed the repartition of B-cell populations from the peritoneum. A decrease in both B-1a and B-2 B-cell proportions was observed in Mb1 cre mice while, in contrast, a decrease in peritoneal B-2 B cells only was visualized in CD21 cre mice (Figures 2f and g). This finding led us to conclude that early during development, autophagy is necessary for the generation of B-1a B cells. The loss of B-2 cells in both mice might be due to sensitivity to cell death of these Ab-secreting cells in the absence of autophagy, or to a defective replenishment from circulating cells.

Altogether, these results show that autophagy is central during development for B-1a B-cell generation. Basal levels of autophagy are necessary to maintain a normal number of B cells in the periphery. B-2 B cells of the peritoneum are particularly sensitive to the decrease in autophagic activity.

Basal levels of autophagy are not necessary for B-2 B-cell development. The survival defects of peripheral B cells found in Mb1 cre mice could be linked to a developmental issue as described in earlier studies using bone marrow fetal liver chimaeras.[5] This does not seem to be only attributable to developmental issues as some defects were also found in CD21 cre mice. We checked the number of precursor cells of the B-cell lineage in the bone marrow of Mb1 cre and CD21 cre mice, and compared the data with WT and littermate mice (Figures 3a and b). As expected, no anomaly in B-cell number or in subset distribution was detected in CD21 cre mice when autophagy deletion occurs later during development (Figures 3c and f). In sharp contrast with previous studies,[5] we did not find any significant decrease in the number of B-cell precursors in Mb1 cre mice. Previous data suggested a developmental block at the

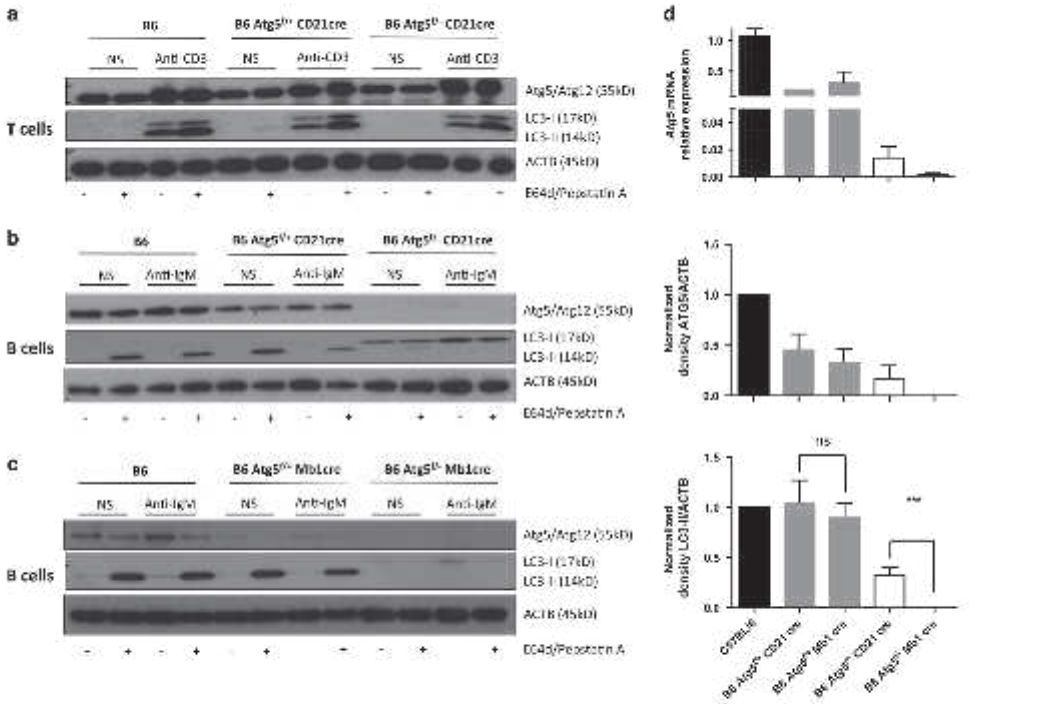

Figure 1 Efficient invalidation of autophagy in B cells from *Atg5*$^{fl/fl}$ CD21 cre mice and *Atg5*$^{fl/fl}$ Mb1 cre mice. *Atg5*$^{+/+}$ CD21 cre, *Atg5*$^{+/+}$ Mb1 cre, *Atg5*$^{fl/fl}$ CD21 cre, *Atg5*$^{fl/fl}$ Mb1 cre, and *Atg5*$^{fl/fl}$ Mb1 cre were generated. B or T cells were purified from the spleen and cultured in vitro in the presence of different stimuli. Lysosomal protease inhibitors pepstatin A and E64d were added (+) or not (−) in the indicated conditions. (**a**) T cells from wild-type C57BL/6 mice (B6), *Atg5*$^{fl/fl}$ CD21 cre, or *Atg5*$^{fl/fl}$ CD21 cre were left unstimulated (non-stim) or stimulated by anti-CD3 Ab for 18 h. Cells were lysed and immunoblots were performed against ATG5, LC3, and ACTB. (**b** and **c**) B cells from wild-type B6, *Atg5*$^{+/+}$ CD21 cre or *Atg5*$^{+/+}$ Mb1 cre, *Atg5*$^{fl/fl}$ CD21 cre (**b**), *Atg5*$^{fl/fl}$ Mb1 cre or *Atg5*$^{fl/fl}$ Mb1 cre (**c**) were left unstimulated (non-stim) or stimulated by anti-IgM Ab for 16 h. Cells were lysed and immunoblots were performed against ATG5, LC3, and ACTB. (**d**) Upper panel: Quantification of Atg5 transcripts in B cells by real-time PCR relative to Gapdh expression. One sample from B6 mouse was used for each plate for normalization and its value is arbitrarily set to 1. Middle and lower panels: Densitometric analysis of ATG5 and LC3-II expressions relative to ACTB. The values corresponding to the non-stimulated condition in the presence of protease inhibitors were collected and normalized to the ratios obtained from B6 wild-type mice on each blot to normalize results. The histograms represent the means with S.E.M. obtained for at least three and seven different blots, respectively, for ATG5 and LC3-II quantification. ns, non-significant; $^{**}P < 0.001$ (Mann–Whitney *U*-test)

transition between the pro- and pre-B cell stages.[6] In this study, normal proportions of pro- and immature B cells were detected in the bone marrow of early ATG5-deleted mice (Figures 3c and d). Distinction of pre-B and immature B cells from pro-B cells by CD43 staining did not reveal any decrease in the proportion of pre-B cells in Mb1 cre mice, but instead showed an increase in this population (Figures 3e and f). Thus, autophagy seems dispensable for the pro- to pre-B cell differentiation. However, as previously described by others[6] using CD19 cre mice, we observed a decrease in the proportion of mature B cells, corresponding to Fraction F defined from Hardy nomenclature (Figures 3c and f).[21] As the overall number of B cells does not vary between Mb1 cre and their littermates, the increased proportion of pre-B cells in these mice could reflect a spatial compensation consecutive to the loss of mature B cells. As these latter cells are described as recirculating cells coming from the periphery, they may not necessarily constitute a final developmental stage occurring in the bone marrow. They have also been considered as cells secreting Abs against blood-borne pathogens.[22] The acute deletion of autophagy in Mb1 cre mice might thus lead to sensitivity to cell death of this B-cell subtype, because of their long-lived nature or of their Ab secretion properties.

Taken together, these results show that autophagy is dispensable for the transition between pro- and pre-B cell stage. However, basal levels of autophagy are necessary to maintain a normal number of mature recirculating B cells in the bone marrow.

Autophagy is dispensable for B-cell activation in response to BCR stimulation. We then assessed in our two models the potential relevance of autophagy activation in B-cell response to BCR stimulation. We found no difference

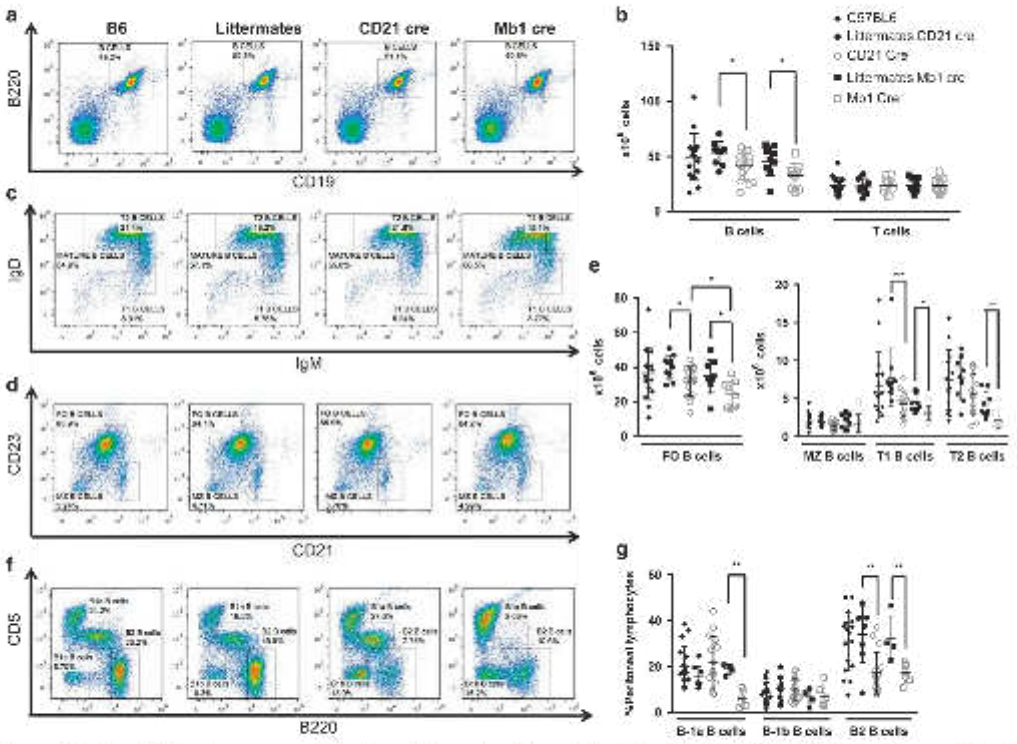

Figure 2 Basal levels of autophagy are necessary for B-cell survival in the periphery. Spleen cells from wild-type C57BL/6 mice (B6), $Atg5^{fl/fl}$ CD21cre (littermates CD21 cre) or $Atg5^{fl/fl}$ Mb1cre (littermates Mb1 cre), $Atg5^{fl/fl}$ CD21 cre (CD21 cre) or $Atg5^{fl/fl}$ CD21 cre (Mb1 cre) were stained with anti-B220 and anti-CD19 Abs (a and b). Representative dot plots obtained after flow-cytometry analysis are depicted in (a) showing the percentages of $B220^+CD19^+$ B cells. (b) Individual values for each mouse tested of absolute numbers of B and T cells, in the spleen obtained from B6 mice $n=16$, littermate CD21 cre mice $n=10$, littermate Mb1 cre mice $n=9$, CD21 cre mice $n=15$, and Mb1 cre mice $n=9$. Means and standard deviations (S.D.) are indicated. (c–e) Expression was assessed for the spleen of the same animals for the expression of (c) IgD, IgM and (d) CD21 and CD23. Representative dot plots after flow-cytometry analysis show the percentages among $B220^+$ cells of mature B cells ($IgM^{int}IgD^+$), transitional 1 and 2 (T1 and T2, respectively, IgM^+IgD^{int} and gM^+IgD^+), follicular (FO) B cells ($CD21^+CD23^{hi}$), and marginal zone (MZ) B cells ($CD21^{hi}CD23^{lo}$). (e) Individual values for each mouse tested of absolute numbers different B-cell populations, in the spleen obtained from B6 mice $n=16$, littermate CD21 cre mice $n=10$, littermate Mb1 cre mice $n=9$, CD21 cre mice $n=15$, and Mb1 cre mice $n=9$. Means and S.D. are indicated. (f and g) Peritoneal lavage was also performed on some animals and the cells obtained were stained by anti-CD5 and anti-B220 Abs. Representative histogram obtained after analysis of cells from the peritoneal lavage by flow cytometry with the different populations indicated. B-2 B cells ($B220^+CD5^-$), B-1a B cells ($CD5^+B220^{int}$), and B-1b B cells ($CD5^-B220^{int}$). (g) Individual values for each mouse tested of percentages of different B-cell populations, in spleens obtained from B6 mice $n=17$, littermate CD21 cre mice $n=8$, littermate Mb1 cre mice $n=4$, CD21 cre mice $n=15$, and Mb1 cre mice $n=6$. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (Mann–Whitney U-test). Hi, high; o, low

in terms of B-cell proliferation in Mb1 cre and CD21 cre mice stimulated by anti-IgM Abs with or without anti-CD40 stimulation (Figures 4a and b) nor in B-cell survival (Figures 4c and d), compared with their respective littermates. These results are in line with previous reports showing that short-term B-cell stimulation by antigen does not require autophagic activity. In contrast, under lipopolysaccharide (LPS) stimulation, both Mb1 cre and CD21 cre mice showed a significantly reduced survival. LPS is known to drive differentiation of mouse B cells into plasmablasts. These findings highlight a preferential role of autophagy in Ab-secreting cell survival rather than in initial B-cell activation. Incidentally, no exclusive annexin-V staining in CD138-negative population was found in these conditions, suggesting that B cells upregulate this plasma cell marker normally, but then fail to survive in the absence of autophagy (Figure 5a). Pengo *et al.*[11] previously described a defect in ER homeostasis, during plasma cell differentiation in the absence of autophagy. Here, we show further that both CD21 cre and Mb1 cre B cells exhibit an increased mitochondrial content after LPS stimulation as shown by mitotracker staining compared with littermates (Figures 5b and c). Furthermore, mitotracker deep red staining experiments revealed the presence in both deficient models of an increased cell population containing damaged mitochondrial content. We thus propose that autophagy participates in early

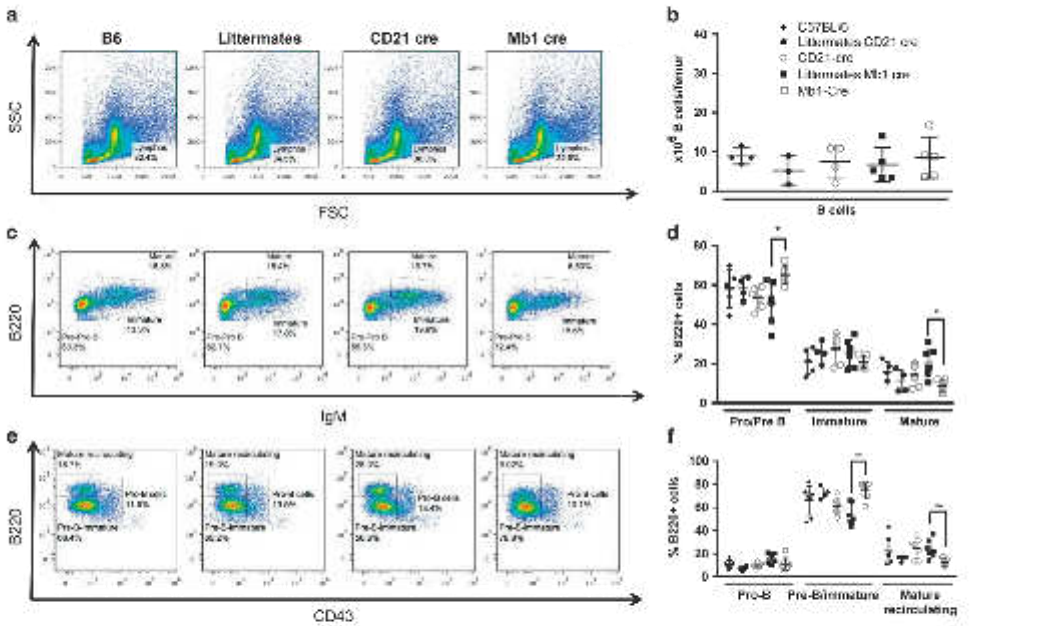

Figure 3 Autophagy is dispensable for B-cell development. Bone marrow cells from one femur of each wild-type C57BL/6 mice (B6), $Atg5^{fl/fl}$ CD21 cre (littermates CD21 cre) or $Atg5^{fl/fl}$ Mb1 cre (littermates Mb1 cre), $Atg5^{fl/fl}$ CD21 cre (CD21 cre) or $Atg5^{fl/fl}$ Mb1 cre (Mb1 cre) were stained with anti-IgM, anti-B220, and anti-CD43 Ace and analysed by flow cytometry. (a) Representative dot plots of FSC and SSC profiles of bone marrow cells. (b) Individual values for each mouse tested of absolute B220+ cell numbers, in the bone marrow obtained from B6 mice $n=4$, littermate CD21 cre mice $n=3$, littermate Mb1 cre mice $n=5$, CD21 cre mice $n=5$, and Mb1 cre mice $n=5$. Means and standard deviations (S.D.) are indicated. (c) Representative dot plots for the expression of B220 and surface IgM, allowing definition of pre-/pro-B cells (IgM^-B220^{low}), immature B cells (IgM^+B220^{low}), and mature B cells (IgM^+B220^{high}). (d) Individual values for each mouse tested of percentages of different B-cell precursors populations depicted in (c), in bone marrows obtained from B6 mice $n=5$, littermate CD21 cre mice $n=4$, littermate Mb1 cre mice $n=7$, CD21 cre mice $n=4$, and Mb1 cre mice $n=5$. Means and S.D. are indicated. (e) Representative dot plots obtained by flow cytometry analysis, showing the percentages among $B220^+$ cells of pre-B/immature B cells ($CD43^-B220^+$), pre-B cells ($B220^+CD43^+$), and mature recirculating B cells ($B220^{high}CD43^-$). (f) Individual values for each mouse tested of percentages of different B-cell precursors populations depicted in (e), in bone marrows obtained from B6 mice $n=7$, littermate CD21 cre mice $n=4$, littermate Mb1 cre mice $n=7$, CD21 cre mice $n=7$, and Mb1 cre mice $n=8$. Means and S.D. are indicated. $^*P<0.05$, $^{**}P<0.01$ significant after Mann–Whitney U-test.

plasmablast survival by limiting the potential damages related to increased and/or damaged mitochondrial content.

We then examined short-term humoral responses against the T cell-dependent model antigen OVA (Figure 6a). No difference was observed in the total levels of IgM and IgG in Mb1 cre and CD21 cre mice compared with controls (Figure 6b). Likewise, the anti-OVA IgG secretion in CD21 cre mice and control mice was similar (Figure 6c). This observation could be explained by the fact that in our immunization conditions, long-lived IgG secreting plasma cell differentiation is normal in the absence of autophagy in B cells, and that survival defects might impact IgG secretion at a longer term. However, a significant drop in anti-OVA IgM secretion was observed in both deficient mice (Figure 6c), which could reflect decreased survival of short-lived plasma cells in the absence of autophagy. Our results do not fully recapitulate the conclusions stated in the work of Cenci's group,[11] where both IgM and IgG secretions were altered in the absence of autophagy. This could be explained by the difference in the immunization protocol. We thus applied a protocol closer to the one used by Pengo and immunized Mb1 cre mice and their littermates only once, without boost. Although a slight decrease in both IgG and IgM levels was observed, the differences did not reach significance (Supplementary Figure S2). We cannot totally exclude that other factors linked to immunization, such as the adjuvant for example, may have influenced the data. The use of Freund's adjuvant for repetitively immunizing mice might effectively amplify the deleterious effect of autophagy deletion in short-lived plasmablasts. However, the results we generated in our experimental settings strongly suggest that although antigen-specific responses are altered, continuous differentiation of naive B cells into plasma cells allows uninterrupted secretion of Ig, as shown by normal levels of total Ig.

Overall, these results demonstrate that in the absence of autophagy in B cells, activation driven by BCR stimulation is largely normal. However, early specific humoral response is compromised, probably due to a defect of short-lived plasma cells, as illustrated by the high mortality rates after stimulating B cells deficient for autophagy by LPS.

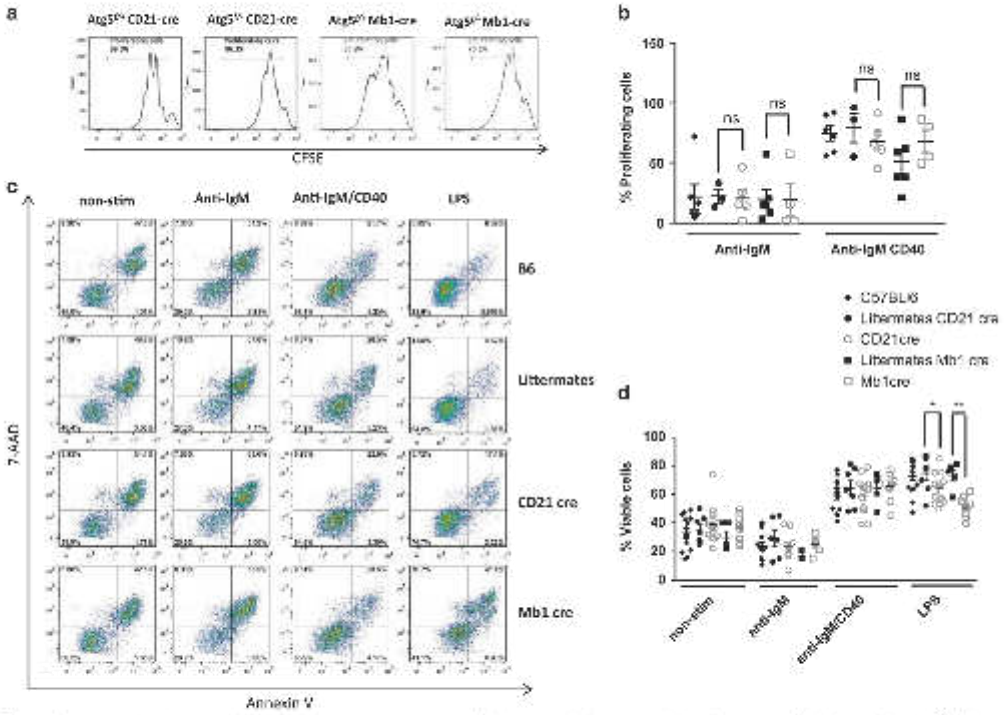

Figure 4 Autophagy is dispensable for B-cell proliferation and survival upon BCR stimulation. Purified splenic B cells from wild-type C57BL/6 mice (B6), $Atg5^{fl/+}$ CD21cre (littermates CD21 cre) or $Atg5^{fl/-}$ Mb1cre (littermates Mb1 cre), $Atg5^{fl/-}$ CD21 cre (CD21 cre), or $Atg5^{fl/-}$ Mb1 cre (Mb1 cre) were cultured without any stimulation (non-stim) or with 5 μg/ml anti-IgM in combination or not with 5 μg/ml anti-CD40 Abs or with LPS (5 μg/ml). (a) Cells from the indicated mice were stained with CFSE before culture, and proliferation was assessed by measuring the dilution of the fluorescent signal by flow cytometry after 3 days of culture. Percentages of proliferating cells are indicated in the histograms, for one representative anti-IgM/CD40 stimulation experiment, for each CD21 cre and Mb1 cre mice with their controls. (b) Mean and standard deviation (S.D.) values of the percentages of proliferating cells obtained in four independent experiments. (c) Alternatively, cells were stimulated as described and cell death was assessed by double annexin-V/7-AAD staining allowing to distinguish viable cells (annexin-V−/7-AAD−), early apoptotic cells (annexin-V+/7-AAD−) and late apoptotic/necrotic cells (annexin-V+/7-AAD+). (d) The mean and S.D. values of viable cell proportions obtained in six independent experiments are indicated. ns, non-significant. *$P<0.05$, **$P<0.01$ (Mann–Whitney U-test)

Autophagy in B cells is integral to the long-term maintenance of autoimmune responses. Both macroautophagy and CMA have recently been shown to be deregulated in B cells from lupus-prone mice and patients with lupus.[19,20] To complete our analysis, we thus asked the question about whether deficiency in *Atg5* could influence autoimmune responses. Owing to the defect in peripheral B-cell homeostasis observed in Mb1 cre mice, these experiments were performed using CD21 cre mice exhibiting a milder phenotype, to be crossed with $B6^{lpr/lpr}$ mice. The $B6^{lpr/lpr}$ strain develops a mild autoimmunity characterized by the secretion of autoAbs directed against nuclear antigens and Ig and complement deposition in kidneys.[23] Crossed mice were born at the expected Mendelian ratio and did not exhibit differential mortality rates before the age of observation. Compared with CD21 cre $Atg5^{fl/+\,lpr/lpr}$ littermate mice (littermates$^{lpr/lpr}$), there was no significant difference in CD21 cre $Atg5^{fl/-lpr/lpr}$ mice (CD21 cre$^{lpr/lpr}$) when total IgM or specific anti-dsDNA IgM levels were measured (Figure 7a). In sharp contrast, however, CD21 cre$^{lpr/lpr}$ mice exhibit a markedly reduced hypergammaglobulinemia and secreted much less anti-dsDNA IgG (Figure 7a). Several sets of experiments were then designed to investigate whether this reduction could be due to a decreased B-cell survival in peripheral lymphoid organs. No change in splenic B-cell proportions was observed in CD21 cre$^{lpr/lpr}$ mice compared with controls (Figure 7b) and no difference was detected in GC area relative to the surface of the spleen section scanned by confocal microscopy (Supplementary Figure S3). These results indicate that in our model of autoimmunity, autophagy does not have any significant role in the early steps of B-cell activation. A slight reduction of splenic plasma cells was noted, but it did not reach statistical significance, consistent with the fact that IgM autoAb levels remain unaffected. These findings may result from a greater replenishment of new short-lived plasma cells in the *lpr* model, compensating their loss in the absence of autophagy.

In contrast to the above observations, a reproducible drop in $CD138^+B220^-$ plasma cell counts was found in the bone marrow of CD21 cre$^{lpr/lpr}$ mice, compared with controls

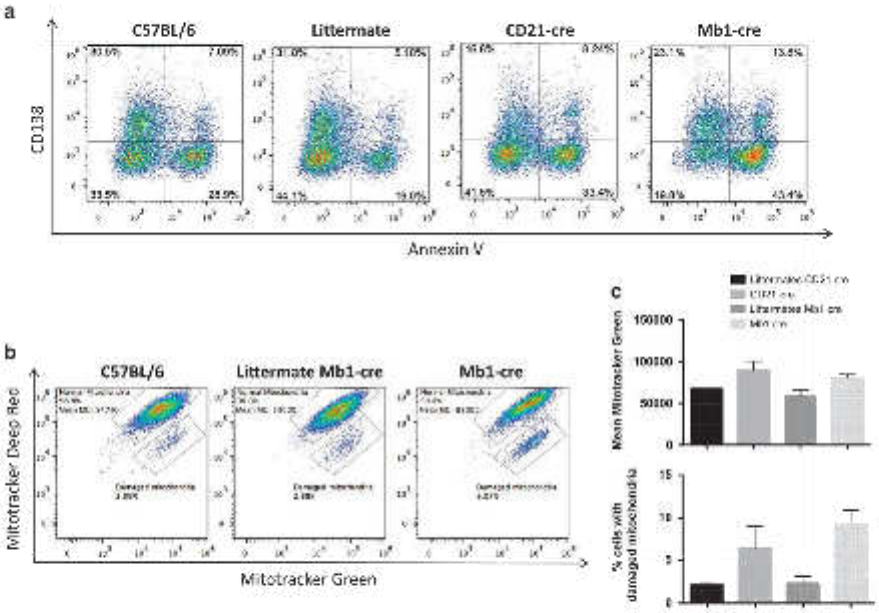

Figure 5 Differentiation of B cells into plasma cells in the absence of ATG5. (a) C57BL/6, $Atg5^{fl/fl}$ CD21 cre (littermate CD21 cre), $Atg5^{fl/fl}$ CD21 cre (CD21 cre), $Atg5^{fl/fl}$ Mb1 cre (littermate Mb1 cre) and $Atg5^{fl/fl}$ Mb1 cre (Mb1 cre) were stimulated by 5 *μ*g LPS for 48 h. Cells were stained by Annexin-V and anti-CD138 antibody, and analysed by flow cytometry. The percentages indicate the proportion of cells among total events after debris exclusion based on FSC/SSC profile, in the corresponding quadrants (b and c) The same cells were stained with mitotracker green and deep red. (b) Representative dot plots obtained after analysis of the indicated samples, after gating on live cells based on FSC/SSC profile, showing the repartition of cells that display proportional staining for both markers indicative of 'normal mitochondria membrane potential (normal mitochondria). The fluorescence mean of mitotracker green in this population is indicated. The proportion of cells with specific decrease in mitotracker deep red, indicative of a disturbed mitochondrial membrane potential is also indicated ('damaged mitochondria'). (c) Histograms showing the means of fluorescence in the population 'normal mitochondria' and the means of cell percentage of damaged mitochondria' population. The results are obtained on $n=2$ mice for each genotype, the bars stand for S.D.

(Figures 6c and d). For some samples, intracellular IRF4 costaining was performed to ensure that the cell population previously identified corresponds to plasma cells. These results fully agree with the above-described diminished levels of IgG secreted by long-lived $CD138^{+}B220^{+}$ plasma cells, which reside in bone marrow niches.

Finally, we investigated the pathophysiological relevance of the decreased autoAb secretion levels observed in $CD21cre^{tg/+}$ $Atg5^{fl/fl}$ mice. In agreement with the above-described results, immunofluorescence imaging of renal glomeruli clearly showed that IgG deposit staining was weaker in CD21 $cre^{tg/+}$ $Atg5^{fl/fl}$ mice compared with littermate controls (Figure 8).

Altogether, these results indicate that in a mild spontaneous model of lupus, autophagy is a central component of the long-term maintenance of autoAb production by plasma cells and Ab deposits in target organs, while B-cell proportion remains unaffected.

Discussion

The main purpose of this study was to provide new insight into the role of autophagy in the development of B cells. It was previously assumed that autophagy was important for the transition from pro- to pre-B cells.[5] These results were obtained using fetal liver chimaeras with ATG5-deficient cells, in which autophagy was deleted at the early stages of embryonic life, and thus before the initial haematopoietic stem cell development took place. Owing to the major role of autophagy in the survival of haematopoietic stem cells and in lymphocyte precursors,[11] it thus remained possible that accumulation of defects in the absence of autophagy in B-cell precursors led to the observed defects on pre-B cell survival. Indeed no such defects were observed using CD19, expressed from the pre-B cell stage, to drive cre expression.[5] It could be argued further that CD19 leads to incomplete ATG5 deletion at this early developmental stage. We thus created a new mouse expressing cre recombinase under the control of the strong Mb1 promoter known to drive very efficient deletion at the pro-B cell stage. In the mice generated in this study, contrary to previous observations, we did not find any developmental blockade at the pro/pre-B cell transition. We thus concluded that autophagy is dispensable for B-cell development in the bone marrow.

However, we found a reproducible decrease in mature recirculating B cells in the bone marrow. These cells

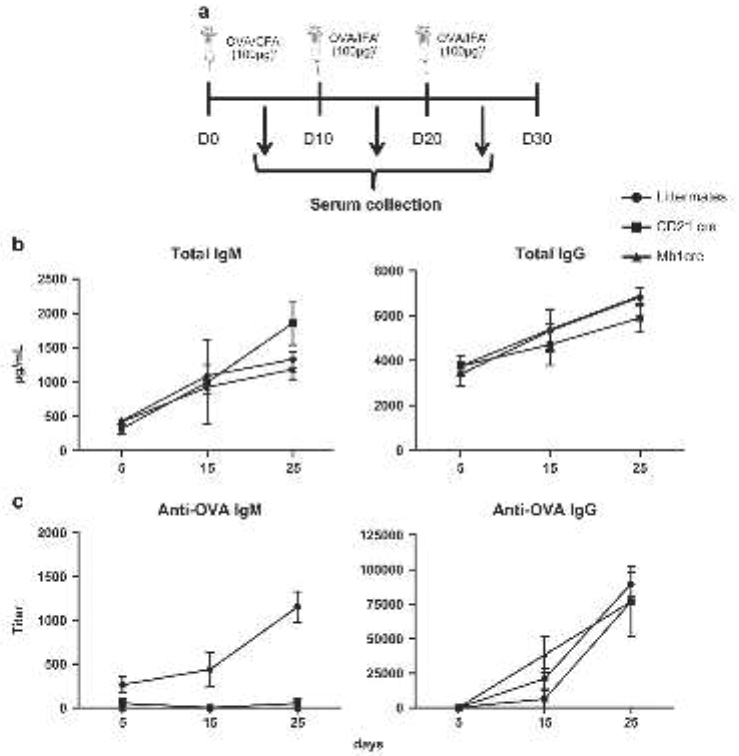

Figure 6 Functional autophagy is important in vivo for short-term humoral response. (a) Schematic representation of the immunization protocol used. C57BL/6 mice (B6), $Atg5^{fl/fl}$ CD21 cre or $Atg5^{fl/fl}$ Mb1cre (littermates), $Atg5^{-/-}$ CD21 cre (CD21 cre) or $Atg5^{-/-}$ Mb1 cre (Mb1 cre) mice were injected i.p. with OVA in the presence of FA at days 0, 10, and 20. Blood was collected at days 5, 15, and 25. (b) Absolute concentrations (+S.E.M. values) of total IgM and IgG in the serum of immunized animals (littermate mice $n=3$, CD21 cre $n=2$, and Mb1 cre mice $n=2$). (c) Measurement of anti-OVA IgM and IgG Ab titres (+S.E.M. values) in the serum from the immunized animals (littermate mice $n=3$; CD21 cre mice $n=2$; Mb1 cre mice $n=2$)

occupying a specific niche in this central lymphoid organ, are described as long-lived cells, secreting Abs against blood-borne pathogens.[22] As CD21 cre mice exhibit no such loss, we cannot totally rule out a developmental issue in B-cell precursors leading to selective defects in this population. It is also possible that the suboptimal deletion of ATG5 in CD21 cre mice is responsible for their normal survival contrary to Mb1 cre mice where the deletion was total. Thus, basal levels of autophagy are particularly necessary for the survival of mature B cells in the bone marrow.

In line with previous reports, we found a strong decrease in B-1a B cells in the peritoneum. Again this loss may be relative to developmental issue, as Mb1 cre mice exhibit this defect but CD21 cre mice did not. Overall, these data support the idea that early precursors of B-1a B cells critically depend on autophagy to generate viable cells at long term.

Interestingly, we also found a diminished survival of B-2 B cells in the peritoneum of both CD21 cre and Mb1 cre mice. This could be explained by the secretory activity of these cells,[23,24] or by their nature of mature recirculating cells. In the latter case and in contrast to what occurs in lymphoid organs, a slower replacement rate of dying cells by new B-cell emigrants issued from the bone marrow could account for these observations.

The survival of naive B-2 cells in lymphoid organs was found to be compromised in both CD21 cre and Mb1 cre mice, in a stronger way for the latter deletion strategy. This difference could be linked to the very efficient deletion of ATG5 in Mb1 cre mice, whereas some basal autophagy remained in B cells from CD21 cre mice. It seems that, contrary to what was previously assumed, basal levels of autophagy are indeed required for the survival of B cells in the periphery. This observation may be related to an impairment of homeostatic maintenance, as proliferation and survival upon BCR signalling were normal in both Mb1 cre and CD21 cre mice. Interestingly, in our study, the only effective stimulus leading to survival defect was the one generated by LPS, and this was observed in both our deletion models. LPS is known to drive

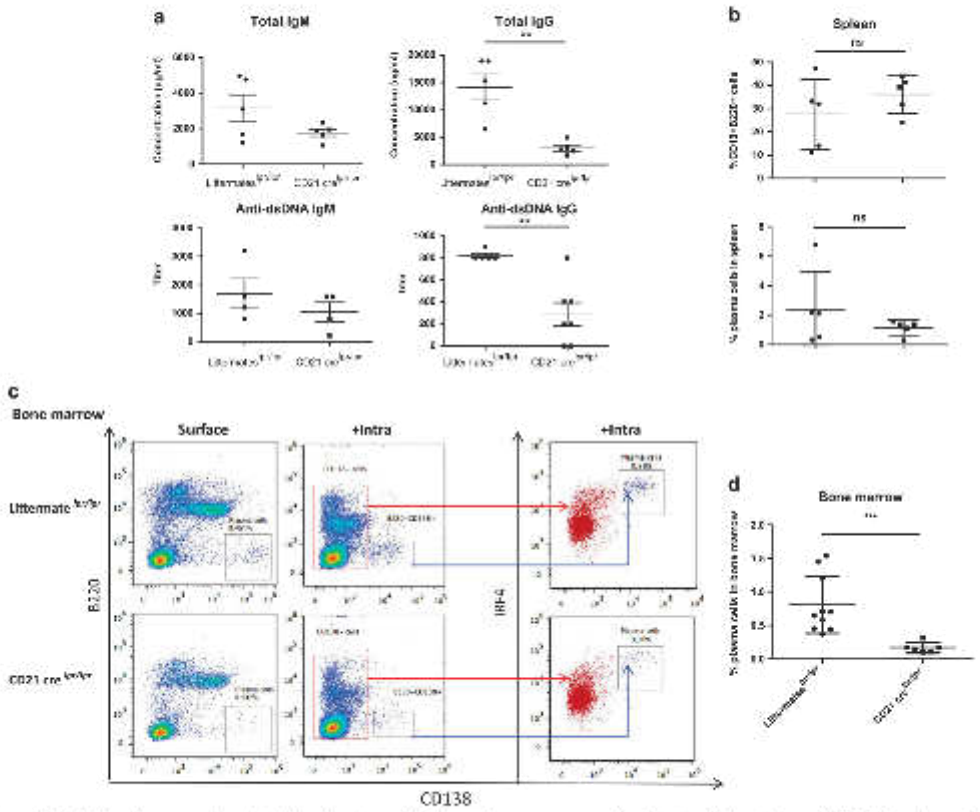

Figure 7 Autophagy is necessary for survival of long-lived plasma cells during autoimmune responses. Mice harbouring the *lpr* mutation on the C57BL/6 background were crossed with $Atg5^{fl/fl}$ CD21 cre mice ($CD21\ cre^{+/tg}$). They were compared with littermate $Atg5^{fl/fl}$ CD21 cre mice, also crossed with *lpr* mice (littermates$^{fl/fl}$), at the age of 9 months. (**a**) Individual values for each mouse assayed for absolute IgG and IgM concentrations in the serum, determined by ELISA and titres of anti-dsDNA IgM and IgG Abs. Means and SEM are represented (at least four mice in each group). (**b**) The spleens of the animals were collected, cells were stained by anti-CD19, anti-CD138, and anti-B220 Abs, and analysed by flow cytometry. Each point represents the value for an individual mouse. The central bar represents the means and the upper and lower bars symbolize the S.D. (**c**) Staining of bone marrow cells collected from one femur per mouse. Each dot plot stands for one representative case for each genotype. On the left, the gate delimiting $B220^{+}CD138^{+}$ cells should indicate the percentages of plasma cells resident in the bone marrow (surface). In the middle, results obtained after an intracellular staining of IRF4 molecule performed after surface staining of the previous markers (+intra). The red population corresponding to $CD138^{+}$ cells is $IRF4^{hi}$ or $IRF4^{int}$. The $CD138^{+}$ blue population corresponding to plasma cells is $IRF4^{+}$. This latter staining was performed on four littermates$^{fl/fl}$ mice and one CD21 $cre^{+/tg}$ mouse. (**d**) Percentage of plasma cells among the bone marrow cells for individual mice analysed as in (**c**). $n = 10$ for littermates$^{fl/fl}$ and $n = 6$ for $CD21\ cre^{+/tg}$. Mean and S.D. are indicated. $^{**}P < 0.01$, $^{***}P < 0.001$ (Mann–Whitney *U*-test)

the differentiation of mouse B cells into plasmablasts. Thus, the selective survival defect could also be linked to the role autophagy plays in the survival of Ab-secreting cells.

A defective IgM production against the T cell-dependent antigen OVA was observed in both Mb1 cre and CD21 cre mice. In contrast, IgG production against OVA appeared to be normal in the absence of autophagy. A possible explanation for this outcome would be the defective survival of short-lived plasma cells in secondary lymphoid organs in the absence of autophagy while long-lived plasma cells could transiently resist the absence of autophagy. In this setting, defective IgG production could be observed on the long term only. It is also possible that selection processes allow autophagy-competent cells only to survive, as it has been described in mice with CD19 cre-mediated ATG5 deletion.[11] This latter argument remains however uncertain, knowing the very efficient ATG5 deletion observed in our models, especially in Mb1 cre mouse B cells.

To study the long-term impact of autophagy deletion in B cells, we used the spontaneous systemic autoimmunity model driven by the *lpr* mutation on a B6 genetic background.[26] This mutation on *Fas* gene leads to secretion of autoAbs directed against nuclear antigens, which can deposit in several organs, especially in the kidneys of affected animals.[33] The pathology

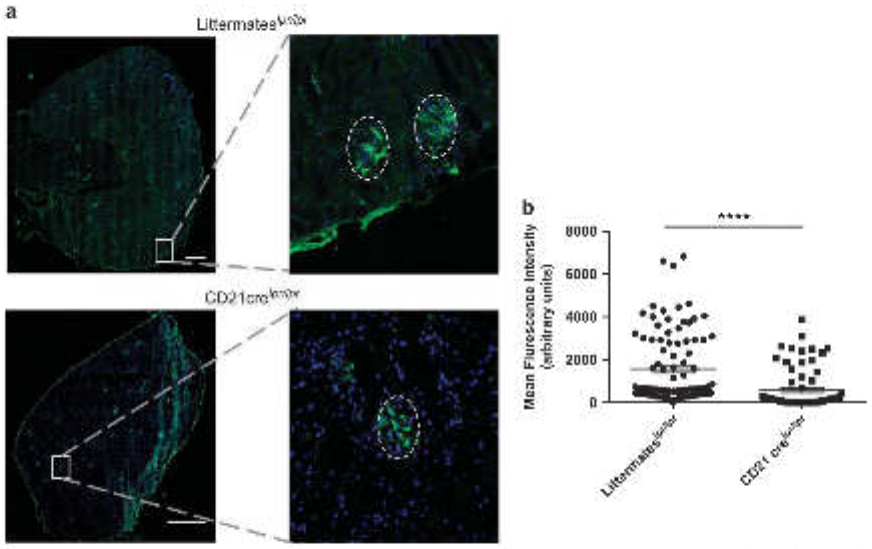

Figure 8 Invalidation of autophagy in B cells reduces IgG deposits in the kidney of lupus-prone mice. Mice bearing the *lpr* mutation on the C57BL/6 background were crossed with *Atg5*$^{fl/fl}$ CD21 cre mice (CD21 cre$^{tg/wt}$) and their kidneys were collected at the age of 9 months. *Atg5*$^{fl/fl}$ CD21 cre littermate mice harbouring the *lpr* mutation were used for comparison (littermates$^{lpr/lpr}$). Organ sections stained with anti-mouse IgG Abs before analysis on a spinning disk confocal microscope. (a) The total section was scanned and a representative image for each genotype is shown on the left. The white bars represent 500 *μ*m. A magnification is indicated on the right to exemplify a glomerulus positive for IgG deposition, delimited by the dotted line. (b) The intensity of IgG staining was determined for each glomerulus as indicated in Materials and methods. Individual values of littermates$^{lpr/lpr}$ ($n = 5$) and CD21 cre$^{lpr/lpr}$ ($n = 4$) ($n = 20$ glomeruli per animal) for each genotype were pooled and plotted on the graph. The bars represent the mean of glomerular IgG staining intensity for each genotype. ****$P < 0.001$ (Mann–Whitney *U*-test)

developed over time, although relatively mild in comparison with MRL/*lpr* mice, for example, mimicking some lupus features. *Atg5*$^{-/-}$ CD21 cre × B6$^{lpr/lpr}$ autophagy-deficient autoimmune mice showed no survival failures in B cells, supporting the minimal role of autophagy in B-cell survival, even in an autoimmune context. In contrast, and in good agreement with data reported in other settings,[11–13] we found a decrease in plasma cell proportion in the bone marrow reflecting selective defects in long-lived cells. This drop in long-lived Ab-secreting cells may be responsible for the reduction of hypergammaglobulinemia and anti-dsDNA Abs observed in our autophagy-defective mice. Note that we cannot formally exclude a possible contribution of the reduced memory B-cell compartment in *lpr* mice deficient for autophagy.[12,13] Interestingly, we also found less IgG deposits in the kidneys of CD21 cre$^{lpr/lpr}$ mice. Considering the above-described findings, we propose that this feature is mainly due to autophagy impairment in plasma cells, and not in initial B-cell activation, as no major defect in GC formation was noticed.

In summary, using three novel mouse models defective for autophagy process, we report here that autophagy is dispensable for B-2 B-cell development contrary to B-1a B cells and that basal levels of autophagy are required in B cells to maintain their normal number in lymphoid organs. We confirm in a new setting that autophagy is a major player of long-lived B cells and Ab-secreting cell survival. We describe further that mice exhibiting signs of lupus autoimmunity and in which autophagy was diminished, show significantly less biological (lower anti-dsDNA IgG Ab levels) and clinical (less IgG deposits in their kidneys) lupus features, without having their whole B-cell compartment adversely affected. Therefore, autophagy modulation appears to be a good therapeutic option to limit autoAb-linked inflammation in systemic autoimmunity.

Materials and Methods

Mice. *Atg5*$^{fl/fl}$ mice, with a flox sequence flanking exon 3 of *Atg5* gene backcrossed on a C57BL/6 (B6) background have been described[27] were a kind gift from Prof. N Mizushima. We also used mice with systemic deletion of exon 3 (*Atg5*$^{-/-}$) to generate *Atg5*$^{-/-}$ mice. *Atg5*$^{fl/fl}$ mice were crossed with *Atg5*$^{-/-}$ CD21 cre[28] or *Atg5*$^{fl/fl}$ Mb1 cre[29] mice for B-cell lineage-specific deletion. In all experiments, B6 *Atg5*$^{-/-}$ CD21 cre or B6 *Atg5*$^{-/-}$ Mb1 cre mice with B-cell specific deletion of *Atg5* were compared with littermates (B6 *Atg5*$^{fl/fl}$CD21cre mice and *Atg5*$^{fl/fl}$ Mb1cre mice). In some experiments, B6 *Atg5*$^{-/-}$ CD21cre mice were crossed with B6 mice bearing the *lpr* mutation affecting *Fas* gene, driving a spontaneous systemic autoimmunity. In this setting, lupus-prone B6$^{lpr/lpr}$ *Atg5*$^{-/-}$ CD21 cre (3 females, 1 male) were compared with age-matched B6$^{lpr/lpr}$ *Atg5*$^{fl/fl}$ littermates (4 females, 1 male). Mice were genotyped for the *Atg5* alleles (NT, with constitutive or induced deletion) with the primers A (exons 3–4): 5′-GAATATTGAAGGCACACCCTGAAATG-3′; B (strat 2): 5′-GTACTGCATAATGGTTTAACTCTTTGC-3′; C (check2): 5′-ACAAC GTCGAGCAGAGCTGCGCAAGG-3′; D (5.2): 5′-CAGGGAATCGTTGTTCCCAC-3′ using PCR (94 °C (4 min); 30 cycles of 94 °C (30 s), 60 °C (30 s), 72 °C (1 min); 72 °C (5 min)). The cre transgene expressed under the control of CD21 promoter was detected with primers cre-F 5′-ACGACCAAGTCACAGCAATG-3′; cre-R 5′-CTCGACCAGTTTAGTTACCC-3′ and for the cre expressed under Mb1 promoter

with primers cre-F 5′-ACCTCTGATGAAGTCAGGAAGAAC-3′; cre-R 5′-GGAGATGTCCTTCACTCTGATTCT-3′ using PCR (94 °C (4 min); 25 cycles of 94 °C (30 s), 60 °C (30 s), 72 °C (1 min); 72 °C (5 min)). The *lpr* allele was identified by PCR using a sense primer, 5′-AGGTTACAAAAGGTCACCC-3′, in intron 2 and two antisense primers, 5′-GATACGAAGATCCTTTCCTGTG-3′ and 5′-CAAACGCAGTCAAATCTGCTC-3′ using PCR (94 °C (4 min); 30 cycles of 94 °C (45 s), 55 °C (1 min), 72 °C (1 min); 72 °C (5 min)). We genotyped mice with the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA). All mice were bred and maintained in accordance with guidelines of the local Institutional Animal Care and Use Committee (CREMEAS).

Flow cytometry. The majority of antibodies (Abs) used for flow-cytometry analyses were purchased from BD Biosciences (Le Pont-De-Claix, France): allophycocyanin (APC)-Cyanin 7-labelled anti-mouse TCR-*β* (clone H57-597, 553139), phycoerythrin (PE)-labelled anti-mouse CD69 (clone H1.2F3, 553236), fluorescein isothiocyanate (FITC), APC or peridinin chlorophyll (PerCP) cyanine 5.5-labelled anti-mouse B220 (clone RA3-6B2, 553087, 553090, and 552771), FITC-labelled anti-mouse CD3ε (clone 145.2C11, 553061), FITC-labelled anti-mouse CD21/CD35 (clone 7G6, 553818), PE-labelled anti-mouse CD23 (clone B3B4, 553139), APC-labelled anti-mouse CD5 (clone 53-7-3, 550035), APC-labelled anti-mouse CD43 (clone S7, 560663), APC-labelled anti-mouse IgD (clone 11-26 c 2a, 560868), APC-labelled anti-mouse CD19 (clone 1D3, 550992), and APC-labelled anti-mouse CD138 (clone 281-2, 558626). FITC-conjugated polyclonal anti-mouse IgM was purchased from Jackson Immunoresearch (Newmarket, UK; 115-095-020) and PE-labelled anti-IRF4 from Biolegend (San Diego, CA, USA; clone 4.3E4, 646404). Cells were incubated with fluorochrome-conjugated Abs and with unlabelled rat anti-mouse CD16/CD32 monoclonal Ab (mAb, clone 2.4G2, 553142) to block Fc receptors, for 15 min at 4 °C in phosphate-buffered saline (PBS) pH 7.4 containing 2% (w/v) fetal calf serum (FCS). Intracellular staining was performed after surface marker labelling using the FoxP3 detection kit purchased from eBiosciences (San Diego, CA, USA; 00-5523-00). Mitochondria content and membrane potential were measured by incubating cells 30 min at 37 °C in complete medium in the presence of mitotracker green and mitotracker deep red (Fisher Scientific, Pittsburgh, PA, USA; M-7514 and M22426) at the recommended concentrations. Data were collected on a Gallios flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analysed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Cell culture and isolation. Splenic B cells were collected from B6 *Atg5*$^{f/-}$ CD21-cre, B6 *Atg5*$^{f/-}$ Mb1-cre, B6$^{lpr/lpr}$ *Atg5*$^{f/-}$ CD21-cre mice or from littermates and immediately cultured at 37 °C, 5% CO_2 in complete RPMI-1640 medium (Lonza BioWhittaker, Levallois, France) containing 10% FCS, 10 *μ*g/ml gentamycin (Lonza BioWhittaker), 10 mM HEPES (Lonza, Basel, Switzerland) and 0.05 mM *β*-mercaptoethanol (Lonza) at a concentration of 5×10^{6} cells/ml. Splenic B cells were purified by negative selection. Briefly, spleen cell suspensions were depleted from monocytes, granulocytes, T cells, and NK cells using the Pan B cell isolation Kit II (130-090-862; Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Resulting TCR-β^{-}/B220^{+} mouse B-cell preparations were > 95% pure as determined by flow cytometry. Cells were stimulated with goat F(ab′)$_2$ anti-IgM (Jackson Immunoresearch; 115-006-020) with or without anti-CD40 Ab (5 *μ*g/ml, BD Biosciences, clone HM40-3, 553721) or LPS alone (5 *μ*g/ml, Sigma, Saint-Quentin-en-Yvelines, France). Alternatively, T cells were sorted from spleen cell suspension with the Dynabeads untouched mouse T cell isolation kit (ThermoFisher Scientific, Illkirch, France; 11413D) and were stimulated by anti-CD3ε Ab (clone 145.2C11, 553057) purchased from BD Biosciences. For the analysis of proliferation, cells were stained with 0.5 *μ*M carboxyfluorescein isothiocyanate succinimidyl ester diacetate (CFSE, Sigma-Aldrich) before the stimulation. Cell death was assessed by Annexin-V-FITC/7-AAD double staining (BD Biosciences, respectively, 556425 and 559419). After acquisition by flow cytometry, proliferation and cell death results were analysed with FlowJo Software.

Immunoblots. The Abs used for western immunoblotting were specific for ACTB (Santa Cruz Biotechnology, Heidelberg, Germany; clone C4, sc-47778), LC3 (MBL, Nagoya, Japan, clone 51-11, ref M115-3) and ATG5 (Polyclonal, Novus, Littleton, CO, USA). In some experiments, lysosomal protease inhibitors E64d and pepstatin A (Sigma-Aldrich; P5318 and E8640) were added at 5 *μ*g/ml each. When indicated, cells were treated with goat F(ab′)$_2$ anti-IgM (5 *μ*g/ml, Jackson Immunoresearch; 115-006-020). To evaluate the autophagosomal membrane load, whole-cell proteins were extracted from cultured cells using Laemmli buffer (125 mM Tris-HCl, pH 6.8; 2% (w/v) sodium dodecyl sulphate (SDS); 10% (v/v) glycerol; 5% (v/v) *β*-mercaptoethanol). Cell lysates were separated using 4–20% gradient gels (Bio-Rad, Hercules, CA, USA) and proteins transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with PBS containing 0.1% (v/v) Tween-20 (PBS-T) and 3% (w/v) non-fat dry milk for 1 h and then incubated overnight at 4 °C with 1 *μ*g/ml anti-LC3 Ab in PBS-T containing 1% non-fat dry milk, or for 1 h at room temperature with 1 *μ*g/ml anti-ATG5 Ab in PBS-T containing 1% non-fat dry milk. After washing with PBS-T, membranes were incubated for 30 min at room temperature with goat anti-mouse IgG Ab (Southern Biotech, Birmingham, AL, USA; 1030-05) conjugated to horseradish peroxidase (HRP). Signal was detected using enhanced chemiluminescence detection reagents (Immobilon Western, Merck Millipore, Darmstadt, Germany; WBKLS0500). When indicated, LC3-II and ATG5 staining was normalized by densitometry to ACTB staining using the ImageJ Software (National Institute for Health, Washington, DC, USA).

Real-time PCR. Total RNA was isolated from 5×10^{6} purified B cells using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France, ref 74106) according to the manufacturer's instructions. After treatment by DNAse (Qiagen, ref 79254) to remove residual genomic DNA, mRNA was retro-transcribed with the Maxima first-strand synthesis kit for cDNA (ThermoFisher, Illkirch, France). Fifteen nanograms of cDNA was used for real-time PCR (RT-PCR) on StepOne apparatus (ThermoFisher). Briefly, *Atg5* and *Gapdh* cDNA was amplified using Taqman Gene Expression Assays provided by ThermoFisher (ref Mm00504340_m1 and Mm99999915_g1). Amplicons and probe were designed to span two exons, limiting the risk of amplifying residual genomic DNA. Relative *Atg5* mRNA quantifications were made by defining ΔC_T (C_T *Gapdh* − C_T *Atg5* where C_T is 'Cycle Threshold') and $\Delta\Delta C_T$ (ΔC_T sample − ΔC_T of one C57BL/6 mouse sample used for each plate) using StepOne software (ThermoFisher). Results shown represent $2^{-\Delta\Delta CT}$ values where one same control sample is used on each plate and arbitrarily equal to 1.

Immunization. Eight- to twelve-week-old mice were injected intraperitoneally (i.p.) at days 0, 10, and 20 with the antigen suspension, and bled on days 5, 15, and 25 after the first immunization. Mice received 100 *μ*g ovalbumin (OVA, Sigma) in complete Freund's adjuvant (CFA, Sigma) for the first injection and 100 *μ*g OVA in incomplete Freund's adjuvant (IFA, Sigma) for the second and third injections. Alternatively, mice were injected only once with 100 *μ*g OVA in CFA and were bled at days 7 and 14 to follow the anti-OVA response.

Antibody detection by ELISA. IgG or IgM titres were measured in serum from immunized or lupus-prone mice. To measure anti-OVA specific Abs, 96-well ELISA Maxisorp plates (NUNC, Fisher Scientific) were coated with OVA (10 *μ*g/ml, Sigma) in 50 mM sodium carbonate buffer (pH 9.6). The wells were blocked with PBS-T 0.1%, 5% (w/v) milk for 1 h at 37 °C and incubated with diluted anti-sera for 1 h at 37 °C. HRP-conjugated anti-mouse isotype-specific Abs (Jackson Immunoresearch) were used to reveal bound Abs. Absorbance was measured at 450 nm, after revelation with tetramethylbenzidine to the wells, and stop of the reaction by 1 M HCl. The same protocol was used for detection of anti-double stranded (ds)DNA Abs except that ELISA plates were coated with 100 ng/ml non-sheared dsDNA (from calf thymus, Sigma-Aldrich) in 25 mM citrate buffer, pH 5. Titres correspond to the inverse of the last dilution giving an absorbance equal to 0.2.

Immunohistofluorescence. Spleens/kidneys were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA; 4583) and snap frozen with liquid nitrogen. To visualize IgG deposits in the kidney, tissue sections were then stained with goat anti-mouse IgG-FITC (Southern Biotech; 1030-02) and DAPI. To detect GC in the spleen, tissue sections were stained with peanut agglutinin (PNA, Vector, Burlingame, CA, USA; FL-1071), IgD-APC and DAPI. Images were acquired with a spinning disk confocal microscope (Zeiss, Oberkochen, Germany) with an A-Plan 20×0.8 Zeiss ×20 objective. Images were analysed with the ImageJ Software. The quantification of IgG deposits in the kidney was performed as follows: glomeruli were identified via typical structures shaped by DAPI staining. A zone comprising 20 glomeruli was delimited for each kidney section. Fluorescence intensity given by the anti-IgG staining was measured in each glomerulus, and background intensity of the delimited zone was subtracted. The quantification for GC in spleen section was performed by delimiting zones positive for PNA staining, measuring the area of the zone, and by normalizing it to the total area of the scanned section.

Statistical analyses. The data were analysed with the Prism software (GraphPad, San Diego, CA, USA) using two-tailed unpaired t-tests or Mann–Whitney U-test. Error bars represent standard deviations (SD).

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

JA, JDF and FG performed and designed experiments. DM and FA performed experiments. SM and FG wrote the article.

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Supplementary Information accompanies this paper on Cell Death and Differentiation website (http://www.nature.com/cdd)

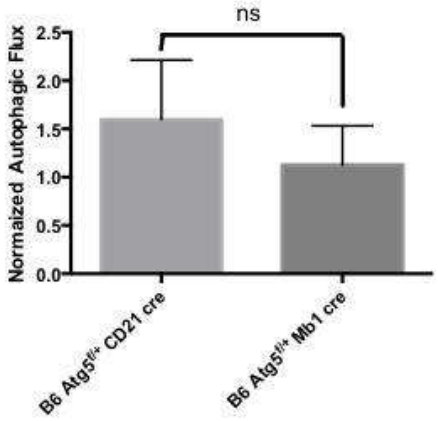

Figure S1: comparable autophagic flux between *Atg5*$^{f/+}$ CD21 cre and *Atg5*$^{f/+}$ Mb1 cre littermates
Autophagic flux measurement obtained from densitometric analysis of the immunoblots presented on figure 1. Flux was measured by dividing the staining density ratios (LC3-II/ACTB) of the non stimulated condition with protease inhibitors by the density ratios without inhibitors for both types of littermates (n=6 *Atg5*$^{f/+}$ CD21 cre, and n=7 *Atg5*$^{f/+}$ Mb1 cre). Histograms represent the mean and the bars stand for SD.

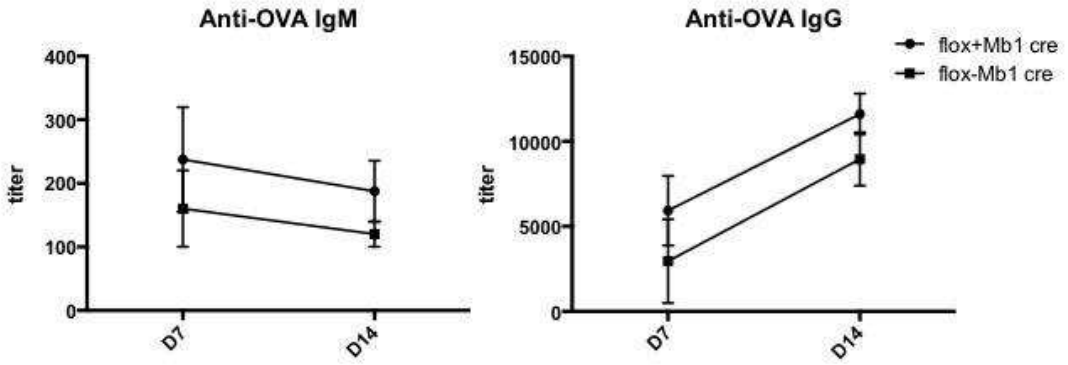

Figure S2: minimal impact of autophagy invalidation in single step immunization
Atg5$^{f/+}$ Mb1cre (flox+ Mb1cre) or *Atg5*$^{f/-}$ Mb1 cre (flox-Mb1 cre) mice were injected i.p. with OVA in the presence of CFA at day 0. Blood was collected at days 7 and 14. Measurement of anti-OVA IgM and IgG Ab titers (+SEM values) in the serum from the immunized animals (flox+Mb1 cre n= 7; Mb1 cre mice n=4).

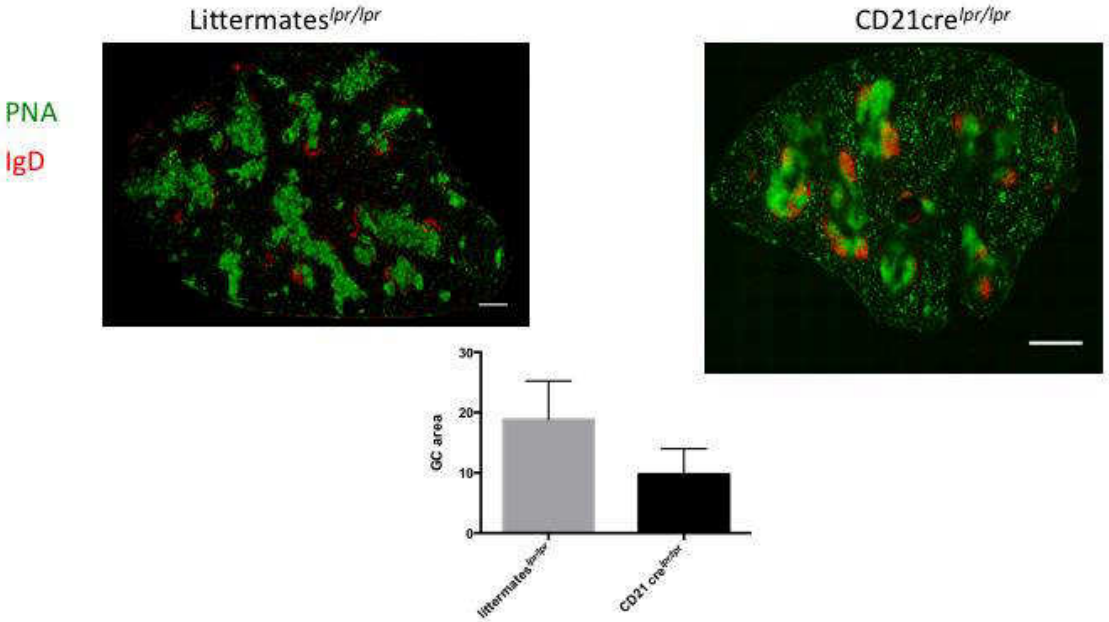

Figure S3: normal generation of germinal centers in lupus-prone mice deficient for autophagy
Spleen sections obtained from Atg5$^{f/-}$ CD21 cre mice (CD21 cre$^{lpr/lpr}$) and Atg5$^{f/+}$ CD21 cre$^{lpr/lpr}$ (littermates$^{lpr/lpr}$) were stained for PNA (green) and IgD (red). Staining overlay appears yellow. Sections were scanned by spinning disk confocal microscopy. The area of PNA^{+} zones was measured and normalized to the total area for each section. The histograms at the bottom show the mean of the relative GC area for n=4 CD21cre$^{lpr/lpr}$ mice and n=5 littermate$^{lpr/lpr}$ mice. No significant difference was found after Mann-Whitney U test comparison. The white bar represents 500 µm.

2 Publication 2

2.1 Forword

Autophagy is integral to CD4 T cell memory maintenance

Diane Murera, Johan Arnold, Florant Arbogast, Sylviane Muller and Frédéric Gros

This work represents the main focus of my thesis and was centered on the role of autophagy in T cell function in periphery. At the start of this project the existing data had mainly allowed to establish that autophagy was induced upon T cell activation and was required for T cell survival and proliferation. T cell autophagy had however barely been investigated *in vivo,* mainly because of the functional defects observed *in vitro* in previous models **(Jia and He, 2011; Pua et al., 2009; Stephenson et al., 2009)**. Since in those models autophagy was deleted early in T cell thymic development we speculated that the observed T cell phenotype could be attributed to autophagy requirements at that stage. Thus we generated a mouse model with a specific autophagy deletion in mature T cells ($Atg5^{ff}$ dLck-cre). This model has allowed us to verify the accuracy of this hypothesis but also to study the impact of T specific autophagy impairment on humoral immune responses. We were able to determine that autophagy requirements in CD4 and CD8 T cells are not the same. Unlike CD8 T cells, CD4 T cells were able to undergo activation and proliferation *in vitro*. Furthermore we saw that autophagy is not required for early but rather long-term humoral responses. Even though recent data on the involvement of autophagy in memory CD8 T cell function has emerged, we demonstrate for the first time the requirement of this process for CD4 T cell memory maintenance.

2.2 Autophagy is integral to CD4 T cell memory maintenance

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Abstract

Autophagy is involved in T cell homeostasis. Previously described mouse models with autophagy deletion early in T cell development, led to functional and survival defects in the periphery. We wanted to delineate the roles of autophagy in CD4 T cells in the periphery by generating mouse models with deletion of autophagy only at a mature stage. In *Atg5*$^{f/f}$ dLck-cre, the invalidation of autophagy impacted CD8 T cell survival but no consequence was observed on CD4 T cell *in vitro* function at short-term. Our mouse model, thus suitable for *in vivo* studies about the role of autophagy in CD4 T cells during humoral responses, was then immunized with the T-dependent antigen ovalbumin (OVA). The early humoral response wasn't significantly different between *Atg5*$^{f/f}$ dLck-cre and control mice. However, immunization at a later time point, showed that autophagy in T cells was required for long-term antibody production. Autophagy allows late survival of antigen stimulated T cells, as shown by adoptive T cell transfer assays in naive mice. Finally, memory CD4 T cells differentiated *in vitro* are shown to depend on autophagy for their survival, and to regulate their mitochondrial homeostasis. Central memory T cells predominantly need autophagy for maintenance. Autophagy in T cells constitutes a seducing target to inhibit long-term humoral immunity. Inhibition of autophagy could be envisaged to treat systemic autoimmune diseases, by impeding chronic memory autoreactive lymphocyte survival, or to improve vaccination efficiency.

Keywords

T cells, autophagy, mitochondria, memory response, humoral immunity

Introduction

Autophagy is a catabolic process, required to produce energy under several stress signals. Moreover, basal autophagy is important to remove protein aggregates, damaged organelles such as defective mitochondria or excess endoplasmic reticulum (ER) in processes respectively called mitophagy and reticulophagy. Basal autophagy has been shown to be crucial in long-lived cells such as neurons or metabolically active cells such as hepatocytes. Immune cells like T lymphocytes exhibit differential energy demands according to their developmental stage or activation status. It is widely accepted that naive T cells require glycolysis early after activation, to quickly mobilize energy. In contrast memory T cells are long-lived cells, that may use differential energy production systems to survive for months or years after the initial activation (1). They are particularly dependent on fatty acid oxidation (FAO) that takes place in mitochondria, to generate ATP. Moreover removal of damaged cellular components may also require autophagy at long-term.

Autophagy has been initially shown to play a role in peripheral T cell homeostasis in mouse chimera models (2). Thanks to several conditional deletion models, it was shown that autophagy was essential for both CD4 and CD8 T cell survival and proper function (3-9). However these models used promoters to drive deletion that are active early during T cell differentiation. These models could thus not allow to distinguish if functional or survival defects were due to early developmental issues, or consequent to real peripheral homeostatic disturbance. One study addressed this issue by deleting with Estrogen Receptor-cre promoter the essential autophagy gene *Atg3*, only after T cells had matured (6). This study brought interesting findings about the autophagy requirement for ER homeostasis, several days after initial activation. However these experiments were driven *in vitro* and no integrated immune response could be studied. More recently two studies addressed this question *in vivo*, for CD8 T cells, by transfer experiments and conditional deletion models active only at the CD8 T cell effector stage. They both concluded that CD8 T cells require autophagy for their survival as memory cells (10, 11), creating an interesting parallel to other studies linking autophagy to the survival of other long-lived cells like neurons.

We generated in this work mice with conditional deletion of *Atg5*, only in mature T cells thanks to the use of the distal Lck promoter-driven conditional deletion (12). We report here new findings about the role of autophagy in peripheral T cell function, in the absence of any developmental issue. We moreover used this model and confirm the essential role for autophagy in memory T cell survival. We describe here, in addition to its proven role in CD8 memory maintenance, a role in humoral immunity, through the long-term memory CD4 T cell survival.

Results

Autophagy is not required for peripheral CD4 T cell homeostasis

Previous conditional deletion models could not totally resolve the question whether or not autophagy was required for mature T cell homeostasis. We thus decided to cross *Atg5*$^{f/f}$ mice with mice expressing CRE recombinase under the control of the distal part of the Lck promoter (dLck-cre), active only in mature T cells. We first assessed the efficiency of the deletion. As shown in figure 1A, no ATG5-ATG12 conjugate was visible by immunoblot in peripheral T cells isolated from *Atg5*$^{f/f}$ dLck-cre mice, in regard to littermates, and to thymocytes from both mice. No conversion from LC3-I to LC3-II was detectable, even under protease inhibitor treatment, confirming the efficiency of autophagy invalidation. We then investigated the impact of this deletion during T cell development. As shown in Figure 1B and contrary to previously described models, no difference in thymic cellularity was found, indicative of an absence of developmental deregulations. No difference in the proportions of thymocyte developmental stages was detected either (Figure 1C, left). These findings are in accordance with the absence of ATG5 deletion in the thymus, as proven by immunoblots shown in figure 1A. We then assessed the proportions of lymphocyte populations in secondary lymphoid organs. In agreement with previous models, we found a decrease in CD8 T cell proportion and number in the spleen (Figures 1B and C right) from *Atg5*$^{f/f}$ dLck-cre mice compared to controls. However, in sharp contrast to all conditional deletions reported earlier, we found no difference in CD4 T cell numbers and proportions among other populations. This finding was also true in other lymphoid organs like lymph nodes (Figure S1).

Autophagy is dispensable for early CD4 T cell activation

We then aimed at understanding the decrease of CD8 T cells in *Atg5*$^{f/f}$ dLck-cre mice compared to control mice. We detected in spleens from *Atg5*$^{f/f}$ dLck-cre mice, an increased cell death only in CD8 T cells, and not in CD4 T cells (Figure 2A). Survival defects could thus explain the previously mentioned decrease in CD8 T cell number. Thus ATG5 contrary to CD4 T cells is essential for CD8 T cell homeostatic survival. We then isolated T cells from *Atg5*$^{f/f}$ dLck-cre mice to activate them. We first measured survival. In all TCR-related stimulations performed, we could not evidence any significant defect in survival, in CD8 nor CD4 T cells (Figure 2B). It thus seems that survival defects in CD8 T cells are mainly related to homeostasis defects. We then assessed the basal mitochondrial load in isolated T cells, as mitophagy was shown to be crucial for T cell homeostasis. First we found that CD4 T cells exhibit a higher mitochondrial mass in control T cells, in comparison to CD8 T cells (Figure 2C). Interestingly, we reproducibly detected more T cells with a high mitochondrial load, or T cells containing mitochondria with decreased membrane potential in CD8 T cells isolated from *Atg5*$^{f/f}$ dLck-cre mice. This could mean

that ATG5 is indeed required after thymus egress, in CD8 T cells and not in CD4 T cells, to reduce mitochondrial mass. This could explain the homeostatic defects observed in CD8 T cells deficient for ATG5 at the mature stage. Finally we could see a specific functional defect *in vitro* after three days of TCR-related stimulations in CD8 T cells but not in CD4 T cells (Figure 3A and B). This confirms that autophagy is required for CD8 T cell homeostasis and function while dispensable for short-term survival and activation for CD4 T cells.

CD4 T cell autophagy increases the efficiency of long-term humoral responses

As CD4 T cells are poorly impacted at early time points by the absence of autophagy in our model, we intended to define precisely the role of autophagy in T cells during humoral responses. We immunized mice with the T-dependent antigen ovalbumin (OVA), and performed a boost at day 10 as indicated in figure 4A. We followed the immune response by sampling blood at several time points during the immunization, and 12 weeks after the start of the experiment. We could not detect any difference in the titers of anti-OVA antibodies between control mice and mice deficient for autophagy in T cells at early time points (Figure 1B). However, from 8 to 12 weeks, we noticed a significantly reduced amount of anti-OVA antibodies in mice with *Atg5* deficiency in T cells. We then performed a second boost to elicit immune memory. We could then observe that the response was weaker in *Atg5*$^{f/f}$ dLck-cre mice. This effect could be due to global immune depression in these mice or early T cell senescence in the absence of autophagy in mature T cells. Interestingly, we could not detect any significant decrease of gammaglobulinemia in *Atg5*$^{f/f}$ dLck-cre mice compared to controls arguing against a global immunosuppression (Figure 1C). We then immunized young mice and compared the results to the ones obtained with mice aged from 20-24 weeks to asses if old mice exhibited a decreased response to first challenges (Figure 1D). We could not observe any significant difference between control mice and mice with autophagy-deficient T cells, corroborating previously cited results. We could not observe either differences immunizing old mice and comparing their production of anti-OVA antibodies to the one from young mice, at short-term. These results show that *Atg5* deficiency in CD4 T cells does not induce early senescence of T cells. They rather suggest a specific effect on the onset of a memory immune response. To further show that an intrinsic defect in CD4 T cell memory is the cause of the decreased long-term humoral response, we performed transfer experiments with antigen-experienced CD4 T cells and B cells in naive mice as shown in Figure 5A. B cells were purified from B6 mice and CD4 T cells from control or *Atg5*$^{f/f}$ dLck-cre mice. We first observed an anti-OVA IgG response in naive mice transferred with antigen-experienced CD4 T cells (Figure 5B) compared with mice that received naïve wild type T cells (not shown). This shows that we are able to transfer memory as IgG are reminiscent of a secondary immune response. Interestingly, the anti-OVA IgG response is weaker when naive mice

were transferred with autophagy-deficient CD4 T cells before the immunization. In contrast, we observed a normal IgM response. These results show that an intrinsic defect in memory CD4 T cell compartment impairs the generation of a memory humoral response.

Autophagy is essential for CD4 T cell central memory survival

We then aimed at understanding at which level autophagy is important for CD4 T cell memory. We purified CD4 T cells from control or *Atg5*$^{f/f}$ dLck-cre mice. We first stimulated them by anti-CD3 and anti-CD28 antibodies. In accordance with our previous findings we could not detect any difference in terms of survival, in the absence or presence of autophagy in CD4 T cells, in the days following this initial activation, if no other survival signal was added (Figure S2). We then aimed at polarizing cells with cytokines, and at culturing them during a long period of time, in the presence of IL-7. The latter cytokine is known to drive and to maintain a memory phenotype. Interestingly, during the first days after activation, the survival rate was irrespective of CD4 T cell genotype (Figure 6A). However, in any of the tested Th polarization, we could observe from 21 days on after the start of the culture until the end of the experiment a significant decrease in survival of CD4 T cells from *Atg5*$^{f/f}$ dLck-cre mice (Figure 6A and B). This decrease was more pronounced in Th2 and Th17 polarized cells. During this experiment, we monitored the acquisition of the memory phenotype by CD44 and CD62L staining. We showed again that initial activation of CD4 T cells is poorly impacted by the absence of autophagy. In fact, considering wild type and littermate cells, the proportion between central memory (CM) and effector memory (EM) cells was normal in cells purified from *Atg5*$^{f/f}$ dLck-cre-mice (Figure 7A and B). However after day 21, *Atg5*-deficient CD4 T cells failed to efficiently maintain a CM phenotype, compared to controls, as shown by the stagnation of the CM/EM ratio (Figure 7A, B and C). The results shown here for Th0 cells, were also observed for other polarizations with the exception of Th1 cells where statistical significance was not reached (Figure S3). We then aimed at understanding the underlying defects leading to impaired survival of memory cells in the absence of *Atg5*. We first observed that from day 0 to day 42, CD4 T cells maintain a relatively high load of mitochondria (Figure 8A and B). We could also observe a significantly increased population of T cells with « damaged mitochondria », meaning cells with depolarized mitochondria, together with a failure to maintain high loads of healthy mitochondria (Figure 8A, B and C). This suggests that survival of memory CD4 T cells is indeed dependent on the maintenance of a high load of healthy mitochondria, and that they may rely on mitophagy for survival. Indeed, when CD4 T cells are deficient for autophagy, T cells exhibit a higher levels of malfunctioning mitochondria, which could explain the increase in cell death observed in memory CD4 T cells from *Atg5*$^{f/f}$ dLck-cre mice.

Discussion

We generated in this work the first model with T cell deletion in all naive T cells, but only from the mature stage on. We could thus bring new information about the precise role for autophagy in T cell homeostasis. As a matter of fact, previous models could not exclude a negative impact of autophagy deletion during T cell development, leading to the reported acute impaired survival and function. The work by Jia et al, used another model to be able to discriminate between developmental defects and break in peripheral homeostasis(6). The use of estrogen receptor promoter enabled to describe defects during long-term cultures after deletion of *Atg3* induced by tamoxifen treatment. However, this model did not allow to study the behaviour *in vivo* of conditionally deleted T cells. We thus report here that CD4 T cells are poorly sensitive to autophagy impairment in the mature stage, contrary to CD8 T cells. It had been reported previously that mitophagy was a major actor for T cell survival. Indeed, thymocytes exhibit a high mitochondrial load. During thymus egress of differentiated cells, mature T cells lose a considerable part of their mitochondrial load. Interestingly, we report that this decrease in mitochondrial load is stronger in CD8 T cells than in CD4 T cells. It could explain the selective survival defect of CD8 T cells in the absence of autophagy. In relation to these findings, we found that *in vitro* CD4 T cell activation, survival and proliferation were not affected by the impairment of autophagy. In contrast, CD8 T cells exhibited proliferation defects when autophagy is absent. We can thus conclude that autophagy is not required for naive CD4 T cell homeostatic survival and short term activation, while naive CD8 T cells require autophagy.

We then validated our model for *in vivo* experiments about CD4 T cell-related immunity, as no major defect was seen in their basal number and function. In the same line as this assessment, we found that autophagy was not required for short-term antibody response against a T-dependent antigen. Moreover, gammaglobulinemia was found to be normal in $Atg5^{f/f}$ dLck-cre mice, confirming that no major humoral immunosuppression occurs. Interestingly, however, we observed that long-term immune response was compromised in the absence of autophagy in CD4 T cells. As we could rule out increased senescence of the CD4 T cell compartment with *Atg5* deficiency, we hypothesized that memory CD4 T cells were selectively impacted by the absence of autophagy. Indeed, we observed that antigen experienced CD4 T cells transferred to a naive host were unable to recapitulate the memory effect. Moreover, memory CD4 T cells differentiated *in vitro* from naive cells, exhibited decreased survival. This defect was more pronounced in Th2 and Th17 cells, and affected primarily central memory T cells. These results are in line with previous reports showing that autophagy was integral to memory CD8 T cell survival. Using transfer experiments with autophagy deficient T cells (10), or mouse lines with autophagy deletion only at the effector stage(11), it has been previously shown that

autophagy allowed the maintenance of the memory CD8 T cell compartment, and was not dispensable for influenza memory cytotoxic response. Puleston and colleagues further showed that memory CD8 T cells required autophagy for limitation of mitochondrial load and generation of reactive oxygen species leading to increased apoptosis.

We report here that memory CD4 T cells also require mitophagy for proper removal of damaged mitochondria. We further report that this process seems more important for CM T cell survival than for effector cells. Memory T cells have been shown to particularly rely on energy produced by mitochondria, such as FAO, for their long term survival. We cannot rule out either an importance of proper ER content control by autophagy as suggested by Jia et al.

In conclusion, we report here the crucial importance of autophagy in CD4 T cell-related immune responses. In addition to the previously described role of autophagy in CD8 T cell memory response and thus cytotoxic mediated effector responses, we can postulate that optimal antibody responses also need autophagy in T cells. These results can also be put in line with the described roles for autophagy in memory B cells and plasma cell long-term maintenance (14-18). Thus it appears that increasing autophagy could optimize the efficiency of humoral responses at several levels, as it was recently shown by Simon's team (10). Moreover, inhibiting autophagy could limit the chronicity of systemic autoimmune responses not only at the level of the B cell lineage as recently described (17), but also by limiting he persistence of pathogenic autoreactive CD4 T cells.

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Author's contribution

DM, JA and FG performed and designed experiments. FA performed experiments. DM, SM and FG wrote the article.

The authors declare no conflict of interest.

Material and Methods

Mice

Atg5$^{f/f}$ mice, with a flox sequence flanking exon 3 of *Atg5,* backcrossed on a C57BL/6 (B6) background, were a kind gift from Prof. N Mizushima (13). *Atg5*$^{f/f}$ mice were crossed with *distal Lck (dLck)*-cre mice for mature T-cell specific deletion (12). In all experiments, *Atg5*$^{f/f}$ dLck-cre mice with T-cell specific deletion of *Atg5* were compared with littermates (B6 *Atg5*$^{f/+}$ dLck-cre mice or *Atg5*$^{f/f}$ mice). Mice were genotyped for the *Atg5* alleles (WT, with constitutive or induced deletion) with the primers A (exons 3–1), 5'-GAATATGAAGGCACACCCCTGAAATG-3'; B (short 2), 5'GTACTGCATAATGGTTTAACTCTTGC-3'; C (check2), 5'-ACAACGTCGAGCACAGCTGCGCAAGG-3'; D (5L2), 5'-CAGGGAATGGTGTCTCCCAC-3' using PCR (94 °C (4 min); 35 cycles of 94 °C (30 s), 60 °C (30 s), 72 °C (1 min); 72 °C (5 min)). The cre transgene expressed under the control of dLck promoter was detected with primers cre-1 5'-ATGGTGCCCAAGAAGAAGAG-3'; cre-2 5'-CAGGTGCTGTTGGATGGTCT-3' using PCR (94 °C (5 min); 35 cycles of 94 °C (30 s), 58 °C (30 s), 72 °C (1 min); 72 °C (10 min)). Mice were genotyped with the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA). All mice were bred and maintained in accordance with guidelines of the local Institutional Animal Care and Use Committee (CREMEAS).

Real-time PCR

Total RNA was isolated from 5 × 10^6 purified T cells using the RNeasy Mini Kit or from 10^5 cells using the RNeasy Micro Kit (Qiagen, Courtabeuf, France, 74103 or 74004) according to the manufacturer's instructions. After treatment by DNAse (Qiagen, 79254) to remove residual genomic DNA, mRNA was retro-transcribed with the Maxima first strand synthesis kit into cDNA (ThermoFisher, Illkirch, France). Fifteen nanograms of cDNA were used for real-time PCR (RT-PCR) on StepOne apparatus (Thermo-Fisher). Briefly, *Atg5* and *Gapdh* cDNAs were amplified using Taqman Gene Expression Assays provided by ThermoFisher (Mm00504340_m1 and Mm99999915_g1). Amplicons and probes were designed to span two exons, limiting the risk of amplifying residual genomic DNA. Relative *Atg5* mRNA quantifications were made by defining ΔCT (CT *Gapdh*− CT *Atg5* where CT is 'Cycle Threshold') and ΔΔCT (ΔCT sample−ΔCT of one C57BL/6 mouse sample used for each plate) using StepOne software (ThermoFisher). Results shown represent $2^{-\Delta\Delta CT}$ values, where one same control sample is used on each plate and arbitrarily equal to 1.

Flow cytometry

Antibodies (Abs) used for flow-cytometry analysis were either purchased from BD Biosciences (Le Pont-De-Claix, France) or from eBioscience (San Diego, CA, USA). For surface stainings: allophycocyanin (APC)-cyanine 7-labelled anti-mouse TCR-β (clone H57-597, BD Biosciences 553139), phycoerythrin (PE)-labelled anti-mouse CD62L (clone MEL-14, BD Biosciences 553151), fluorescein isothiocyanate (FITC), APC or peridinin chlorophyll (PerCP) cyanine 5.5-labelled anti-mouse B220 (clone RA3-6B2, BD Biosciences, 553087, 553092, and 552771), FITC-labelled anti-mouse CD3ε (clone 145-2C11, BD Biosciences 553061), APC cyanine7, PE or PerCP cyanine 5.5, APC-labelled anti-mouse CD4 (clone GK1.5,552051, eBioscience 12-0041 or clone RM4-5, BD Biosciences, 550954, 553051), PerCP cyanine 5.5-labelled anti-mouse CD8 (clone 53-6.7, BD Biosciences, 551162), APC, APC cyanine 7 or FITC-labelled anti-mouse CD44 (clone IM7, BD Biosciences 559250, 103027, 553133), PE-labelled CD127 (IL-7R, clone A7R43 eBioscience 12-1271-82). For intracellular stainings: PE-labelled anti-mouse IL-4 (clone BVD4-1D11, BD Biosciences, 554389), PE-labelled anti-mouse IL-17A (clone eBio17B7, 12-7177-81), FITC-labelled anti-mouse IFN-γ (cone XMG1, BD Biosciences, 554411), Alexa-647-labelled anti-mouse/human TBET (clone O4-46, BD Biosciences, 561267), PE cyanine7-labelled anti-mouse GATA3 (clone L50-823, BD Biosciences, 562683), PerCP cyanine 5.5-labelled anti-mouse RORγT (cloneQ31-378, BD Biosciences, 560405). Splenocytes or lymph node cells were incubated with fluorochrome-conjugated Abs and with unlabeled rat anti-mouse CD16/CD32 monoclonal Ab (mAb, clone 2.4G2, BD Biosciences, 553142) to block Fc receptors, for 15 min at 4 °C in phosphate-buffered saline (PBS) pH 7.4 containing 2% (v/v) fetal calf serum (FCS). Surface stainings were performed for 15 min at 4° in PBS pH 7.4 containing 2% FCS. Intracellular stainings were performed after surface marker labelling using the intracellular fixation and permeabilization set from eBioscience (88-8824-00). Cells were incubated 30 min in the fixation buffer, followed by a 10 min incubation with anti-CD16/32 antibody in buffer furnished by supplier and were finally stained for 45 min with antibodies indicated above. Between each step, cells were washed with supplier's buffer. Mitochondria content and membrane potential were measured by incubating cells 15 min at 37 °C in complete medium in the presence of mitotracker green and mitotracker deep red (Fisher Scientific, Pittsburgh, PA, USA; M-7514 and M22426) at 1 µM each. Cell survival was assessed using FITC or APC-labelled Annexin V (BD Bioscience, 556419, 550475) in combination with propidium iodide (PI) at 1µg/mL (Sigma-Aldrich, Saint-Quentin, France; P4170) or 7-aminoactinomycin D (7-AAD, BD Pharmingen, 559925) following suppliers indications. Data were collected on a Gallios flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analysed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Western Blot

The Abs used for Western immunoblotting were specific for ACTB (Santa Cruz Biotechnology, clone C4, sc-47778), LC3 (MBL, clone 51–11, ref M115–3) and ATG5 (Rabbit Polyclonal, Novus, NB110-53818). In some experiments, lysosomal protease inhibitors E64d and pepstatin A (Sigma-Aldrich, P5318 and E8640) were added at 5 µg/mL each. When indicated, cells were stimulated with hamster anti-mouse CD3e (5 µg/ml, clone 145-2C11, 55305, BD Pharmingen), hamster anti-mouse CD28 (5 µg/ml, and Clone 37.51, 553294, BD Pharmingen), Phorbol-12-myristate 13-acetate (PMA, 50ng/mL, Sigma) or ionomycin (1µM, Sigma). To evaluate the autophagosomal membrane load, whole cell proteins were extracted from cultured cells using Laemmli buffer (Tris-HCl 125 mM pH 6.8; 2% (w/v) sodium dodecyl sulfate (SDS); 10% (v/v) glycerol; 5% (v/v) β-mercaptoethanol). Cell lysates were separated on 4-20% gradient gels (Biorad) and then transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with PBS containing 0.1% (v/v) Tween 20 (PBS-T) and 3% (w/v) non-fat dry milk for 1h and then incubated overnight at 4°C with 1 µg/mL anti-LC3 antibody in PBS-T containing 1% non-fat dry milk, or for 1h at room temperature with 1µg/ml anti-ATG5 antibody in PBS-T containing 1% non-fat dry milk. After washing with PBS-T, membranes were incubated for 30 min at room temperature with goat anti-mouse IgG antibody (Southern Biotech, Birmingham, Alabama, 1030-05) conjugated to horseradish peroxidase (HRP). Signal was detected using enhanced chemiluminescence detection reagents (Immobilon Western, Merck Millipore, Darmstadt, Germany, WBKLS0500).

Immunizations

Eight to twelve week-old mice were injected intraperitoneally (i.p.) on day 1 and 10. A third immunization was performed 12 weeks after the first one. Mice were bled on days 5 and 15, on weeks 8 and 12 + 5 days. Mice were injected with 100 µg OVA (Sigma) in complete Freund's adjuvant (CFA, Sigma) for the first injection and with 100 µg OVA in incomplete Freund's adjuvant (IFA, Sigma) for the second and third injections. Detection of anti-OVA IgM and IgG was assessed by ELISA.

Antibody detection by ELISA

IgG, IgM absolute quantities and titers were measured in serum from immunized mice. To measure anti-OVA specific antibodies, 96-well ELISA Maxisorp plates (NUNC, Denmark) were coated with OVA (10 µg/ml, Sigma) in 50mM sodium carbonate buffer (pH 9.6) over-night at 4°C. The wells were blocked with PBS-0,1 % tween 5% (w/v) milk for 1h at 37°C and incubated with diluted sera for 1 h at 37°C. HRP-conjugated anti-mouse isotype-specific antibodies (polyclonal antibodies purchased from Jackson Immunoresearch) were used as revealing antibodies. Absorbance was measured at 450 nm, after revelation with tetramethylbenzidine to the wells, and stop of the reaction by 1M HCl. Titers were

determined as the last dilution giving an absorbance equal or superior to 0,2. IgG and IgM absolute quantifications were performed using the IgM/IgG quantification kit from Bethyl laboratories following manufacturer's indications (Bethyl, Montgomery,TX, USA, E90-101/E90-131). IgM/IgG concentration of the different samples was evaluated following a standard curve.

Cell isolation and culture

Spleens were collected from B6, dLck-cre *Atg5*$^{f/f}$ mice or from littermates. Splenic CD4 or CD8 T cells were purified by negative selection. T cells were sorted from spleen cell suspension with the Dynabeads untouched mouse CD4 or CD8 cells isolation kit (ThermoFisher Scientific, Illkirch, France, respectively 11415D and 11417D). Cells were >90% pure according to TCRβ$^+$/CD4$^+$/B220$^-$ or TCRβ$^+$/CD8α$^+$/B220$^-$ population quantifications by cytometry. When indicated, cells were stimulated with hamster anti-mouse CD3ε (5 µg/ml, clone 145-2C11, 553057, BD Pharmingen), hamster anti-mouse CD28 (5 µg/ml, and Clone 37.51, 553294, BD Pharmingen), Phorbol-12-myristate 13-acetate (PMA, 50 ng/mL, Sigma) or ionomycin (1 µM, Sigma). For the analysis of proliferation, cells were stained with 0,5 µM carboxyflurescein isothiocyanate succinimidyl ester diacetate (CFSE, Sigma) before stimulation.

CD4 T cell polarization and long-term culture

After isolation, splenic CD4 T cells were cultured at 37°C, 5% CO_2 in RPMI 1640 medium (Lonza BioWhittaker) supplemented with 10% FCS, 10 mg/mL gentamycin (Lonza BioWhittaker), 10 mM HEPES (Lonza BioWhittaker) and 0.05 mM β-mercaptoethanol (Lonza BioWhittaker) with the according cytokine/antibody cocktail for each T helper cell subset. For the non-polarized condition (Th0), cells were only stimulated with anti-mouse CD3 and CD28 (clone 145.2C11, 553057, and Clone 37.51, 553294) at the same concentration as for previous cell cultures (see cell isolation and cell culture). In addition to the anti-CD3/CD28 stimulation, anti-mouse IL-4 (10µg/mL, clone 11B11, 554432) and recombinant mouse IL-12 (2ng/mL, p70, 554592) were added for Th1 polarization, anti-mouse IFN-ɣ (10µg/mL, clone XMG1.2, 554408) and recombinant mouse IL-4 (15ng/mL, 550067) for Th2 polarization and finally anti-mouse IL-4, anti-mouse IFN-ɣ, recombinant mouse IL-6 (10ng/mL, 554582) and recombinant mouse TGF-β1 (1 ng/mL, R&D Systems, 7666-MB-005) for Th17 polarization. The cells were cultured at a density of 10^5 cells/well in a 96 well plate for four days. Then, cell polarization was assessed by flow cytometry, cell medium was changed and recombinant IL-7 (5 ng/mL, 407-ML-005, R&D Systems) was added every 3-4 days and the medium changed every 7 days. The CD4 T cells were in culture for 42 days. The majority of cytokines/antibodies used were purchased from BD Biosciences except if otherwise specified.

Adoptive transfer

The donor mice (B6, dLck cre *Atg5*$^{f/f}$ mice or littermates) were immunized with 100 µg OVA/CFA injected i.p. Four weeks later, the mice were sacrificed and CD4 T cells were isolated from the spleen from each mouse. Memory B cells were isolated from the spleen of one B6 immunized mouse. Positive Memory CD4 T cell isolation was accomplished using the CD4 (L3T4) MicroBeads cell isolation kit (130-049-201; Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Resulting $CD4^+/CD8\alpha^-/B220^-$ mouse CD4 T cell preparations were >85% pure (~20% were however neither CD8+ nor B220+) as determined by flow cytometry. Memory B Cells were negatively isolated using the CD43 (Ly-48) MicroBeads cell isolation kit (130-049-801, Miltenyi) according to manufacturer's instructions. Resulting CD4-/CD8-/B220+ B cells were >90% pure. The CD4 T cells and the B cells were co-injected in recipient B6 mice in the proportion of $2x10^6/2x10^6$ cells. 7 days after adoptive transfer the recipient mice were immunized with 100µg OVA/CFA and 7 days later they were bled. Detection of anti-OVA IgM and IgG response was assessed by ELISA.

Figures and Legends

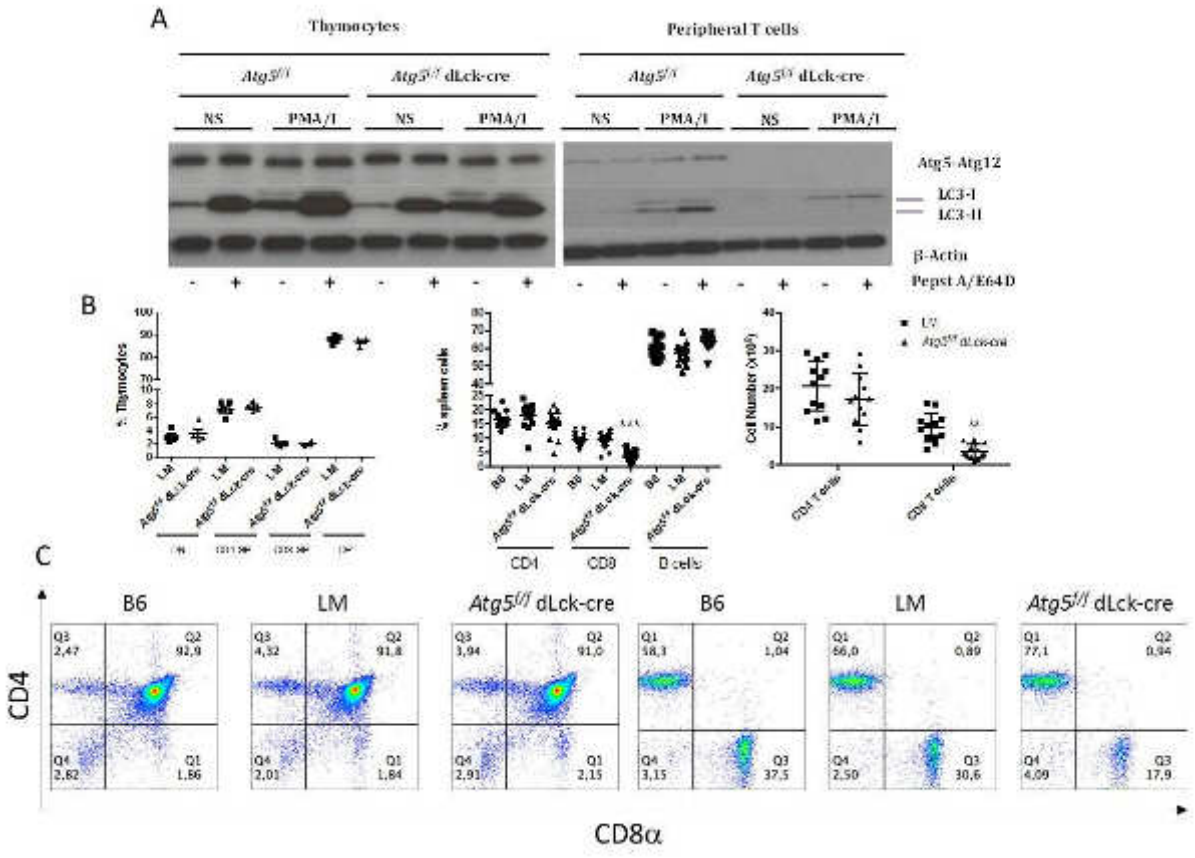

Figure 1: autophagy is dispensable for CD4 T cell homeostasis

(A) Thymocytes or peripheral T cells were isolated from spleens of littermate or *Atg5*$^{f/f}$ dlck-cre mice. Cells were stimulated by 50 ng/mL PMA and 1 µg/mL Ionomycin for 18 hours or not (NS). During the last 4 hours of stimulation, cells were treated (+) or not (-) by pepstatin A and E64d. Cell lysates were then processed by SDS-PAGE and blotted against ATG5 and LC3. Representative experiment of at least three replicates.

(B) Left: double negative (DN), CD4 single positive (CD4 SP), CD8 single positive (CD8 SP) or double positive (DP) cells proportion among thymocytes (n=5 Littermates and n=4 *Atg5*$^{f/f}$ dlck cre mice). Middle: percentages of CD4, CD8 T cells, and B cells among spleen cells (n=13 B6, n=17 littermate and n= *Atg5*$^{f/f}$ dlck-cre mice). Right: absolute CD4 and CD8 T cell number in spleens (n=12 for each genotype). Each point represents a measure for one mouse. Littermate (LM) and *Atg5*$^{f/f}$ dLck cre mice are represented and compared by Mann Whitney U Test. *** p<0,001.

(C) Representative dot plots of thymocytes stained by anti-CD4 and anti-CD8 antibodies (left) and spleen cells gated on TCRβ^{+}B220^{-} cells (right) from C57BL/6 (B6), Littermate (LM), *Atg5*$^{f/f}$ dLck cre mice.

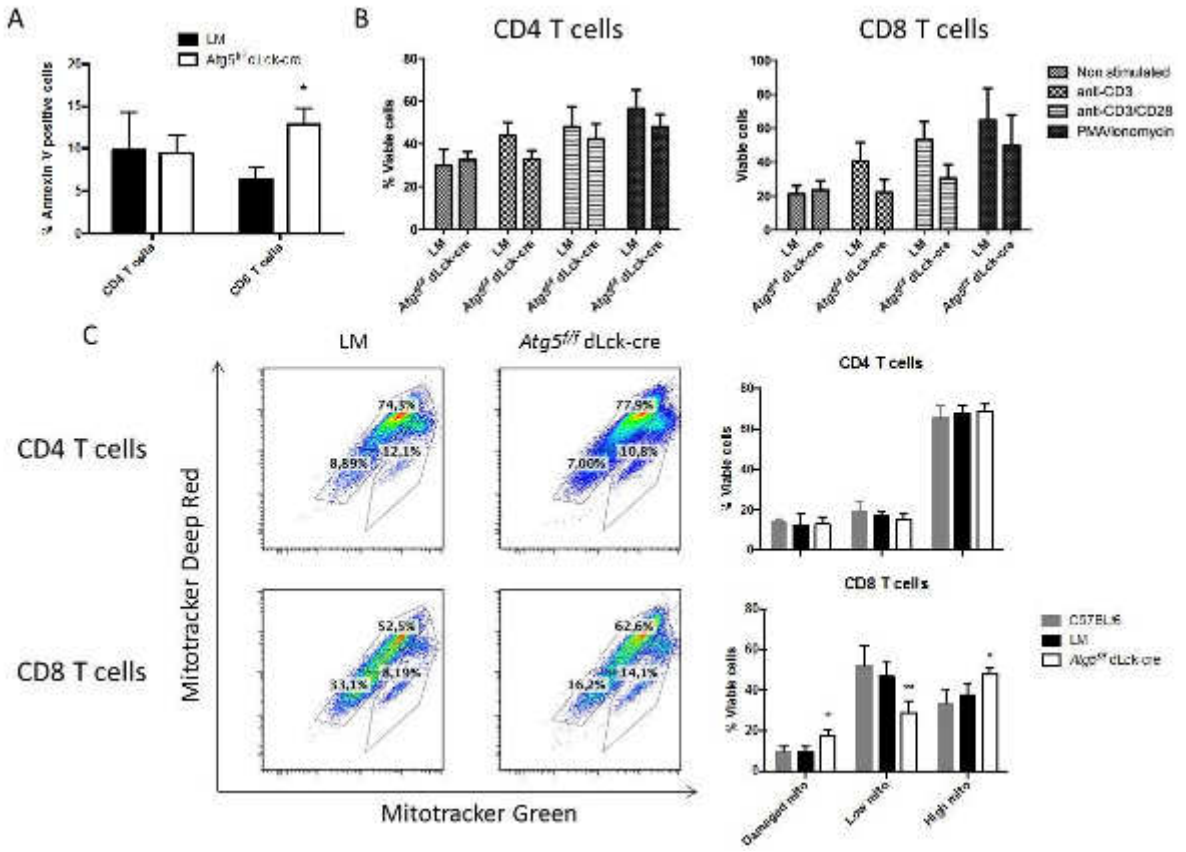

Figure 2: *Atg5* is necessary for mitochondrial homesostasis in mature CD8 T cells

(A) Percentage of dead cells measured by Annexing V staining *ex vivo* from spleen cells isolated from littermate (LM) and *Atg5*$^{f/f}$ dLck-cre mice, after gating on TCRβ^{+}CD4^{+} cells (left) or TCRβ^{+}CD8^{+} cells (n=1 for B6 mice and n=3 for LM and *Atg5*$^{f/f}$ dlck cre mice).

(B) percentage of viable cells defined as annexing V^{-}/ PI^{-}, defined after stimulation of purified CD4 (left) or CD8 T cells (right) under the indicated conditions (n=4 for each genotype).

(C) Staining of CD4 (up) or CD8 T cells (low) purified from LM or *Atg5*$^{f/f}$ dLck-cre mice by mitotracker deep red and mitotracker green. Gates could be defined for cells with high load of mitochondria (mitotracker deep red^{hi}, mitotracker green^{hi}), cells with low mitochondrial content (mitotracker deep red^{low}, mitotracker green^{lo}) and cells with damaged mitochondria (mitotracker green^{hi}, mitotracker deep red^{low}). On the right, histograms showing results on several experiments of percentages obtained as described above (n=4 for each genotype). Littermate (LM) and *Atg5*$^{f/f}$ dLck-cre mice are represented and compared by Mann Whitney U Test. ** p<0,01. * p<0,05

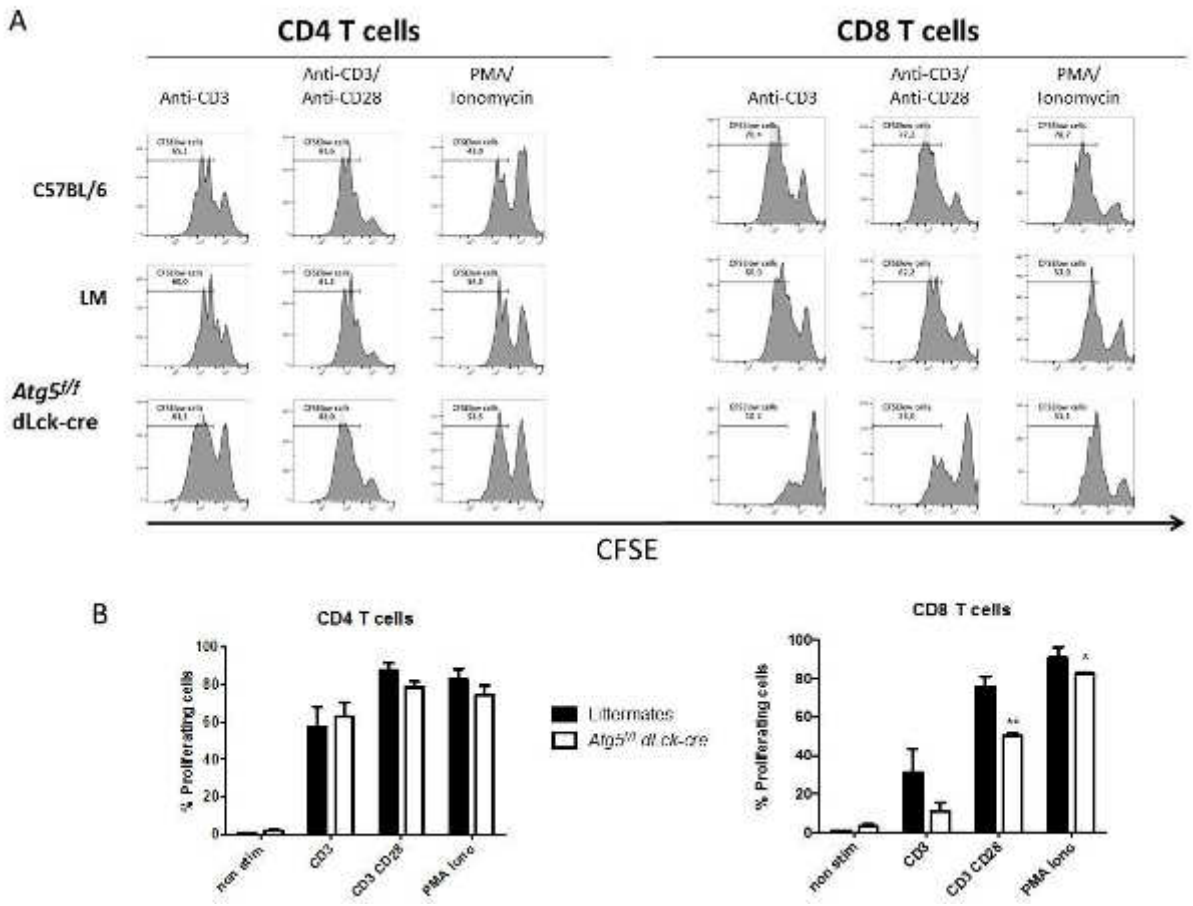

Figure 3: *Atg5* is dispensable for CD4 T cell activation *in vitro*

(A) Evaluation of proliferation monitored by CFSE staining after stimulation by anti-CD3 antibody, a combination of anti-CD3 and anti-CD28 antibodies or 50 ng/mL PMA and 1 µg/mL ionomycin. Percentages shown indicate $CSFE^{low}$ cells, i.e. cells that have proliferated.

(B) In the lowest panels, results obtained on several experiments are synthesized in histograms (n=5 for each genotype). Littermate (LM) and *Atg5*$^{f/f}$ dLck cre mice are represented and compared by Mann Whitney U Test. ** p<0,01. * p<0,05

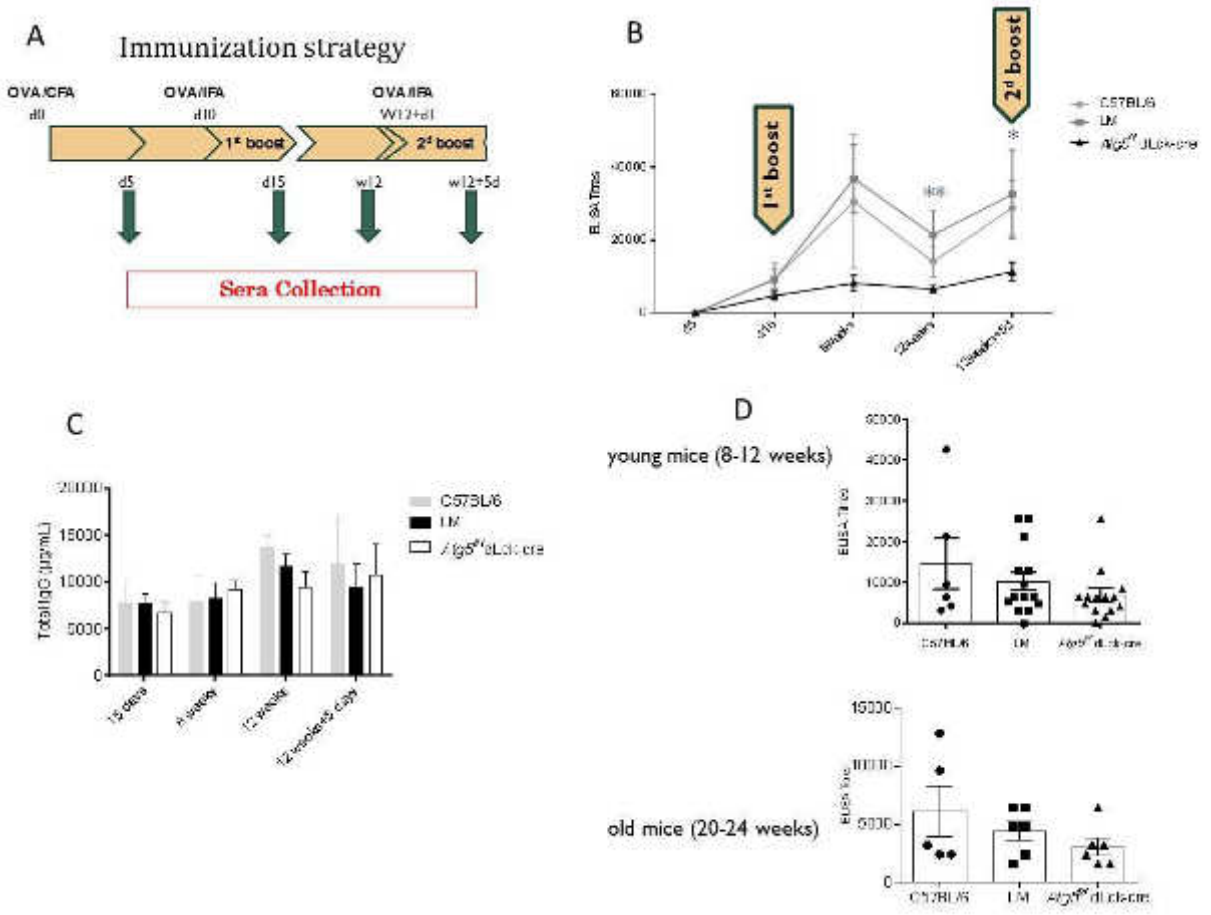

Figure 4: *Atg5* is dispensable for CD4 T cell activation *in vitro*

(A) Immunization protocol used to show the impact of T cell autophagy on long term humoral response. Mice were immunized at day 0 by OVA in CFA and at day 10 with OVA in IFA. Mice were then immunized at week 12+1 day with OVA in IFA. Serum were collected at days 5, 15, at week 12 and week 12+5 days.

(B) Longitudinal monitoring of anti-OVA antibody titers measured by ELISA (n=6 B6, n=12 LM and n=14 *Atg5*$^{f/f}$ dlck cre mice).

(C) Total IgG levels measured by ELISA at different time points after the immunization (n=2 B6, n=6 LM and n=6 *Atg5*$^{f/f}$ dlck cre mice).

(D) Histograms showing results of anti-OVA antibodies measured by ELISA obtained after one immunization at day 0 and one boost at day 10. Each point corresponds to a measure for one mouse. Titers were measured at day 15. Young mice (8-12 week-old, (n=6 B6, n=12 LM and n=14 *Atg5*$^{f/f}$ dlck cre mice) and old mice (20 to 24 week-old, n=5 B6, n=6 LM and n=6 *Atg5*$^{f/f}$ dlck cre mice) from different genotypes were compared.

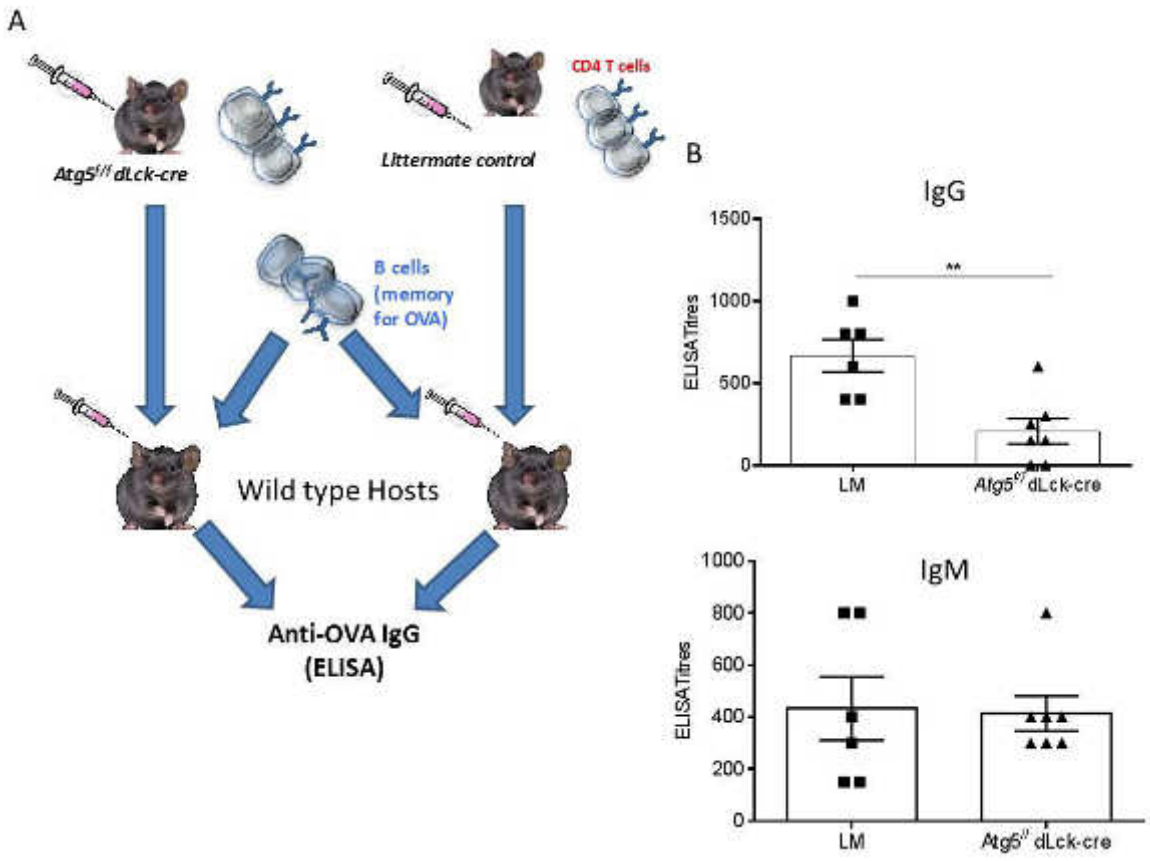

Figure 5: CD4 T cell autophagy is intrinsically needed for humoral memory

(A) Transfer experiment protocol that show intrinsic defect in CD4 T cell memory in the absence of autophagy. Wild type, littermate or *Atg5*$^{f/f}$ dLck cre were immunized with OVA in CFA. B cells were isolated from wild type mice and CD4 T cells isolated from littermate or *Atg5*$^{f/f}$ dLck cre mice. B cells and CD4 T cells were transferred into naive wild type hosts that were then immunized by OVA in IFA. **(B)** Measurement by ELISA of anti-OVA IgG antibodies in serum, 5 days after immunization of naive hosts. Each point represents an individual mesure, histograms stand for means and bars represent standard deviation (n=6 transfers with LM T cells and n=7 with *Atg5*$^{f/f}$ dlck cre T cells). ** p<0,001 by Mann Whitney U Test.

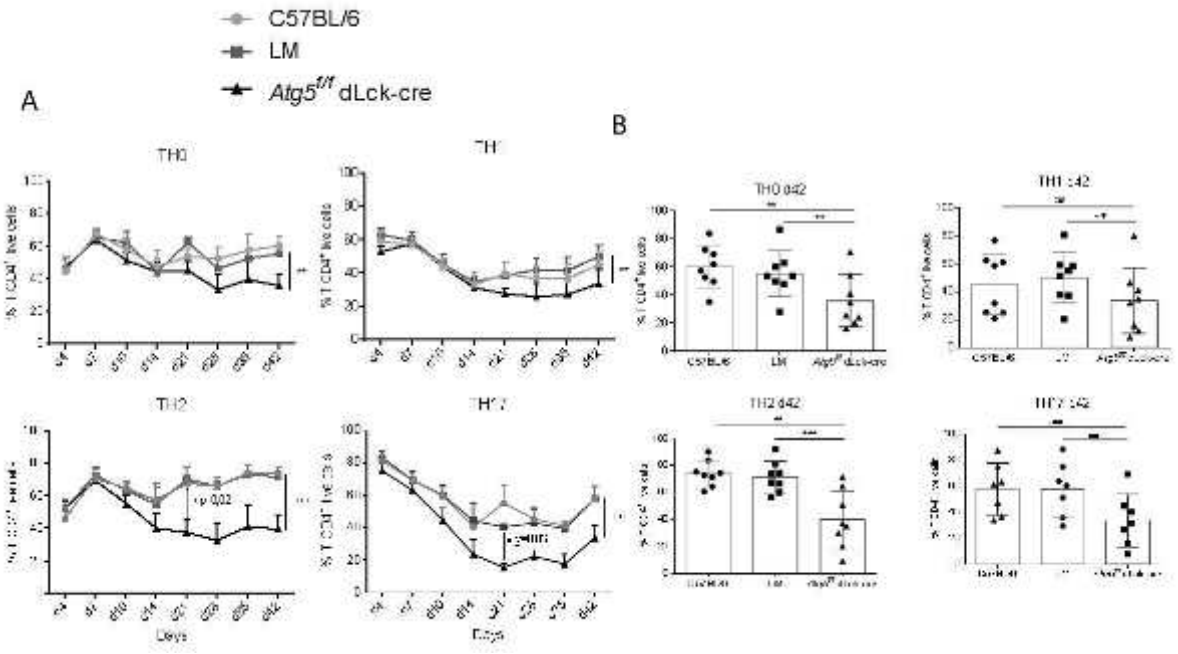

Figure 6: Autophagy is needed for memory CD4 T cell survival

CD4 T cells were isolated from indicated genotypes and stimulated by anti-CD3 and anti-CD28 antibodies for 7 days in the presence of polarizing cytokines to differentiate cells into Th0, Th1, Th2 or Th17 cells.

(A) Longitudinal study of cell viability by flow cytometry measurement of AnnexinV/PI negative cells at indicated days for Th0, Th1, Th2, and Th17 cells.

(B) Results obtained on several individual experiments for cell survival at the end of the protocol, at day 42. Each point represents an individual mesure, histograms stand for means and bars represent standard deviation. *Atg5*$^{f/f}$ dLck cre mice are represented and compared by Mann Whitney U Test with LM mice (n=8 experiments). *** p<0,001. ** p<0,01

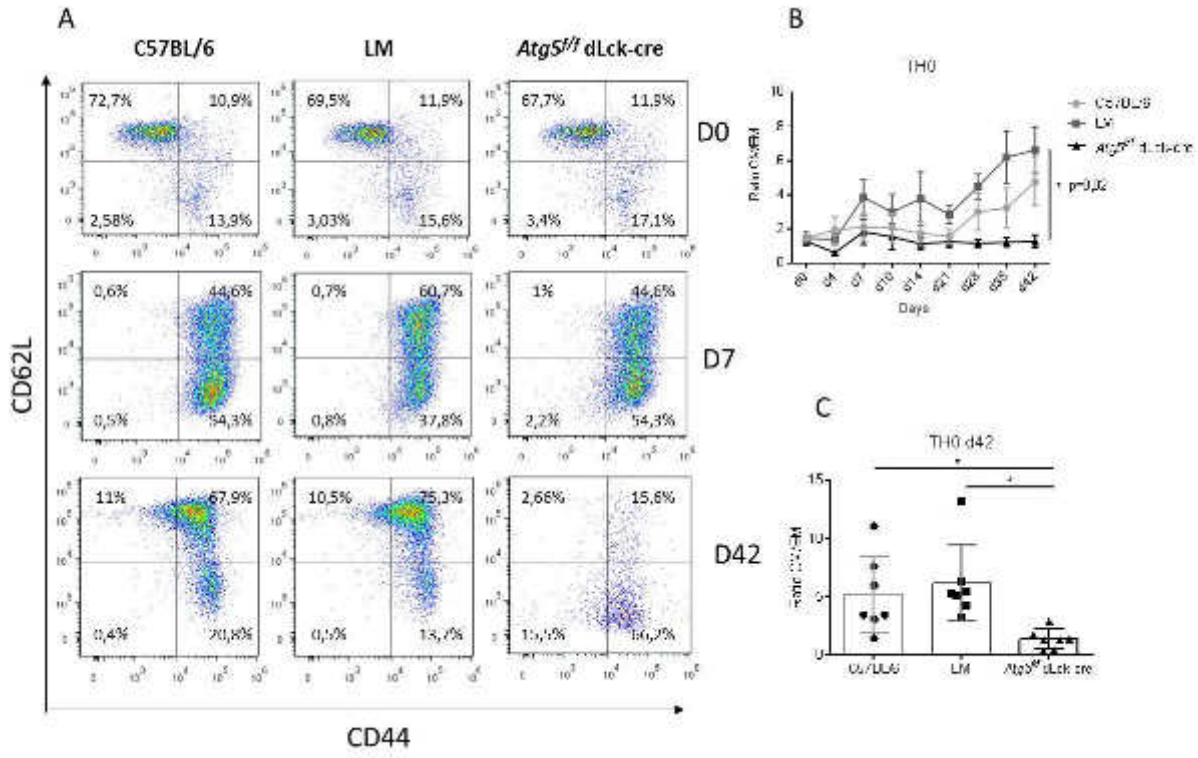

Figure 7: Autophagy is needed for central memory CD4 T cell maintenance

CD4 T cells were isolated from indicated genotypes and stimulated by anti-CD3 and anti-CD28 antibodies for 7 days in the presence of polarizing cytokines to differentiate cells into Th0, Th1, Th2 or Th17 cells.

(A) At indicated days (0, 7 and 42) cells were stained to detect CD44 and CD62L expression, and analysed by flow cytometry. A representative experiment is shown for each genotype (B6, LM and *Atg5*$^{f/f}$ dLck cre). Central memory T cells (CM) were defined as CD44^{hi}CD62L^{hi}, effector memory T cells (EM) were defined as CD44^{hi}CD62L^{lo} and naive cells were defined as as CD44^{lo}CD62L^{hi}.

(B) Longitudinal study of cell viability by measurement by flow cytometry of the ratio between percentages of CM T cells and EM T cells, at indicated days for Th0 cells.

(C) Results obtained on several individual experiments for cell survival at the end of the protocol, at day 42. Each point represents an individual mesure, histograms stand for means and bars represent standard deviation. *Atg5*$^{f/f}$ dLck cre mice are resented and compared by Mann Whitney U Test with LM mice (n=7 experiments). * p<0,05

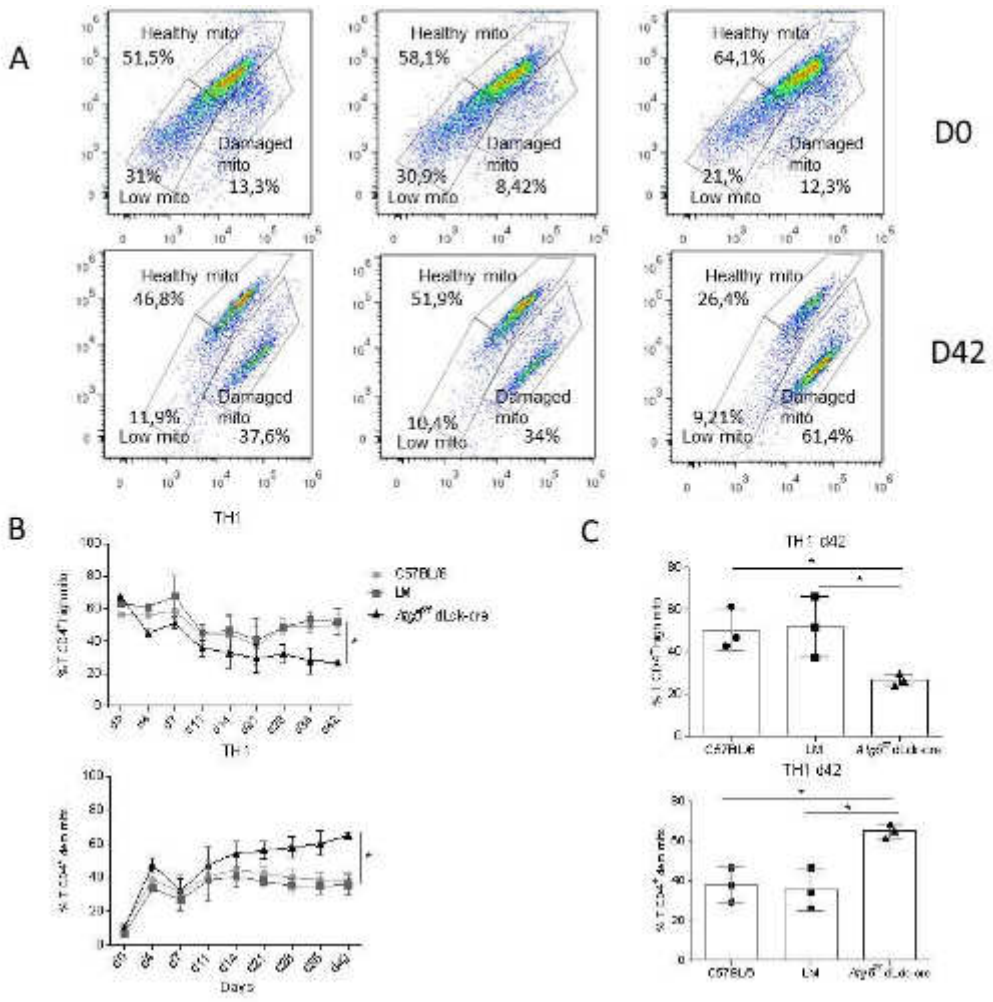

Figure 8: Autophagy is needed for mitochondrial homeostasis in memory CD4 T cells

CD4 T cells were isolated from indicated genotypes and stimulated by anti-CD3 and anti-CD28 antibodies for 7 days in the presence of polarizing cytokines to differentiate cells into Th0, Th1, Th2 or Th17 cells.

(A) At indicated days (0 and 42), cells were stained by mitotracker deep red and mitotracker green. Gates could be defined for cells with high load of mitochondria (mitotracker deep red^{hi}, mitotracker green^{hi}), cells with low mitochondrial content (mitotracker deep red^{low}, mitotracker green^{lo}) and cells with damaged mitochondria (mitotracker green^{hi}, mitotracker deep red^{low}). A representative experiment is shown.

(B) Longitudinal study of percentages of CD4T cells with high mitochondria content (up) or damaged mitochondria (bottom).

(C) Results obtained on several individual experiments for percentages of CD4T cells with high mitochondria content (up) or damaged mitochondria (bottom) at day 42. Each point represents an individual mesure, histograms stand for means and bars represent standard deviation. *Atg5$^{f/f}$* dLck cre

mice are represented and compared by Mann Whitney U Test with LM mice (n=3 experiments). * p<0,05

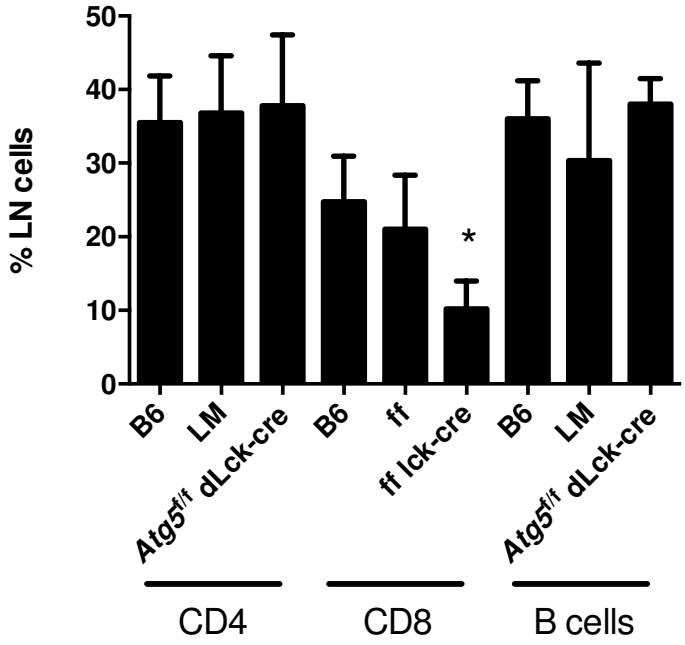

Figure S1: Autophagy is dispensable for CD4 T cell homeostasis in lymph nodes.

Histograms showing percentages of CD4 ($TCR\beta^+CD4^+$), CD8 ($TCR\beta^+CD8\alpha^+$) and B cells ($TCR\beta^-B220^+$) among lymph node cells. C57BL/6 (B6), Littermate (LM), *Atg5$^{f/f}$* dLck cre mice. are shown and compared by Mann Whitney U Test with LM mice (n=8 experiments) . * p<0,05.

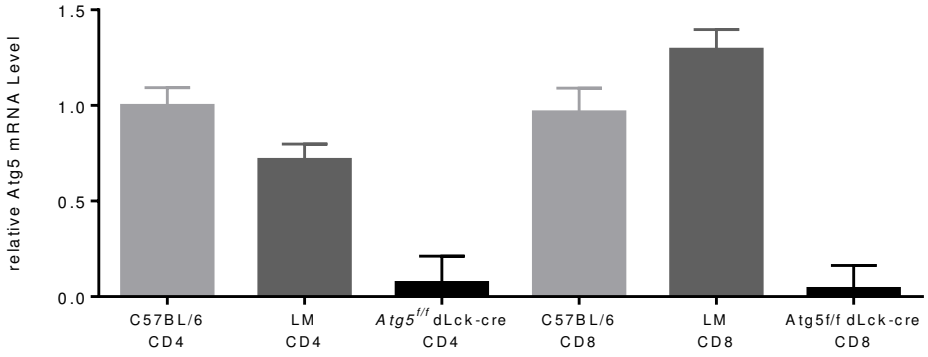

Figure S2: Atg5 deletion is equally efficient in CD4 and CD8 T cells from *Atg5$^{f/f}$* dLck cre mice

Histograms showing relative quantities of *Atg5* transcripts in isolated CD4 or CD8 T cells according to the indicated genotypes: C57BL/6 (B6), Littermate (LM), *Atg5$^{f/f}$* dLck cre mice. Means obtained on several experiments are shown and bars represent standard deviation (n=3).

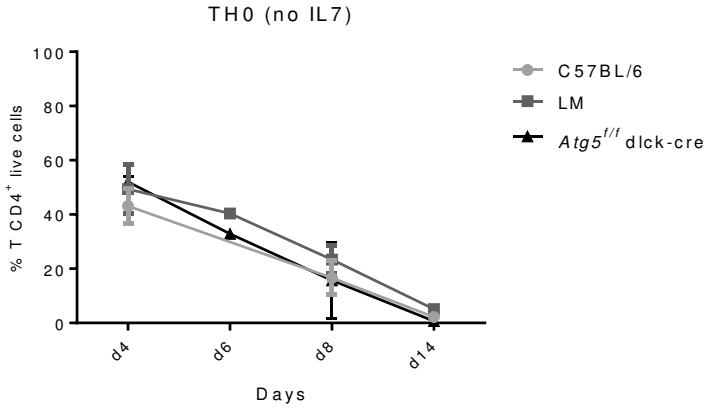

Figure S3: Autophagy is dispensable survival early after TCR stimulation.

CD4 T cells were isolated from indicated genotypes and stimulated by anti-CD3 and anti-CD28 antibodies for 7 days in the presence of polarizing cytokines to differentiate cells into Th0, Th1, Th2 or Th17 cells. Longitudinal study of cell viability by measurement by flow cytometry of annexing V propidium iodide negative cells at indicated days for Th0 cells (n=2).

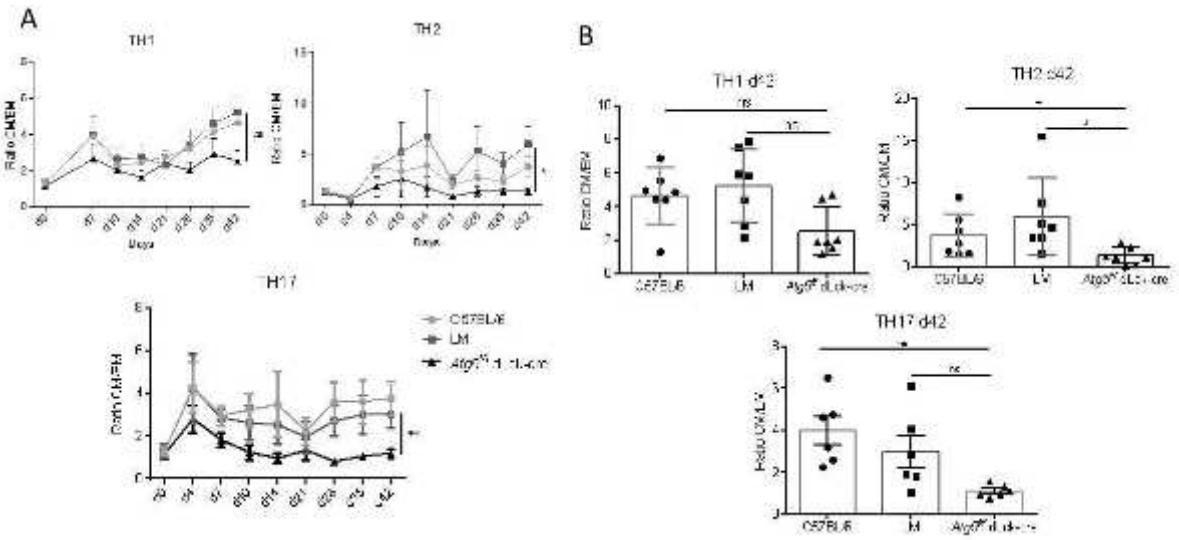

Figure S4: Autophagy is necessary for polarized memory CD4 T cell survival

CD4 T cells were isolated from indicated genotypes and stimulated by anti-CD3 and anti-CD28 antibodies for 7 days in the presence of polarizing cytokines to differentiate cells into Th1, Th2 or Th17 cells. (A) Longitudinal study of cell viability by measurement by flow cytometry of the ratio between percentages of CM T cells and EM T cells, at indicated days for Th1, Th2 and Th17 cells. (B) Results obtained on several individual experiments for cell survival at the end of the protocol, at day 42. Each point represent an individual measure, histograms stand for means and bars represent standard deviation. *Atg5$^{f/f}$* dLck-cre mice are resented and compared by Mann Whitney U Test with LM mice (n=7 experiments) . ** p<0,01. * p<0,05

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3 Project 3

3.1 Forword

Signaling pathways inducing autophagy in response to TCR stimulation (preliminary results)

Diane Murera, Johan Arnold, Nico Reusch, Florent Arbogast, Sylviane Muller and Frédéric Gros.

A part of my thesis work was dedicated to the investigation of the pathways that initiate autophagy upon TCR activation. This subject was of particular interest since it was poorly understood when I started my PhD. The fact that my team and others have uncovered that this process is upregulated in a systemic lupus erythematosus (SLE)-context, raised even more the question on the regulation leading to these events **(Gros et al., 2012; Alessandri et al., 2012; Clarke et al., 2014)**. Furthermore, autophagy has been identified as essential for T cell homeostasis, which regulation is crucial in controlling adaptive immunity. Their tolerance breakdown is implicated in the development of autoimmune diseases like SLE.

My work so far has allowed to establish that autophagy induction is mainly mediated by the calcium pathway for *in vitro* TCR-stimulated healthy and pathological T cells. Since inhibiting calcineurin downstream of the calcium signaling pathway led to down regulation of LC3-II, we speculated that autophagy induction could be regulated by the transcription factor NFAT. Transcription studies revealed however rather a downregulation than an upregulation of *Atg* transcripts, suggesting that the process is controlled by other mechanisms. We hypothesized that this process could be translationally regulated and in fact inhibition of translation with a pharmacological inhibitor led to blockade of the autophagic flux. These results need to be confirmed and this project still requires to be investigated in more details. In this part of the manuscript I will discuss the data collected so far and the possible perspectives to bring to light the exact mechanisms responsible for autophagy induction in T cells.

3.2 Signaling pathways inducing autophagy in response to TCR stimulation (preliminary results)

Introduction:

Macroautophagy more commonly called autophagy, is a catabolic lysosomal degradation process of cytoplasmic content. It allows the recovery of metabolites that can be reused in cellular functions and degradation of dysfunctional organelles. This process is conserved among eukaryotes and is active in every cell at basal levels. It can however be triggered in various conditions such as starvation or through activation of cellular receptors. Autophagy has been shown to be highly involved in the regulation of the immune system. Autophagy participates in fact in pathogen clearance upon infection, in the establishment of inflammatory events as well as in the maintenance of lymphocyte homeostasis.

In T lymphocytes this process is induced upon TCR stimulation and it has been shown to be important for their survival and activation in the periphery as demonstrated by several works **(Pua et al., 2007; Stephenson et al., 2009; Willinger and Flavell, 2012)**. Our team was also able to show that T cell activation with the pharmacological activators PMA and ionomycin, resulted indeed in increased levels of LC3-I into LC3-II both in healthy and lupus prone mice ($MRL^{lpr/lpr}$ and (NZB/W)F1). The increase was more pronounced in lupus T cells. The further accumulation of LC3-II in the presence of protease inhibitors was reflective of an active autophagic flux in both cases. T cells from SLE patients displayed enhanced formation of double membrane vesicles in comparison to T cells from healthy subjects. Other teams have also observed an upregulation of the autophagic markers in SLE lymphocytes **(Alessandri et al., 2012b; Clarke et al., 2015)**. This and the fact that single nucleotide polymorphisms of the core autophagy machinery gene *Atg5* were found in a lupus context have led to envisage that autophagy could contribute to the maintenance of pathologic T lymphocytes **(Zhang et al., 2013, 2015b; Zhou et al., 2011b).** Thus it has raised a number of questions about the mechanisms leading to the induction of this process in this specific cell type, and why the increase is more important in an SLE context.

We found that the calcium pathway is the main inducer of LC3-II. This phenomenon is transcription independent and translation depedent. It reflects a sustained autophagosome production with constant lysosomal degradation.

Results:

Autophagy is upregulated both in CD4 and CD8 T cells from lupus-prone $MRL^{lpr/lpr}$ mice.

As shown by our previous work, autophagy is deregulated in T lymphocytes from SLE patients and lupus-prone mice. We wanted to establish if this was the case in both T cell subtypes, which is why we

isolated CD4 and CD8 T cells from the spleen, and treated them with pharmacological activators phorbol myristyl acetate (PMA or P) and ionomycine (Iono or I), stimulating the protein kinase C (PKC) pathway and the calcium pathway respectively. We carried out LC3 conversion immunoblots in order to assess the autophagic activity. We observed that the autophagic activity was increased upon activation in both cell types (comparison LC3-II detection between condition without (-) and with (+) protease inhibitors pepstatin A and E64D). This was the case in control CBA/J mice as well as in MRL$^{lpr/lpr}$ mice, with a higher autophagic activity in MRL$^{lpr/lpr}$ CD4 and CD8 T cells. The basal autophagic activity in lupus-prone mice was however already higher in comparison to control mice, especially in CD8 T cells **(Fig 1)**.

TCR stimulation induces autophagy in T cells and more intensely in MRL$^{lpr/lpr}$ mice

We wanted then to investigate if TCR activation by antibodies had the same effect on autophagy. For this purpose, we stimulated splenic T cells with both anti-CD3 and anti-CD28 agonist antibodies. Anti-CD3 activates the TCR while anti-CD28 serves as the co-stimulatory signal required for full T cell activation and survival. This resulted in a potent induction of autophagy markers in the case of TCR stimulation alone in T cells from CBA/J control mice as well as in MRL$^{lpr/lpr}$ T cells, while co-stimulation alone had no or little impact as LC3-II levels remained at the basal level. Co-activation with both anti CD3 and anti-CD28 did not have a synergistic effect on LC3-II expression either. The absence of increased LC3 detection after anti-CD28 activation could paradoxically be explained by the fact that the stimulation results in AKT pathway activation, which leads to mTOR activation a well know autophagy inhibitor. This might also be a question of the antibody concentration used, since Botbol and colleagues observed an increase in LC3-II when stimulating CD4 T cells with lower anti-CD28 concentrations (0,5µg/mL) **(Botbol et al., 2015)**. Nevertheless the TCR signal alone seems to be strong enough to mediate autophagy induction in our settings. As expected, autophagic activity was way higher in lupus T cells suggesting an underlying defect in autophagosome generation and/or degradation **(Fig 2)**.

Calcium pathway activation induces autophagy in MRL$^{lpr/lpr}$ T cells.

TCR stimulation induces various signaling pathways leading to the expression of differentiation, growth and survival factors. The main pathways induced upon TCR stimulation are the PKC pathway, the MAPK pathway and the calcium pathway. We thus wanted to explore if any of these pathways could possibly be the signal to autophagy induction in T cells or if it requires an interplay of the different pathways. The pharmacological activators PMA and ionomycin as already mentioned, activate down-stream TCR-induced signals namely the PKC and the calcium pathways respectively. Since they revealed to be quite efficient autophagy inducer when combined we were interested in their effect when used separately

to activate splenic T cells. We observed that stimulating the PKC pathway led to conversion of LC3-I into LC3-II but that the calcium pathway induction was even more efficient for this purpose, especially in lupus T cells **(Fig 3)**. This is in agreement with the fact that the intracellular calcium store has been shown to be aberrantly regulated in T lymphocytes of lupus patients and lupus-prone mice. Interestingly, the use of EGTA an extracellular calcium chelator had a potent inhibitory effect on the autophagic activity of T cells for both mice, confirming further the dominant implication of calcium signaling in autophagy activation. Incubating T cells with EGTA resulted indeed in the decrease of LC3-II in both conditions without and with protease inhibitors in a dose-dependent manner, suggesting that the autophagosome generation is affected by the absence of extracellular calcium influx. The autophagic flux was however still active as we could see an accumulation of LC3-II in the presence of protease inhibitors. The inhibition threshold is lower in CBA/J T cells than in MRL$^{lpr/lpr}$ T cells for which a higher concentration of this inhibitor was required to completely block the autophagic activity **(Fig 4A, B)**. In contrast, inhibition of the PKC pathway by bisindolylemaleimide (BIM) upon TCR stimulation with anti-CD3, only resulted in a slightly reduced autophagic flux in T cells from control mice and lupus T cells, suggesting a possible interplay of different pathways in autophagy completion **(Fig 5A, B, C).**

Calcineurin inhibition decreases the amount of autophagic structures and autophagic flux in T cells. Seeing the impact of EGTA on the autophagic activity we wanted to explore further the calcium pathway. As a matter of fact, extracellular calcium influx leads to the activation of the phosphatase calcineurin, required to induce translocation of the transcription factor NFAT into the nucleus where the latter regulates gene expression. We used a well-known calcineurin inhibitor, cyclosporine A (CsA) at different concentrations to establish if previous observations were linked to this pathway in particular. We observed an almost complete inhibition of the autophagic activity in CBA/J T cells treated with 100ng/mL CsA **(Fig 6).** In T cells from lupus prone mice, the autophagic activity could still be detected at the same concentrations of CsA, but it had more an impact on the flux. Since only high concentrations of CsA were required to inhibit autophagy, we verified the viability of the T cells after treatment in order to assess that the observed decrease wasn't due to cell toxicity of CsA. Even at the highest concentrations with protease inhibitors there was no significant increase in pre-apoptotic or dead cells in CBA/J and MRL$^{lpr/lpr}$ T cells (not shown).

***Atg* expression decreases upon TCR stimulation**.

Since calcineurin directly interacts with the transcription factor NFAT, which is also over activated in T cells from SLE patients, we speculated that NFAT could regulate the expression of some *Atg*. In lupus T cells the enhanced calcium flux leads in fact to increased activation of calmodulin and calcineurin and thus to increased translocation of NFAT into the nucleus. We thus decided to investigate first in normal T cells from C57BL/6 mice if activating or inhibiting the TCR signal could have an influence on

the transcription program of some chosen core *Atg* (*Beclin1, Atg7, Atg5, Maplc3b*). Surprisingly we predominantly observed a decreased expression of the tested *Atg* as demonstrated by the kinetic qPCR experiments **(Fig 7)**. The gene expressions of *Beclin1, Atg5* and *Atg7* were evaluated 8h, 21h and 48h after activation and were shown to be decreased under every condition except for the stimulation with ionomycin. In this case the expression of *Beclin1* was slightly increased (1.5-fold) after 21h and 48h while for *Atg5* the expression went back to the basal levels after 48h stimulation with ionomycin. *Maplc3b* expression was also decreased upon TCR stimulation, while calcineurin abrogated its repression **(Fig 8)**. These results are in contradiction with LC3-II protein levels observed by western blot, and suggest that the increase of LC3-II is not due to an increase in the transcription of *Atgs* in response to the TCR signaling.

Autophagy induction is mainly post-transcriptionally regulated.

Based on the qPCR results we postulated that autophagy might not be transcriptionally regulated but might rather depend on other factors and events occurring post-transcriptionally. To establish that we decided to use inhibitors of transcription (actinomycin D) and translation (cycloheximide). We observed in fact in a preliminary experiment a decrease in the generation of autophagic membranes and in the flux in T cells treated with cycloheximide while no effect was seen under actinomycin D treatment. If confirmed, these data will indicate that autophagy is post-transcriptionally and possibly translationally regulated upon TCR induction **(Fig 9)**.

Discussion:

Our study has allowed to establish that upon TCR engagement, the calcium pathway is required to induce autophagy in T cells. This is demonstrated by the fact that stimulating or inhibiting this pathway respectively results in an increased or decreased autophagic activity. T cell autophagy has already been linked to calcium signalling by the regulation of calcium mobilization via the maintenance of endoplasmic reticulum (ER) homeostasis. Jia and colleagues demonstrated in fact that in autophagy deficient T cells the ER was expanded leading to increased calcium stores and thus to a defective calcium influx **(Jia et al., 2011)**. Furthermore it appears that increased cytosolic calcium leads to apoptosis **(Zhong et al., 2006)**. In this context autophagy is required to regulate both ER stress and intracellular calcium mobilization. Upon TCR activation the release of these intracellular calcium stores is required to mediate the activation of the CRAC channel allowing the entry of extracellular calcium into the cell, where it acts as a potent second messenger initiating T cell differentiation, effector functions, proliferation and survival. Inversely, the role of calcium as a trigger for autophagy has been first investigated in cancer cell lines. When treated with calcium mobilization agents such as ionomycin, vitamin D or thapsigargin, several cell lines generated autophagosomes through the activation of AMPK **(Høyer-Hansen et al., 2007).** This was due once again to raised intracellular calcium concentration. However, since this phenomenon is tightly linked to ER stress, it is not easily discernible if autophagy induction is directly linked to calcium or to ER stress in these conditions. To our knowledge no study has been investigating calcium-dependent autophagy induction specifically in T lymphocytes.

An article published recently proposed another way of autophagy induction in CD4 T cells from C57BL/6J mice: the common γ-chain cytokine receptor signaling pathway **(Botbol et al., 2015)**. The authors postulate that cytokines such as IL-2, IL-4, IL-7 and IL-15, which bind the common γ-chain receptors, induce autophagy in different T helper subsets, in a JAK-dependent mechanism. We speculate however that the calcium signalling might be upstream of or complementary to this mechanism. Furthermore, our studies were performed on healthy as well as lupus-prone MRL$^{lpr/lpr}$ mice. It is a well-known fact that the calcium flux is overactivated in lupus T cells. This has been linked to the fact that in T cells from lupus patients and lupus prone-mice, the ζ chains of the TCR/CD3 complex are replaced by the FcRγ chain, leading to the recruitment of the spleen tyrosine kinase (Syk) instead of ZAP 70 **(Kyttaris et al., 2007; Tsokos et al., 2003)**. Syk signalling has in fact been associated to an enhanced calcium influx. This observation is in agreement with the upregulated autophagic activity previously observed by our team in MRL$^{lpr/lpr}$ mice as well as in lupus patients **(Gros et al., 2012b).** We confirmed these results in this study, when the T cells were stimulated with PMA and ionomycin.

TCR stimulation with anti-CD3 certainly increased the autophagic activity, even more in MRL$^{lpr/lpr}$ T cells. However, no major difference of flux in activated T cells between both mice can be evidenced. It could be speculated that TCR stimulation leads to an accumulation of autophagic structures due to a blockade of lysosomal degradation, as it has been recently proposed by Xu and colleagues **(Xu et al., 2014)**. This blockade is only partial as demonstrated by our conversion assays of LC3-I to LC3-II assed by immunoblotting in normal T cells as well as in lupus T cells. Upon TCR engagement we could still see an accumulation of LC3-II in the presence of lysosomal protease inhibitors, meaning that autophagy is still being initiated and that some autophagosomes are degraded. The differences between their study and ours could be related to the fact that they studied autophagy T cell function in an *in vivo* context while we have been focusing exclusively on *in vitro* studies so far. Moreover, they suggest that more read-outs might be required in order to properly assess the nature of the autophagic activity. Thus it would be relevant to inquire in our experiments, if p62 expression is also affected. Other methods like electron microscopy morphological studies or quantifications of LC3-GFP dots in transgenic T cells could allow to quantify different steps of the autophagy process. In any case, it is possible that TCR stimulation leads to a disequilibrium between autophagosome synthesis and degradation. Another signal would then be required to lead to the complete process, meaning the autolysosome formation. This could be the survival signal brought by common γ-chain receptor cytokines. This would explain why autophagy is poorly required at the first moments of T cell activation as described in part 2. This would also propose a mechanism allowing cells that received cytokine signalling to be long-lived, with the help of autophagy triggering. IL-7 could be a good candidate for both memory induction and autophagy.

Apart of cytokine signalling, calcium seems necessary for the continuation of autophagosome generation. This effect seems to be calcineurin dependent. Since the main demonstrated purpose of calcineurin is to induce translocation of NFAT into the nucleus where it mediates the transcription of numerous genes essential for the function of activated T cells, it seemed logical to suspect an implication of this protein in *Atg* gene expression as well. Establishing the transcription levels of various *Atg* genes*,* led us however to reject this hypothesis. QPCR kinetic experiments showed that neither pharmacological nor anti-CD3-induced T cell activation led to an increased expression of *Atg*. These results go along with former ones in our team that demonstrated a decrease of *Map1lc3a* in CBA/J and MRL$^{lpr/lpr}$ mice upon PMA/ionomycin activation **(Gros et al., 2012b)** and results by Xu et. al and Botbol et al. that showed a decrease in *Map1lc3b* upon T cell activation **(Xu et al., 2014b**). Since we see an enhancement of the autophagic activity upon TCR stimulation, at least in terms of LC3 protein expression, we propose various explanations for these contradictory observations which aren't necessarily mutually exclusive. First of all, the decline of *Atg* expression through TCR activation might

be explained by a negative regulation loop upon autophagy induction. The high level of autophagy may lead to a self-regulating process in which the expression of several *Atg* genes is downregulated and vice-versa.

The induction of autophagy through a transcriptional regulation seems unlikely as inhibiting this process with actinomycin D does not particularly affect LC3 protein expression. This leads us to hypothesize that the upregulation of autophagy is induced post-translationally in a calcium-dependent mechanism. This assumption is reinforced by the fact that treatment of T cells with a translation inhibitor cycloheximide, cancels the increased autophagic activity induced after TCR engagement. We can thus propose that calcium influx after TCR engagement directly leads to autophagosome formation and/or to flux inhibition, leading to the observed accumulation of LC3-II.

In conclusion we agree that various paths need to be explored in order to clearly establish the mechanisms leading to autophagy induction upon TCR stimulation. Moreover it will be interesting to investigate the differences observed between healthy and pathological T lymphocytes and how this influences autophagy and subsequently T cell biology. Interestingly CsA used in our experiments is also used to treat lupus patients. Thus understanding the implication of calcineurin and the broadly signalling laeding to the activation of autophagy could contribute to a better understanding of the function of this drug. The experiments depicted here have allowed to gather preliminary data about the possible regulation of autophagy upon TCR activation. Supplementary information is required to confirm the results already obtained. The different hypothesis we have proposed here will also need to be thoroughly explored in order to determine the mechanism behind TCR-induced autophagy both in normal T cells as well as in lupus conditions.

Materials and Methods

Mice

MRL$^{lpr/lpr,}$, CBA/J and C57BL/6 mice were purchased from Harlan. All mice were bred and maintained in accordance with guidelines of the local Institutional Animal Care and Use Committee (CREMEAS).

T cell Isolation and culture

Splenic T cells were collected from MRL$^{lpr/lpr}$, CBA/J or C57BL/6, mice and immediately cultured at 37°C, 5% CO2 in RPMI 1640 medium (Lonza BioWhittaker) supplemented with 10% FCS, 10 mg/mL gentamycin (Lonza BioWhittaker), 10 mM HEPES (Lonza BioWhittaker) and 0.05 mM β-mercaptoethanol (Lonza BioWhittaker) at a concentration of $5x10^6$ cells/mL. Splenic T cells were purified by negative selection. Briefly, spleen cell suspensions were depleted from monocytes, granulocytes, B cells and NK cells using Dynal T cell Negative Isolation Kit (Dynal-Life Technologies, 114-13D) according to the manufacturer's instructions. CD3+CD4-CD8- double-negative T cells that are frequent in MRLlpr/lpr mice, were discarded by anti-B220 Abs included in the commercial preparation. Splenic CD4 or CD8 T cells were purified by negative selection as well with the Dynabeads untouched mouse CD4 or CD8 cells isolation kit (Dynal-Life Technologies, respectively 11415D and 11417D). Resulting TCR-β+/B220-, TCRβ$^+$/CD4$^+$/B220$^-$ or TCRβ$^+$/CD8α$^+$/B220$^-$ mouse T cell preparations were >90% pure as determined by flow cytometry.

Western Blot

The Abs used for western immunoblotting were specific for β-actin (Santa Cruz Biotechnology, clone C4, sc-47778) and LC3 (MBL, clone 51–11, ref M115–3). In some experiments, lysosomal protease inhibitors E64d and pepstatin A (Sigma-Aldrich, P5318 and E8640) were added at 5 mg/mL each. When indicated, cells were stimulated with hamster anti-mouse CD3ε (5 µg/ml, clone 145-2C11, 553057, BD Pharmingen), hamster anti-mouse CD28 (5 µg/ml, and Clone 37.51, 553294, BD Pharmingen), 50 ng/mL PMA (Sigma-Aldrich, P8139) and 1 mM ionomycin (Sigma-Aldrich, I0634). For inhibition experiments the following molecules purchased from Sigma-Aldrich and were used at the concentrations indicated in the corresponding figures: BIM IV (B3306), EGTA (E3889), Cyclosporin A (30024), Actinomycin D (A1410), cycloheximide (C7698). To evaluate the autophagosomal membrane load and monitor autophagic flux, whole cell proteins were extracted from cultured cells using Laemmli buffer (TRIS-HCl 125 mM pH 6.8; 2% (w/v) sodium dodecyl sulfate (SDS); 10% (v/v) glycerol; 5% (v/v) β-mercaptoethanol). Cell lysates were separated using 4–20% gradient gels (Bio-Rad, Hercules, CA, USA) and proteins transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with

PBS containing 0.1% (v/v) Tween-20 (PBS-T) and 3% (w/v) non-fat dry milk for 1 h and then incubated overnight at 4 °C with 1 µg/ml anti-LC3 Ab in PBS-T containing 1% non-fat dry milk or for 1 h at room temperature with 1 µg/ml anti-b-Actin Ab in PBS-T containing 1% non-fat dry milk. After washing with PBS-T, membranes were incubated for 30 min at room temperature with goat anti-mouse IgG Ab (Southern Biotech, Birmingham, AL, USA; 1030-05) conjugated to horseradish peroxidase (HRP). Signal was detected using enhanced chemiluminescence detection reagents (Immobilon Western, Merck Millipore, Darmstadt, Germany; WBKLS0500). When indicated LC3-II staining was normalized by densitometry to ACTB staining using the ImageJ Software (National Institute for Health, Washington, DC, USA).

Real-time PCR

Total RNA was isolated from 5 × 10^6 purified T cells using the RNeasy Mini Kit (Qiagen, Courtabeuf, France, 74103) according to the manufacturer's instructions. After treatment by DNAse (Qiagen, 79254) to remove residual genomic DNA, mRNA was retro-transcribed with the Maxima first strand synthesis kit for cDNA (ThermoFisher, Illkirch, France). Fifteen nanograms of cDNA were used for real-time PCR (RT-PCR) on StepOne apparatus (Thermo-Fisher). Briefly, *Beclin1, Atg5, Atg7* and *Gapdh* cDNAs were amplified using Taqman Gene Expression Assays provided by ThermoFisher (Mm01265461m1, Mm00504340_m1, Mm00512209_m1 and Mm99999915_g1). Amplicons and probes were designed to span two exons, limiting the risk of amplifying residual genomic DNA. The *Map1lc3b* gene expression was evaluated by SYBR Green using the following primers: forward 3'-CACTGCTCTGTCTTGTGTAGGTTG-5', reverse 3'-TCGTTGTGCCTTTATTAGTGCATC-5'.

GAPDH (forward 3'-TGACGTGCCGCCTGGAGAAA-5', reverse 3'-AGTGTAGCCCAAGATGCCCTTCAG-5') was used as a house-keeping gene to normalize the results. Relative *Beclin1, Atg5 Atg7* and *Map1lc3b* mRNA quantifications were made by defining ΔCT (CT Gapdh− CT Atg5 where CT is 'Cycle Threshold') and ΔΔCT (ΔCT sample−ΔCT of one C57BL/6 mouse sample used for each plate) using StepOne software (ThermoFisher). Results shown represent $2^{-\Delta\Delta CT}$ values, where one same control sample is used on each plate and arbitrarily equal to 1.

Figures and legends:

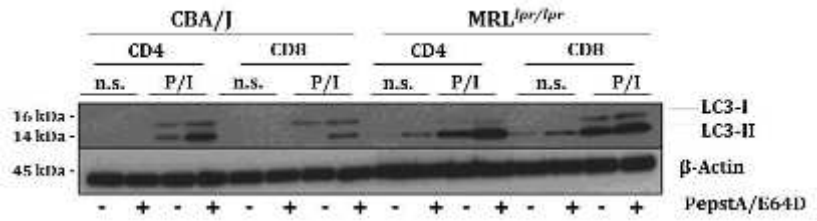

Figure 1: Autophagy is upregulated both in CD4 and CD8 T cells from lupus prone MRL$^{lpr/lpr}$ mice.

LC3 conversion was assessed by western immunoblotting. CD4 and CD8 T cells were isolated from spleens of control CBA/J and lupus prone MRL$^{lpr/lpr}$ mice and stimulated for 21h with P/I (50ng/mL and 1 µM). When indicated, cells were treated (+) or not (-) during the last 4h of culture with 5mg/mL pepstatin A and 5mg/mL E64D to block protease-mediated lysosomal degradation. Cell lysates were resolved by SDS-PAGE, transferred onto PVDF membranes before staining with anti-LC3 antibody (Ab). Loading controls were performed by staining actin β-chain. Depicted here a representative immunoblot of three experiments with identical results. P=phorbol meristate acetate, I=ionomycine, n.s.= no stimulation.

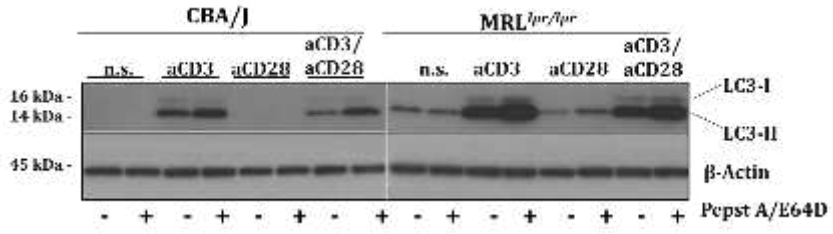

Figure 2: TCR stimulation induces autophagy in T cells and more intensely in MRL$^{lpr/lpr}$ mice.

LC3 conversion was assessed by western immunoblotting. T cells were isolated from spleens of 12 week-old control CBA/J and lupus prone MRL$^{lpr/lpr}$ mice and stimulated for 21h with plate bound anti-CD3 Ab and/or anti-CD28 Ab. When indicated, cells were treated (+) or not (-) with 5mg/mL pepstatin A and 5mg/mL E64D during the last 4h of culture to block protease-mediated lysosomal degradation. Cell lysates were resolved by SDS-PAGE, transferred onto PVDF membranes before staining with anti-LC3 Ab. Loading controls were performed by staining actin β-chain. Depicted here a representative immunoblot of three experiments with identical results. n.s.= no stimulation.

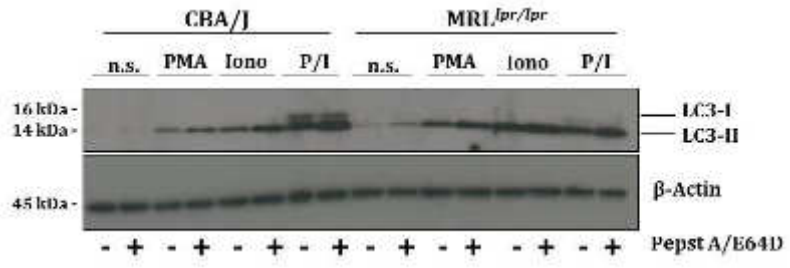

Figure 3: Calcium pathway activation induces autophagy in MRL$^{lpr/lpr}$ T cells.

LC3 conversion was assessed by western immunoblotting. T cells were isolated from spleens of 12 week-old control CBA/J and lupus prone MRL$^{lpr/lpr}$ mice and stimulated for 21h with 50ng/mL PMA or 1µM ionomycin (Iono) or both (P/I). When indicated, cells were treated (+) or not (-) with 5mg/mL pepstatin A and 5mg/mL E64D during the last 4h of culture to block protease-mediated lysosomal degradation. Cell lysates were resolved by SDS-PAGE, transferred onto PVDF membranes before staining with anti-LC3 Ab. Loading controls were performed by staining actin b-chain. Depicted here a representative immunoblot of two experiments with identical results. n.s.= no stimulation

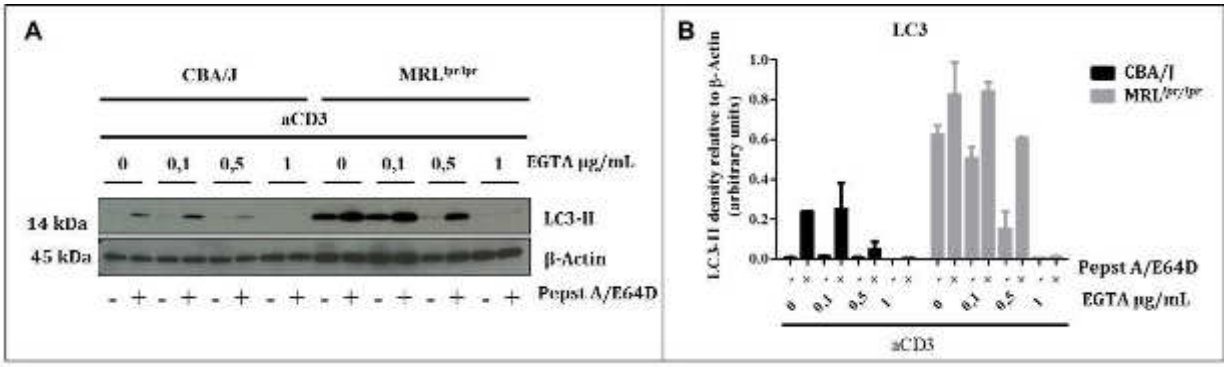

Figure 4: Chelating extracellular Ca^{2+} during TCR activation decreases the autophagic activity.

T cells were isolated from the spleen of CBA/J control mice or lupus-prone MRL$^{lpr/lpr}$ mice and activated with immobilized anti-CD3 antibodies for 21h. When indicated, different concentrations of the calcium chelator EGTA were added to the medium. During the last 4h of culture the cells were treated (+) or not (-) with 5µg/ml E64D and 5µg/ml Pepstatin A to block lysosomal protease degradation.

(A) shows an exemplary Western blot for the staining of LC3 and β-Actin.

(B) LC3-II-levels were evaluated by densitometry and normalized to β-Actin band intensities. Histogram bars represent the means of individual experiments with standard errors. (n=2, mice were 12-13 weeks old).

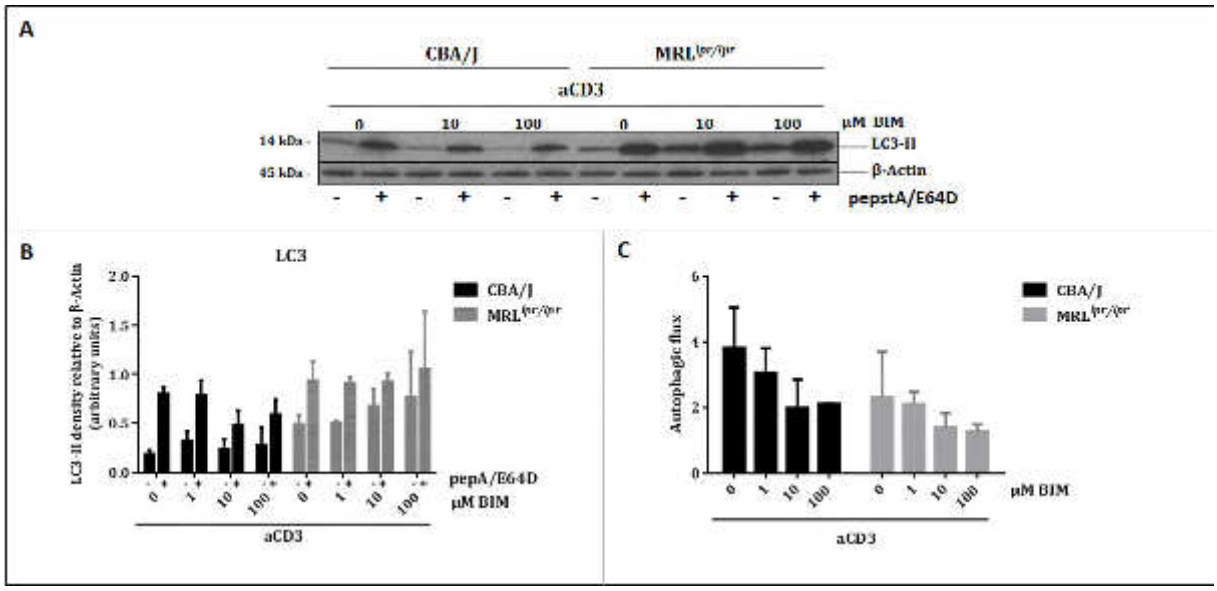

Figure 5: PKC pathway inhibition blocks the autophagy flux in CBA/J and MRL$^{lpr/lpr}$ mice T cells.

(A) LC3 conversion was assessed by western immunoblotting. T cells were isolated from spleens of control CBA/J and lupus prone MRL$^{lpr/lpr}$ mice and stimulated for 21h with plate bound anti-CD3 Ab and the PKC pharmacological inhibitor bisindolylemaleimide IV (BIM), added at the indicated concentrations. When indicated, cells were treated (+) or not (-) with 5mg/mL pepstatin A and 5mg/mL E64D during the last 4h of culture to block protease-mediated lysosomal degradation. Cell lysates were resolved by SDS-PAGE, transferred onto PVDF membranes before staining with anti-LC3 Ab. Loading controls were performed by staining actin β-chain. Depicted here a representative immunoblot.

(B) LC3-II levels were evaluated by densitometry and normalized to β-actin band intensities for at least three other independent experiments.

(C) Autophagic flux measurement consists on a ratio between the values with and without protease inhibitors (= autophagic flux). Histogram bars represent the means of individual experiments with standard errors. (n=3, mice were 10-13 weeks old).

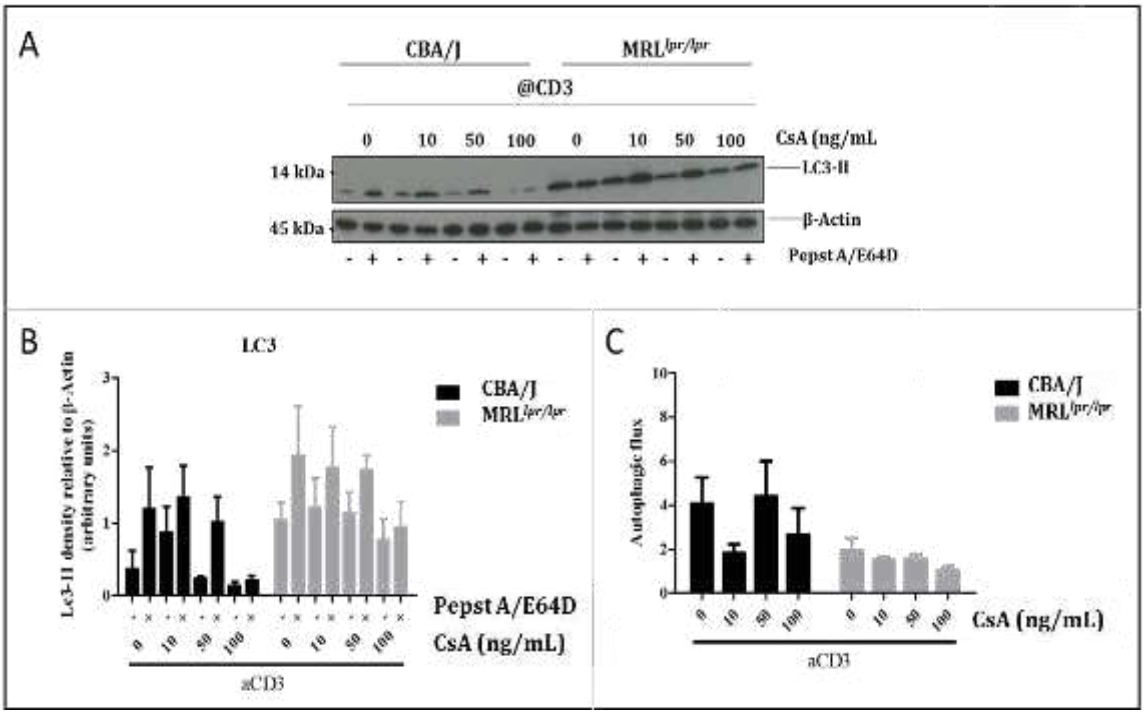

Figure 6: Calcineurin inhibition decreases the amount of autophagic structures and autophagic flux. T cells were isolated from the spleen of CBA/J control mice or lupus-prone MRL$^{lpr/lpr}$ mice and were in culture for 21h. When indicated cells were activated with immobilized anti-CD3 Ab and with different concentrations of Cyclosporin A (CsA) that inhibits the activity of calcineurin. During the last 4h of culture the cells were treated with (+) or not (-) 5µg/ml E64D and 5 µg/ml Pepstatin A to block lysosomal degradation.

(A) shows an exemplary Western blot for the staining of LC3 and β-Actin.

(B) LC3II-levels were evaluated by densitometry and normalized to β-Actin band intensities.

(C) Autophagic flux was measured by defining the ratio of LC3-II levels with or without treatment with E64D and Pepstatin A. Histogram bars represent the means of individual experiments with standard errors. (n=3; mice were 10-13 weeks old).

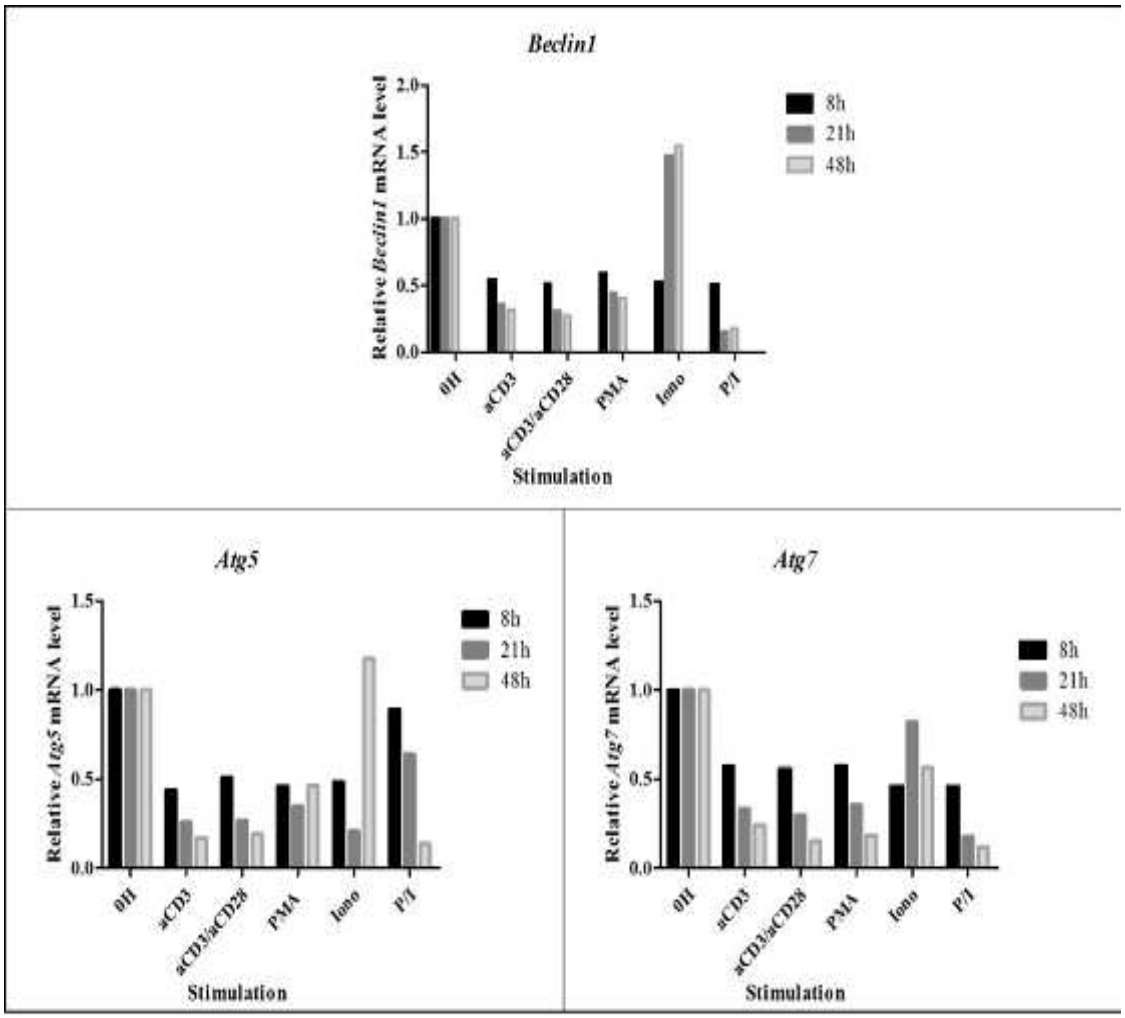

Figure 7: The gene expression of autophagy-related genes decreases upon TCR activation or Ca^{2+} influx. T cells were isolated from spleens of C57BL/6 mice and were stimulated with immobilized anti-CD3 agonist antibodies with or without anti-CD28 agonist antibodies or 50ng/ml PMA, 1μM Iono or both (P/I) for 8h, 21h and 48h. RNA was extracted and retrotranscribed into cDNA. Quantitative PCR was performed for measurement of *Beclin1, Atg5* and *Atg7* transcripts and the measurement was normalized to *Gapdh*. mRNA levels are relative to one non-stimulated mouse at the starting point of incubation that was arbitrarily set to 1. (n=1, mice were 12 weeks old)

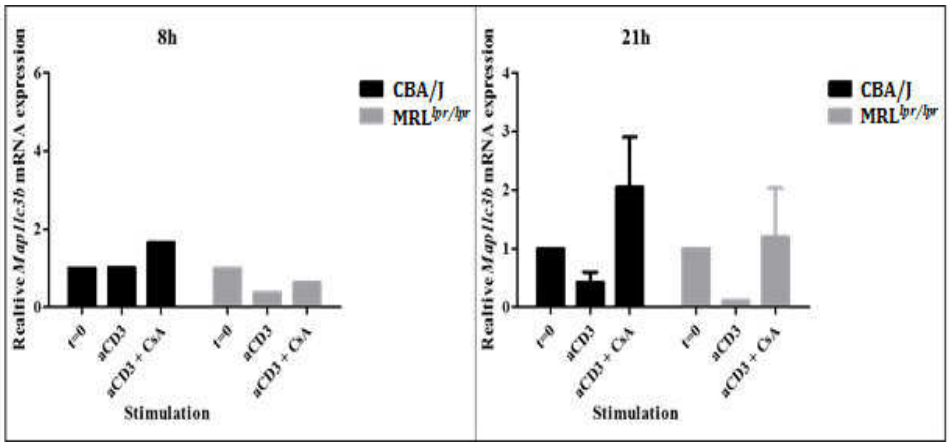

Figure 8: *Maplc3b* gene expression is increased upon inhibition of calcineurin during T cell activation.

T cells were isolated from spleens of CBA/J and MRL$^{lpr/lpr}$ mice and were stimulated with plate bound anti-CD3 agonist antibodies or anti-CD3 antibodies together with 100ng/ml Cyclosporin A (CsA) for 21h. RNA was extracted and retrotranscribed into cDNA. Quantitative PCR was than performed for measurement of *Map1lc3b* transcripts and the measurement were normalized to *Gapdh*. mRNA levels are relative to one non-stimulated mouse at the starting point of incubation that was arbitrarily set to 1. (n=1 for 8h, n=2 for 21h, mice were 10-13 weeks old).

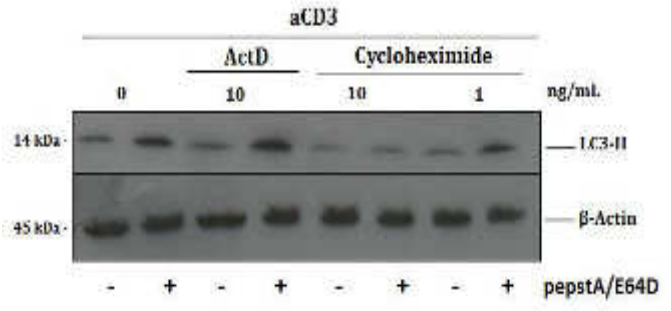

Figure 9: Inhibiting translation blocks autophagosome generation in TCR stimulated T cells.

LC3 conversion was assessed by western immunoblotting. T cells were isolated from spleens of C57BL/6 mice and stimulated for 12h with plate bound anti-CD3 and the pharmacological transcription inhibitor Actinomycin D (ActD) or translation inhibitor Cycloheximide, added at the indicated concentrations. When indicated, cells were treated (+) or not (-) with 5mg/mL pepstatin A and 5mg/mL E64d during the last 4 h of culture to block protease-mediated lysosomal degradation. Cell lysates were resolved by SDS-PAGE, transferred onto PVDF membranes before staining with anti-LC3 Ab. Loading controls were performed by staining actin β-chain. Depicted here a representative immunoblot. (n=1, mouse 12 weeks old).

1 Discussion and Perspectives

1.1 Context of the study

Because of its function as a guardian of cellular homeostasis, autophagy is a process essential for the survival of eukaryotic cells. Depending on the cell type and environmental triggers, autophagy can moreover play various roles. Hence, autophagy has emerged as a central player in the function of the immune system seeing that it mediates pathogen clearance, regulates inflammatory responses and participates in antigen presentation. Furthermore, autophagy has been shown to be involved in B and T lymphocytes homeostasis. These cells are mediators of the adaptive immunity. Their dysfunctions have often been associated to autoimmunity. Thus understanding every aspect of their biology appears to be crucial in order to delineate the events leading to tolerance break-down. It is with this purpose in mind that our team has engaged in investigating the role of autophagy in B and T lymphocytes.

A number of data point in fact towards an involvement of autophagy in autoimmune and autoinflammatory disease development. Autophagy has been proposed to shape the T cell repertoire by participating in thymic T cell selection. The study by Nedjic and colleagues demonstrated indeed that transplanting *thymi* from *Atg5*-deficient mice into athymic *nude* mice, led to development of colitis due to a break-down of intestinal immune homeostasis **(Nedjic et al., 2008)**. Moreover autophagy appears to be a gatekeeper of inflammatory events because of its capacity to down-regulate the inflammasome response and type I interferon secretion **(Nakahira et al., 2011)**. Considering the role of inflammation in autoimmune disease development, it strengthens the idea of this process being implicated in these disorders. It appears from recent studies that the role of autophagy in autoimmunity and autoinflammation is complex. While autophagy might seem to play mostly a protective role in Crohn's disease after the identification of *Atg16L1 open-reading frame* as a susceptibility locus, the role of autophagy is less clear in other disorders like SLE for instance **(Murthy et al., 2014)**. SNPs in *ATG* such as *ATG5* and *DRAM1* have also been associated to risk factors for SLE development and drugs known to induce autophagy such as hydroxychloroquine are routinely used to treat this disease (Thomson et al., 2009). On the other hand, rapamycine, that upregulates this process have also been shown to be an efficient treatment in clinical trials **(Gros and Muller, 2014)**. The need to inhibit autophagy during SLE is in agreement with the fact that autophagy has been shown to be increased in some immune cell types in a lupus context. In activated macrophages of lupus patients and lupus-prone mice the expression of *Atg5, Atg12* and *Beclin1* was found to be augmented **(Li et al., 2014)**. In addition, we and others have observed an upregulation of the autophagic markers in lupus T

cells **(Alessandri et al., 2012; Gros et al., 2012)**. Another team identified that autophagy was deregulated in B cells as well **(**(Alessandri et al., 2012b; Clarke et al., 2015; Gros et al., 2012a)**).** In light of these observations it seems that autophagy is a fine-tuned and complex process that can lead to or protect from disease pathogenesis in different contexts. Hence studying autophagy in lupus B and T lymphocytes is of great interest especially because of the central role of these cells in the break-down of immune tolerance at the origin of autoimmunity. Furthermore prior to the first publication on this subject by our team **(Gros et al., 2012)**, except for the GWAS, no information was available about the role of autophagy in SLE. The results from that first study led the path to further investigations aiming to better understand the role of autophagy both in B and T lymphocytes in a normal context as well as in pathological conditions.

Studying autophagy in B cells was of special interest for various reasons. First, as described previously, the precise role of autophagy in B cell development remained not totally resolved. Moreover, the importance of autophagy in B cell responses, when we started the project, was far from clear. The possibility remained that B cells might not require autophagy for their function. Indeed, at the time, only few data on the role of this process in B cell biology was available. In 2008 Watanabe and colleagues demonstrated that autophagy could be induced in response to BCR stimulation **(Watanabe et al., 2008)**. Another study concluded that autophagy was required for the survival of the B-1a B cell subpopulation as well as for the transition from pro- to pre-B cells **(Miller et al., 2008**). A third study by Susan Pierce's team had shown that autophagic activity favoured BCR/TLR9 co-signalling. Thereby some knowledge gaps needed to be filled especially in terms of autophagy requirements in B cell development. Indeed, Miller's findings were based on *Atg5* deficient chimeras with a possibility that the conclusions drawn could be due to an earlier defect in B cell progenitor generation. Furthermore, *in vivo* functional studies on the role of B cell autophagy were clearly missing. This information seemed crucial as defects in B cell autophagy were later identified in pathological contexts. It is in this context that the project which gave rise to the first publication found in this manuscript.

1.2 Autophagy and long-term humoral immunity

In the first study described in this manuscript we generated three mice models in order to answer the following interrogations:

- Does autophagy play a role in B cell development?
- Is autophagy required for peripheral B cell function?
- What is the contribution of B cell autophagy in an autoimmune context?

To address all these issues, we generated three mouse models with a specific invalidation of autophagy specifically in B cells. In the first model, autophagy was deleted in early phases of B cell development. For this purpose, we took advantage of the cre-lox system and deleted *Atg5* under the control of the Mb1 promoter and obtained *Atg5*$^{f/-}$ Mb1-cre mice. In order to abrogate autophagy in peripheral mature B cells we used the same deletion strategy but with a B cell specific promotor expressed at mature stages. The mice obtained were *Atg5*$^{f/-}$ CD21-cre. Contrary to most studies, we used the Mb1 promotor to delete autophagy in B cell progenitors, rather than CD19's one, which has emerged to be a leaky promoter leading to incomplete autophagy invalidation, complicating the interpretation of some results. The Mb1 promoter revealed to be an efficient inducer of *Atg5* deletion, allowing to be confident in our further conclusions, about the role of autophagy in B cell development. The comparison of their phenotype with the one using CD21 promoter to drive the deletion, helped to establish the requirement of the autophagic machinery in B cell development and functions. In both models the distribution of B cell subpopulations in secondary lymphoid organs such as the spleen or lymph nodes remained unchanged. In terms of B cell absolute numbers however those were reduced in the spleen of *Atg5*$^{f/-}$ Mb1-cre mice. That fact that the Mb1-cre is quite efficient in deleting targeted genes might have contributed to this phenotype suggesting the requirement of minimum levels of autophagy for B cell survival. Strikingly, no alteration of the transition from pro- to pre-B cell stage in the bone morrow could be observed. Thus contrary to previous observations autophagy doesn't seem to be necessary for B-2 B cell development. Peritoneal B-1a B cells were however highly decreased as were B-2 cells in *Atg5*$^{f/-}$ Mb1-cre mice. We conclude that autophagy is strictly required during development, for B-1a generation since this defect was not observed in *Atg5*$^{f-}$ CD21-cre mice. However, the survival of B-2 B cells in the peritoneum requires autophagy also after development as shown by their diminished number in *Atg5*$^{f-}$ CD21 cre mice. The lack of autophagy strikingly had no impact on splenic B cell function, after BCR activation with or without co-stimulation since they were able to proliferate and revealed no major survival defect. Only LPS stimulation supposed to drive B cell differentiation into plasmablasts affected B cell survival in both mouse models, indicative of the requirement of autophagy in the maintenance of these antibody secreting cells, as suggested by other studies published before ours **(Cenci, 2014; Pengo et al., 2013)**.

As B cell functionality *in vitro* remained intact and the B-2 B cell compartment was poorly impacted *in vivo*, we performed immunizations of our mice, to inquire after the role of autophagy in humoral responses and thus on peripheral B cell function. Except of a defective response IgM response against the model antigen OVA, both *Atg5*$^{f/-}$ Mb1-cre and *Atg5*$^{f/-}$ CD21-cre mice were able to produce antigen specific IgGs in short-term humoral responses. The lack of IgM could be explained by the fact that short lived plasma cells cannot be preserved in the absence of autophagy.

The fate of long-term responses was addressed in the third model, which harboured an autoimmune-prone mutation (C57BL/6$^{lpr/lpr}$). Interestingly, deleting autophagy in these mice (C57BL/6$^{lpr/lpr}$ *Atg5*$^{f/-}$ CD21-cre) had no impact on B cell proportions, showing further the low involvement of this mechanism in mature B cell homeostasis. In contrast, even after 6 months, no hypergammaglobulinemia nor IgGs directed against dsDNA were detectable, contrary to their controls (C57BL/6$^{lpr/lpr}$). Moreover, immune complex deposition in the kidneys was reduced. These are in fact hallmarks of autoimmunity, that were attenuated in the absence of autophagy in B cells, although B cells were not affected. Plasma cells in the bone marrow, however, were strongly reduced. All these results suggest that autophagy is required for the maintenance of long-term humoral response and for the survival of these long-lived cells which is in agreement with our previous *in vitro* results obtained with LPS stimulated B cells, but also with the work by Cenci's team **(Pengo et al.2014)**. According to two other studies by Chen and colleagues, autophagy is needed to initiate an efficient memory response which could explain the reduced autoimmune response in our experimental settings **(Chen et al., 2014, 2015)**. This last point should be addressed in more details.

In regard to our results and to those of other teams autophagy exerts a minor influence on B cell short term functions and thus is not required for primary B cell activation. Indeed, B cells still pass through germinal centers, undergo class-switch recombination as demonstrated by the normal secretion of antigen specific IgGs **(Conway et al., 2013; Pengo et al., 2013)**.

It is however possible that autophagy might be participating in early B cell activation in specific situations. It has indeed been suggested that upon stimulation, the BCR can be internalized and co-localize with autophagosome-like structures, as well as LC3 **(Ireland and Unanue, 2011; Chaturvedi et al., 2008; Watanabe et al., 2008)**. This trafficking pathway may have an impact on BCR signalling after coactivation with TLRs for example. It could also favour B cell activation through the call for T cell help, thanks to MHC presentation. In that case, the nature of the antigen may dictate whether the autophagic machinery is required or not. Ireland and Unanue showed for instance that autophagy was required for presentation of citrullinated antigens contrary to non citrullinated ones **(Ireland and Unanue, 2011).** Thereby our team has undertaken to investigate if indeed, the nature of the antigen matters and has been able to show that autophagy is required for the presentation of particulate BCR bound exogenous antigens but not for soluble antigens.

My thesis work was mainly dedicated to the study about the role of autophagy in T cell function. Based on the discoveries by our team about the dysregulation of this process in lupus T cells, my research was split in two parts. The first project was centred on studying the impact of autophagy in T cell

function and the second project aimed at understanding how autophagy is induced upon T cell activation.

For the first part of the investigation on T cell autophagy, we proceeded the same way as for the study about the role of autophagy in B cells. As a matter of fact, we generated a mouse model with T cell specific *Atg5* deletion. Contrary to B cells, the implication of this process in T cell function had been already intensively studied. The groups that were interested in the subject, mainly identified the requirement of this process for T cell survival and function in periphery. The resulting studies showed indeed that autophagy mediated T cell homeostasis by regulating the clearance of excess and/or defective mitochondria and by limiting ER expansion. Most mouse models used at that time displayed in fact dramatic T cell number decrease in the periphery, and for some, even already in the thymus. This was accompanied by massive cell death and defective survival after activation *in vitro,* as mentioned previously, and made these mice unsuitable to study T cell function *in vivo*. As for most models autophagy deletion occurred early in thymus development, we hypothesized that this could influence T cell development and lead throughout time to an accumulation of defects, that would induce cell death right after T cell activation. Thus with the intention to overcome this obstacle and to study the requirement of this process in T cell function *in vivo*, we used the distal Lck promoter to delete *Atg5* only in peripheral T lymphocytes.

Our model (*Atg5*$^{f/f}$ dLck-cre) displayed no specific decrease in CD4 T cell numbers or proportion and did not show any major defect in CD4 T cell survival, contrary to previously described phenotypes obtained with other models. In contrast, the phenotype of CD8 T cells was largely reminiscent of the other published models. It appears that these cells require autophagy for their peripheral maintenance contrary to CD4 T cells. CD8 T cells were reduced in the spleen and the lymph nodes and when the few cells left were stimulated *in vitro*, they failed to proliferate optimally. We were able to detect that their load in mitochondria was high when compared to littermate controls, and also that more mitochondria showed membrane potential defects. It had been established that mitochondrial content is developmentally regulated by autophagy **(Pua et al., 2009; Stephenson et al., 2009; Willinger and Flavell, 2012)** and that when T cells leave the thymus, a reduction in mitochondria is essential for their survival in periphery because of the changes in oxygen availability, which is higher in periphery. In lymphocytes containing a high load of mitochondria, this could lead to excessive ROS production and subsequently to cell death. Considering the impairment in survival and function of the CD8 T cells, it suggests that in these cells, the mitochondrial load needs to be tightly controlled even in periphery.

Nevertheless, CD4 T cells did not require autophagy for proper short-term *in vitro* functions which led us to use our model to study their function *in vivo*. Thanks to this model we have been able to show

that same as for B cells, autophagy is not involved in early T cell mediated humoral responses since the mice were able to generate IgM and IgG, directed against the T-dependent antigen OVA, in the same proportions as the controls. Long-term immunization however led to decreased humoral responses. We postulated that in our conditions the CD4 T cells were not capable to mediate a memory response in absence of autophagy. In order to establish that these observations were intrinsic to autophagy-deficient CD4 T cells and not to a bystander effect due to the reduced CD8 T cells for instance, we performed transfer of antigen primed CD4 T cells into recipient mice that we re-immunized with the same antigen as the host mice. We noticed that the mice that had been injected with autophagy deficient CD4 T cells displayed a reduced memory humoral response. Recreating a memory phenotype by *in vitro* stimulation and polarization of CD4 T cells in the presence of IL-7, resulted in increased cell death throughout the culture time. The analysis of their status in mitochondrial load revealed that with time there was accumulation of dysfunctional mitochondria which could explain the increase in cell death. Memory T cells have been shown to rely on mitochondria to generate energy from FAO, necessary for their long-term maintenance **(Verbist et al., 2012)**. Rafi Ahmed's group who showed a defect in memory CD8 T cell survival in the absence of autophagy, investigated the metabolic profile of the T cells in their model (*Atg7*$^{f/f}$ Gzmb-cre). They performed metabolomics analysis of LCMV infected autophagy deficient CD8 T cells and discovered dysregulations in carnitine shuttle and di-unsaturated FAO in comparison to wild type CD8 T cells. They proposed that autophagy might be required to generate lipid substrates for FAO and in general to maintain metabolic homeostasis allowing to mediate the transition from effector CD8 T cells to memory T cells. As a matter of fact, in our case autophagy-deficient CD4 T cells failed to undergo the transition from effector memory (CD44^{high} CD62L^{low}) to central memory (CD44^{hi} CD62L^{high}) T cells *in vitro*. The observed alterations in memory CD4 T cell survival are most probably due to a combination of both dysfunctional mitochondrial and altered metabolic pathways which are in any case linked to one another. We intend to investigate the metabolic profile in our experimental settings as well in order to establish if the requirements in metabolites and autophagy are the same as those proposed for CD8 T cells. Studying Glut1 expression for instance could be an interesting read-out to evaluate the metabolic state of the CD4 T cells. Glut1 being a glucose transporter, its increased expression has been specifically correlated to an effector phenotype (Macintyre et al., 2014).

Long-term cultured autophagy deficient memory CD4 T cells are CD44^{high} and CD62L^{low}. This specific phenotype has been attributed to T cells undergoing homeostatic proliferation but for *in vivo* settings only since it is due to lymphopenia **(Surh and Sprent, 2000)**. We do not see any difference in our hand in terms of CD44 expression *ex vivo*, with or without autophagy, ruling out increased CD4 T cells homeostatic proliferation occurring in our mice. Thus the increased CD44 expression we quantify *in*

vitro may indeed be attributable to an activation-induced memory phenotype. Even though we were able to exclude the possibility of early senescence being the reason for reduced long-term humoral response, we haven't investigated this possibility *in vitro* by staining the cells for specific senescence markers such as CD57 **(Ng et al., 2015)**.

Our work so far has allowed to shed some light on some questions that had not been addressed yet by other researchers. The generated mouse model has allowed to discriminate between the observations that are linked to the role of autophagy in T cell function *per se* and those that are the consequence of accumulated developmental issues. It is now described that autophagy contributes to memory CD8 T cell survival. This has only been established recently. When we started the project nothing about the role of autophagy in memory T cells was known, yet especially not in *in vivo* settings, since the models used previously were hardly directly suitable for those kind of investigations. Jia and He had however given a first hint in this direction as they demonstrated that tamoxifen-induced *Atg3* deletion, *in vitro,* had no impact on T cell survival in the presence of IL-7 in short periods of culture showing survival defects only after >24 days of culture **(Jia and He, 2009)**.

CD4 T cell specific requirements for the autophagic machinery had not been investigated yet. In consideration of the new findings on memory CD8 T cell biology, it could be suspected that autophagy plays here a comparable role since these two T lymphocyte subsets share some similarities. But seeing that CD4 and CD8 T cells do not accomplish the same tasks in adaptive immune responses it was essential to investigate their status independently from one another. Moreover, as shown by our model their need for autophagy diverges in terms of basal homeostasis as demonstrated by the increased cell death of the CD8 T cells even though they could develop normally in the thymus and autophagy was deleted only in periphery. The findings on the importance of autophagy in memory lymphocytes (B cells, CD8 and CD4 T cells) and plasma cells, open compelling perspectives in regard to autoimmunity. The maintenance of autoreactive memory T and B cells contributes to the chronicity of those pathologies as it is the case for SLE. Thus, reducing autophagy could contribute to the amelioration of disease symptoms.

Modulating autophagy however is a complex matter that needs to be considered with caution. Rapamycin for instance is proposed to treat SLE patients **(Fernandez et al., 2006)**. This drug known to inhibit mTOR and thus to induce autophagy also favours CD8 T cell differentiation into memory cells at least in mice infected with lymphocytic choriomeningitis virus **(Araki et al., 2009)**. While this feature is in agreement with our findings about CD4 T cells, it might appear surprising in regard to the effect of rapamycin in SLE treatment, that improving the survival of memory T cells does not aggravate the disease. This is can probably be explained by the pleiotropic effect of rapamycin as an immune-

suppressive drug as it limits DC maturation and function and reduces T cell proliferation **(Thomson et al., 2009)**. The efficacy of rapamycin in SLE treatment could be autophagy-independent. Moreover, it has been recently described that low doses of rapamycin promote memory T cell differentiation and can lead to autoimmune symptom exacerbation **(Zhang et al., 2012)**, whereas high doses had the opposite effect. It would be interesting to treat lupus-prone mice with different doses of rapamycin and follow the progression of the disease, and to correlate it with the autophagy status.

In any case, knowing the role of memory lymphocytes in the maintenance of an autoimmune phenotype, designing new drugs specifically targeting those memory cells could contribute to develop fine-tuned treatments that would have decreased side effects since they would affect less early adaptive immune responses, unlike immune-suppressive drugs. Autophagy, to that respect is a good candidate. Nevertheless, targeting the memory compartment is of course not entirely harmless. This could indeed render the patients more susceptible to re-occurring infections. But SLE patients are often young and would have the possibility to reconstitute a competent memory compartment after treatment. However, in order to evaluate the impact of T cell autophagy in autoimmune settings it would require, same as for the B cell study, the generation of transgenic mice with a T cell specific autophagy deletion on an autoimmune genetic background. This could give first insights on this sensitive matter.

Another interesting aspect of these discoveries about the role of autophagy in memory lymphocytes is that it could help to find new strategies to improve vaccines but also to boost memory responses in elderly, as suggested by Puleston and colleagues. The use of spermidine, a naturally occurring autophagy inducer, significantly ameliorated the immune responses to vaccination **(Puleston et al., 2015).**

1.3 Autophagy and T cell signalling

As already mentioned, the project investigating the signalling pathways leading to autophagy induction after T cell activation, was initiated as the continuation of our study that had revealed that autophagy is dysregulated in lupus T cells. Thus we wanted to figure out why this process was specifically upregulated in a lupus context. We first considered that one of the intrinsic differences between normal and autoreactive lupus T cell resides in their signalling capacity. Since the signalling pathways leading to autophagy induction in T lymphocytes had been barely studied we undertook to investigate this issue in normal and autoimmune conditions in parallel to the work we were doing with the T cell specific autophagy-deficient mice.

We were able to identify that autophagy markers were mainly induced upon TCR engagement in agreement with the literature **(Pua et al. 2007)**. TCR signalling dissection brought to light the involvement of the calcium pathway and more precisely, calcineurin in autophagy induction. This induction does not seem to be transcriptionally regulated despite that fact that calcineurin activation results in NFAT diphosphorylation, and translocation into the nucleus. NFAT regulates a number of genes required for T cell activation and differentiation. We thought that NFAT might regulate *Atg* transcription as well. We could not detect any upregulation of any of the *Atgs* upon TCR activation in the quasi totality of the conditions tested. These results need however to be confirmed. Furthermore, in order to completely exclude a regulation of autophagy at the transcriptional level it would be interesting to down-regulate NFAT or TFEB, the latter being another transcription factor that has been shown to depend on calcineurin for its activation as well. The fact that TFEB has been shown to regulate not only *Atgs*, but in general lysosomal activity, makes this transcription factor actually the most suitable candidate to be studied in our experimental settings **(Medina et al., 2015)**. Down-regulating these proteins by siRNA could allow to see if it leads to a change in the autophagic activity. The results would have to be considered carefully because the transcription factors could still have an indirect effect on autophagy induction through the regulation of other factors that could have an influence on autophagy, such as cytokines for instance. In any case, in our hands, a transcriptional regulation of autophagy modulation in response to TCR signalling is unlikely as our first actinomycin D treatments did not change the level of autophagic activity in response to TCR stimulation. This last point is in accordance with Botbol and colleagues' study **(Botbol et al., 2015)**. It seems thus that calcium-dependent, post-translational mechanisms govern the modulation of autophagy in response to TCR stimulation.

In lupus T cells the calcium pathway also induces autophagy markers, even more than in control mice. The fact that lupus T cells exhibit increased calcium signalling, strengthens even more our hypothesis that this pathway is the main responsible for the increase of LC3-II expression. The study by Macian's team suggests however that autophagy induction in CD4 T cells depends more on common γ-chain cytokine signalling **(Botbol et al., 2015)**. In the context of lupus T cells, it remains improbable because in the case of IL-2, which is one such cytokine, a down-regulation of its secretion is characteristic of lupus T cells. This indicates that at least in these cells a different signalling is required**.** But since this disease leads to the accumulation defects in various cells among which aberrant T cell signalling, it cannot be excluded that their hypothesis applies to normal T cells but not necessarily to lupus T cells. In regard to memory T cells, Botbol's study could explain the described phenomenon, since common chain-γ cytokines are involved in the generation and maintenance of memory T cell. The autophagy inducer function of these cells is in fact in agreement with our results and those of others showing that

autophagy is important for the survival of memory CD4 and CD8 T cells respectively, particularly in response to IL-7 stimulation, which is a cytokine dependent on common γ-chain receptor.

There is however one pathway that we have not discussed yet, the PKC pathway leading to the NF-kB activation. Autophagy regulation by NF-kB has been shown to be context-dependent. It can either induce or inhibit autophagy **(Trocoli and Djavaheri-Mergny, 2011)**. In our conditions it appears that activation of this pathway with PMA, which mimics DAG activity, slightly induces the generation of autophagic membranes in T cells from lupus-prone mice. Furthermore, inhibiting this pathway with a pharmacological inhibitor led to the reduction of the flux. Since in normal T cells we didn't see any increase of autophagy when activating this pathway, and because in lupus conditions NF-kB signalling has been shown to be mainly defective, we speculate that the effect we see could be related to aberrant activation, that could lead to secondary raise in intracellular calcium. The reason why inhibiting this pathway induces a decreased flux is however unclear.

Moreover, even though the autophagic membrane load is high upon TCR activation in lupus T cells, as shown by increased detection of LC3-II with and without protease inhibitors, it is not the case for the overall autophagic activity, meaning the flux. This is an observation which has to be further investigated. The hypothesis of a blocked autophagic flux in a lupus context has been suggested by Pierdomonicci's team. Alessandri and colleagues showed indeed that T cells from lupus patients were resistance to serum-mediated autophagy induction and that those T cells had an increased expression of negative regulators of autophagy such as B-cell lymphoma 2 (*Bcl2*), v-akt murine thymoma viral oncogene homolog 1 (*Akt1*) but mostly *alpha-synuclein* which expression has been shown to inhibit autophagosome generation **(see section 2.1.1) (Alessandri et al., 2012)**. More recently Caza and colleagues also demonstrated that the autophagic flux might be impacted in lupus T cells. Those cells displayed an accumulation of dysfunctional mitochondria due to decreased mitophagy. They identified that HRES-1/Rab4, a small GTPase which has been shown to be over-expressed in T cells from lupus patients and lupus-prone mice at basal levels, was even more upregulated upon T cell activation and that this led to the depletion of dynamin-related protein 1 (Drp1) **(Caza et al., 2014)**. Drp1 resides in the cytosol but when translocated to mitochondria this protein initiates mitophagy. Drp1 appears to be decreased before onset of lupus symptoms in lupus prone mice and is associated to mitochondria accumulation. As for HRES-1/Rab4 it has been shown to increase autophagosome formation while inhibiting mitophagy **(Talaber et al., 2014)**. This is in agreement with our observations suggesting that the autophagic pathway is activated but fail to mediate organelle degradation. This is a theory that we need to investigate in more detail also in regard to calcium signaling.

When comparing the deletion model to our results on signalling there seems to be some contradiction. On one hand we saw that autophagy was dispensable for CD4 T cell activation in *in vitro* settings and for early humoral responses and on the other hand we see an increased autophagic activity upon TCR activation. The questions asked in the two studies certainly aren't the same but it appears surprising that T cell activation seems to induce autophagy while it doesn't seem to be essential for their function. It can however be speculated that autophagy might be necessary to progressively remove damaged organelles such as mitochondria or ER to prevent accumulation of defects which can later on lead to defective maintenance of memory T cells as shown by our study on memory CD4 T cell function as well as by the two recent inquisitions on memory CD8 T cells by Puleston et al. and Xu et al. respectively. In that regard it is possible that the autophagic flux might be inhibited in early stages of activation, as suggested by Ahmed's study and that the TCR signal still leads to autophagosome generation while waiting for a second signal to initiate degradation to respond to energy demands and maintain organelle homeostasis at later times. This second signal could indeed be induced through cytokine signalling which would be in agreement with the observations by Macian's team who suggested that autophagy could be induced by common γ-chain cytokines. This stimulation could lead to autolysosome formation and to the completion of the process, necessary for future survival. Thus the regulation of these events needs to be investigated further.

1.4 General conclusion: autophagy as a therapeutic target

Autophagy is an essential mechanism in cellular maintenance and function and as such it harbours a broad role in a vast panel of pathogenesis. Thus it has been suggested that autophagy could serve as a therapeutic target in a number of situation, either to be inhibited or to be activated.

Studies on autophagy have led to better understand the aetiology of some neurodegenerative diseases such as Alzheimer's, Parkinson's or Huntington's disease. These pathologies are characterized by the accumulation of protein aggregates that become toxic for neurons and lead to their death. Autophagy is defective in most of those disorders. Moreover, these pathologies usually develop in elderly and autophagy has been shown to decrease with age **(Carroll et al., 2013)**. Manipulating this process to treat neurodegenerative patients has been proposed, but currently the therapeutic approaches have mainly been tested in animal models where they revealed to be quite efficient. These are only some pathological conditions where autophagy has been shown to be involved. Because of its dominant role in metabolism, autophagy is of course involved in the development of metabolic disorders such as type II diabetes or obesity.

Even though autophagy was first associated to cell survival, its role appears to be much more complex and to be highly dependent on the cell type and the environmental context. Cancer cells illustrate this complexity quite well. As mentioned in the introduction, autophagy plays a dual role in tumorigenesis. In the first stages of cancer cell development, autophagy according to some studies, contribute to their death in an attempt to avoid their persistence. But once the tumors are established they use the autophagic machinery to their advantage in order to satisfy the metabolic demands and to decrease oxidative stress. Autophagy has also been linked to resistance of tumours to chemotherapy. On the other hand, molecules that have been shown to be efficient in some cancer treatments have also been shown to induce autophagy. Hence the choice to induce or activate autophagy in cancer treatments could indeed be an excellent strategy but has to be specifically adapted to each tumour type and probably according to its grade. This duality probably also applies on immune-related pathologies.

Autophagy participates in pathogen clearance but can also be used by some pathogens for their replication. Nevertheless, this process mostly harbours advantages for the immune system since it leads to a better response to PRR stimulation and to a more efficient degradation of intracellular microbes. Hence in these contexts inducing autophagy could potentially serve to increase the efficiency of some drugs already used to treat infectious diseases or even inspire the design of new treatments. Furthermore, in consideration of the new observations about the role of autophagy in plasma cells, in memory CD8 and CD4 T cells, it gives some new insights on how to ameliorate vaccines by increasing the survival of those long-lived cells. Enhancing autophagy on the systemic level could contribute to the maintenance of a potent memory adaptive immunity even in older age, as proposed by Puleston and colleagues.

But then again this could possibly lead to other issues. As discussed in the introduction of this manuscript and also shown by us and others for SLE, autophagy seems to be upregulated in most autoimmune pathologies. In RA, augmented secretion of TNF-α, a feature of this disorder, leads to an increased autophagic activity in fibroblasts and osteoclasts **(Connor et al., 2012)**. *Atg7* invalidation in monocytes or treatment with pharmacological autophagy inhibitors (Bafilomycin A) was shown to reduce bone disruption *in vitro* **(Lin et al., 2013)**. In MS patients, *ATG5* expression was found to be increased. Furthermore in EAE mice this increase was correlated with disease severity while in Kovacs study these mice were resistant to EAE induction when deficient for *Beclin 1* in T cells **(Alirezaei et al., 2009; Kovacs et al., 2012)**. In psoriasis and vitiligo, two autoimmune skin diseases, polymorphisms in *ATG16L1* and *UVRAG* respectively have been associated to these pathologies. In T cells from RA patients however autophagy was found to be decreased due to the down-regulation of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3 (PFKFB3) wich seems to contribute to disease progression. Also in CD decreased autophagy seems to be greatly involved in disease pathogenesis.

Thus under certain conditions and for some pathologies, activating autophagy would seem more beneficial for the patients even though requirements for autophagy can varie from one disorders to another and even from one cell type to another in the same pathology.

In light of the discoveries made so far about the role of autophagy in cellular homeostasis and in disease pathogenesis, it seems that finding strategies to modulate this process is of great interest but has to be extremely fine-tuned. Thus knowing all the players involved and understanding every aspect of the autophagic machinery is essential. A lot of knowledge about autophagy has been collected during this last decade but many questions still remain, even though the more we research the more complex this mechanism appears.

But as Albert Einstein said: *"The important thing is to not stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day".*

1 Publication 3

Autophagy in Chronic Inflammation

Johan Arnold, Diane Murera, and Frédéric Gros

Abstract Autophagy is a catabolic process consisting in the formation of cytoplasmic vacuoles, fusing with lysosomes and leading to the degradation of their content. Part of the autophagy machinery is also involved in specialized forms of endocytosis and vesicle trafficking. The role of autophagy, initially described as a response to energetic stress, has now been extended to other stress signals like tissue damage and infection. Autophagy is indeed deeply involved in the regulation of inflammation and in the biology of immune cells. Autophagy regulates cell metabolism and integrates it to the elimination of microorganisms, to the fine-tuning of inflammation and to the activation of the adaptive immune system. The inflammatory response aims at controlling pathogen invasion and at initiating tissue repair. If unrestricted, inflammation can become chronic and be the source of the so-called autoinflammatory and autoimmune pathologies. These complex disorders result from a combination between genetic and environmental factors. A clear genetic link between Crohn's disease and autophagy deregulation has been demonstrated. Autophagy deregulation provoked by environmental triggers like nutrient excess or by aging, are also linked to low-grade inflammation observed during metabolic syndrome, especially in the case of type II diabetes and atherosclerosis. Both genetic causes and environmental triggers could also link autophagy deregulation to autoimmune pathologies like rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis. The emerging causality between autophagy deregulation and chronic inflammation, subject of intense studies as it could lead to new therapeutic options, will be described in this chapter.

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Abbreviations

AMPK AMP	Activated protein kinase
APC	Antigen presenting cells
ATG	Autophagy-related genes
ATP	Adenosine tri-phosphate
CD	Crohn's disease
CDS	Cytosolic DNA sensor
CMA	Chaperone mediated autophagy
DCs	Dendritic cells
DSS	Dextran sodium sulphate
EAE	Experimental autoimmune encephalomyelitis
ER	Endoplasmic reticulum
FFA	Free fatty acids
FoxO	Forkhead homeobox type protein O
GWAS	Genome-wide analysis studies
HFD	High fat diet
HM	Hypomorphic
HMGB1	High-mobility group 1 protein
IAPP	Islet amyloid peptide
IBD	Inflammatory bowel disease
IFN	Interferon
ILC	Innate lymphoid cells
IRGM	Immunity-related GTPase M
LAP	LC3-associated phagocytosis
LC3	Light chain 3 standing for microtubule-associated protein 1 light chain 3B
LDL	Low density lipoproteins
LPS	Lipopolysaccharide
MAMP	Microbe-associated molecular pattern
MHC	Major histocompatibility complex
MiR	Micro RNA
MS	Multiple sclerosis
NET	Neutrophil extracellular trap
NLRP3	NACHT, LRR and PYD domains-containing protein 3: IL, interleukin
NOD	Nucleotide oligomerization domain
NRV	Norovirus
PBMC	Peripheral blood mononuclear cells
pDCs	Plasmacytoid dendritic cells
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PRR	Pattern recognition receptors
RA	Rheumatoid arthritis
RASF	RA synovial fibroblasts
RLR	Retinoic acid induced gene (RIG)-like receptors

ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
STING	CDS-activated proteins stimulator of IFN gene
TBK1	TRAF family member-associated nuclear factor-κB activator-binding kinases abbreviated
TCR	T cell receptor
TEC	Thymic epithelial cell
Th	t helper
TIID	Type II diabetes
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
Treg	Regulatory T cell
UPR	Unfolded protein response
VSMC	Vascular smooth muscle cells

1 Autophagy in the Regulation of Inflammation

Inflammation is a physiological process, shared by higher animal eukaryotes, triggered by several stress signals. First, entry of microorganisms in otherwise sterile tissues, changes in the composition of the bacterial flora on epithelia, for example in the gut, can initiate inflammation. Secondly, inflammation can also be triggered by cytokines, themselves inducible by infection, or by danger signals, like the ones delivered by intracellular components released in the extracellular milieu (nuclear components like high mobility group 1 (HMGB1) protein or adenosine triphosphate (ATP) release). Oxygen stress induced by intra or extracellular reactive oxygen species (ROS) is also a potent stimulant of inflammation. Regulated inflammation leads to the initiation of an immune response aiming at controlling infection, and to tissue repair. Inflammation and the subsequent immune response, which can further participate in inflammation, must be down regulated at the end of the process. If not, chronic inflammation can lead to disorders linked to aberrant tissue remodelling, excessive cell death and tissue damage, sometimes associated to an autoimmune reaction. Inflammation is thus at the crossroads between metabolic-stress, control or elimination of pathogens by the immune response, and tissue homeostasis.

Autophagy is a process linked to self-digestion by cells of their own components via lysosomal degradation. Several forms of autophagy coexist in animal cells. Chaperone-mediated autophagy (CMA), and microautophagy both allow direct translocation of cytosolic material inside lysosomes. The relations of CMA and microautophagy with inflammation are plausible although not yet proven and will thus not be discussed in this chapter.

Macroautophagy, the best-characterized form of autophagy, will be hereafter called autophagy. It involves the formation of double membrane vesicles fusing with lysosomes leading to degradation of their content. The proteins encoded by autophagy-related genes (*ATG*), play a major role in canonical autophagy but also in other processes like endocytosis and vesicle trafficking [17]. Autophagy was initially described as a catabolic mechanism involved in metabolic stress response, like deprivation of amino acids. It is becoming increasingly clear that in higher eukaryotes autophagy is also involved in other cellular stress responses, like ROS reaction [26], hypoxia [22], unfolded protein response (UPR) and endoplasmic reticulum (ER)-stress [20], genotoxic stress [90] and pathogen recognition [85]. All these responses are main actors in the inflammation process and sometimes impact the subsequent immune response. It is thus not surprising that autophagy is a fundamental player in the inflammation process.

A very ancestral function of autophagy, beyond its role as sensor of energy stress, is probably the elimination of pathogens, particularly intracellular ones. This very particular aspect of autophagy is called xenophagy, when autophagic machinery directly engulfs pathogens or facilitates their translocation and degradation into lysosomes [23]. Autophagy is triggered in that case by metabolic stress induced by pathogen invasion, or by direct recognition of microbe associated molecular patterns (MAMP) via pattern recognition receptors (PRR). In that case autophagy is induced upon inflammation, and contributes to its resolution, by eliminating pathogens.

Interestingly, autophagy is tightly linked to mitochondrial homeostasis. Mitochondria are issued from ancestral proteobacterias, having adopted intracellular life in compartmented cells. It is a very seducing concept that evolution, initially dedicating autophagy to controll invasive pathogens, drove this degradative pathway toward the regulation of symbiotic organelle homeostasis. With respect to the regulation of inflammation, the maintenance of well functioning mitochondria is of great interest. First, balanced autophagic activity limits ROS produced by damaged mitochondria [98], dampening pro inflammatory stimulus. Secondly, mitochondria removal leads to the degradation of PRR associated to their membrane, like retinoic acid induced gene-like receptors (RLR). As a consequence, inhibition of autophagy can lead to hyper-responsiveness to cytosolic double stranded RNA in terms of type I interferon (IFN) secretion [34].

Autophagy also regulates the recognition of cytosolic DNA. In certain circumstances, mitochondrial DNA can be released in the cytosol, especially of PRR-stimulated macrophages [59]. In the previsouly cited publication, an unidentified cytosolic DNA sensor (CDS) leads to the activation of the NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome and to the subsequent production of the pro-inflammatory cytokines interleukin (IL)-1ß and IL-18. Another publication reported a contribution of toll-like receptor (TLR) 9 to that respect [63].

Considering the growing literature on this subject, autophagy is more widely linked to the regulation of cytosolic DNA recognition. Interestingly, not only mitochondria

associated anti-viral PRR can be regulated by autophagy machinery. ATG9a protein activity and microtubule associated protein light chain 3, often abbreviated as LC3, both associate with the CDS-activated proteins stimulator of IFN gene (STING) and prevents its trafficking from ER to compartments containing tumour necrosis factor (TNF) receptor-associated factor (TRAF) family member-associated nuclear factor-κB activator-binding kinases, abbreviated as TBK1 [80]. This non-canonical autophagic function of ATG9a contributes to limit inflammatory responses toward cytosolic DNA.

Type I IFNs are secreted early after viral infection by both immune and non-immune cells. Autophagy, as introduced above, can also regulate pro-inflammatory cytokine secretion, especially in phagocytes. One of the first reports showing a link between inflammasome and the autophagy machinery was published by Saitoh and colleagues [81]. They showed that *Atg16L1*-deficient macrophages secreted higher amounts of IL-1ß after TLR stimulation than controls. They showed that this deregulated secretion was linked to a higher activity of the inflammasome. Kehrl's group showed more recently that the assembled inflammasome was subjected to ubiquitination and targeted to autophagy-related lysosomal degradation via sequestosome1/p62 binding [82]. It was also shown that ATG16L1 activity might indirectly control TRAF6 level via p62, then downregulating the intensity of IL1-ß receptor signalling [43]. Autophagy also limits inflammasome activity by preventing ROS production by damaged mitochondria [98]. Interestingly, a recent publication showed that during viral infection by influenza, receptor-interacting serine-threonine kinase 2 and nucleotide oligomerization domain 2 (NOD2) mediated degradation of damaged mitochondria by autophagy, limiting the activation of NLRP3 inflammasome by intracellular ROS [50].

On the onset of an immune response, innate immunity is the main first actor in the induction of immunity. Although the subsequent adaptive immune response is dependent on this first wave of pro-inflammatory signals, adaptive immune cells can also contribute to the maintenance of inflammation by the cytokines they secrete and by the tissue damage they induce. In certain circumstances, in addition to the initial inflammation, an antigen, sometimes encoded by self-genetic information, can be recognized by cognate T or B lymphocytes. These immune reactions mediated by antibodies or by cytotoxic cells, accompanying inflammation, can be responsible for tissue degradation. Autophagy can contribute to the abnormal activation of the adaptive immune system and to the maintenance of inflammation. First autophagy contributes to endogenous and exogenous/antigen presentation to T cells [79], and thus probably also to autoantigen presentation. Autophagy machinery as discussed later, may play a role for dead cell clearance limiting the access to autoantigen [54]. Moreover, autophagy is a key player in the regulation of lymphocyte survival and activation [71] and by this mean is suspected to regulate inflammation related to the activity of adaptive immunity.

Considering the increasing evidence that autophagy is a key player in immunity, it does not seem surprising now that the link between human immune diseases and autophagy deregulation is emerging.

2 Autophagy and Crohn's Disease

The first era of identification of candidate genes for autoinflammatory and autoimmune diseases, before genome-wide analysis studies (GWAS), strongly relied on linkage disequilibrium studies. One of them identified three variants of *NOD2* genes, strongly associated with Crohn's disease development (CD, [30]): one frameshift mutation, also identified by another independent study [62], and two nonsense variations, altering the leucine-rich repeat domain. In several independent studies, *NOD2* polymorphisms, are strongly linked to the development of inflammatory bowel disease [97]. The most common mutated variants are characteristic of patients suffering from CD rather than ulcerative colitis. IBD in general and CD in particular, are related to hyper inflammation caused by environmental factors like an abnormal commensal flora or abnormal reaction against the flora. Genetic predispositions participate in the inflammation process. Loss-of-function mutations of NOD2 lead to a decrease of IL-6, IL-8 and TNF-α response against cytosolic microbial products probably implying an impaired clearance of pathogens [61, 89]. This diminished inflammatory acute response is suspected to lead paradoxically to a chronic immune response at term. This could be due to pathogen overload linked to changes in the composition of the flora, but also to skewing of the adaptive immune response towards excessive T helper (Th)1/Th17 patterns. Indeed, loss of function of NOD2 mutants in peripheral blood mononuclear cells (PBMC) from CD patients showed impaired IL-10 production leading to the hypothesis that NOD2 participates to the global down regulation of Th cell activity [61]. In the same line, reduced regulatory T (Treg) cell numbers and survival have been found in CD patients with NOD2 loss of function mutation [74]. NOD2 is also highly expressed by innate lymphoid cell (ILC) population from the intestine. These ILC may contribute to homeostasis via their cytolytic activity against infected cells but also by the production of regulatory cytokines like IL-22, known to decrease inflammatory symptoms in a mouse model of colitis. Finally NOD2 mutations are linked to a decrease in antibacterial peptides secretion by Paneth cells in response to bacterial invasion [92]. This decrease in defensin production is eager to contribute to the increased bacterial load in the intestine, favouring hyper inflammation.

GWAS greatly contributed to the identification of new candidate genes for complex chronic inflammatory diseases, often of polygenic origin. One of the first genetic evidence pointing out a role for autophagy machinery in chronic inflammation was found in the context of CD. These studies confirmed *NOD2* as a candidate gene for CD development as single nucleotide polymorphisms (SNPs) were found to be strongly associated with the disease. Strikingly, these studies identified SNPs in the *ATG16L1* gene region, strongly correlated with CD development [6, 25, 77]. The more frequent coding variant of ATG16L1 leads to a substitution of a threonine to an alanine (T300A) and is located at the vicinity of the WD-repeat domain. Another candidate gene was also described: the immune related GTPase M [56, 67]. The latter protein is described as an inducer of xenophagy, particularly important in IFN-γ induced response towards mycobacteria and viruses [69]. Interestingly, one of the exonic variants for IRGM, although conservative in terms of amino acid sequence, leads to an increased sensitivity to down-regulation by micro RNA (MiR) 196 that is overexpressed in CD [8].

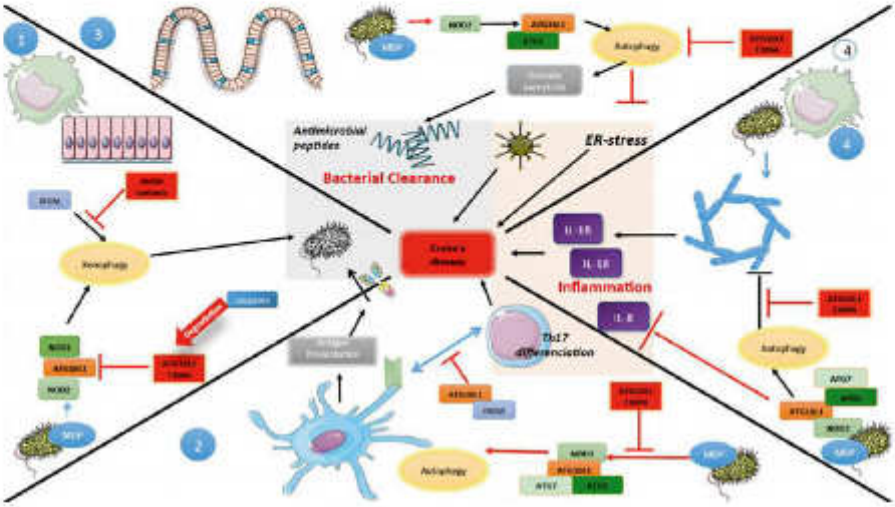

Fig. 1 Possible involvement of autophagy in CD development. *1* During bacterial infection of phagocytes or epithelial cells, ATG16L1 is recruited to the plasma membrane and can associate with NOD1 or NOD2. This induces the elimination of the bacteria through xenophagy. NOD-dependent xenophagy is compromised when ATG16L1^{T300A} protein variant is expressed. ATG16L1^{T300A} variant is more sensitive to degradation by caspase 3 and interacts less with NOD1/NOD2. The *IRGM*, identified as a candidate gene in CD development, encodes a protein known to also induce xenophagy in response to mycobacterial infection. IRGM variants may also be involved in xenophagy impairment and thus in decreased bacterial clearance. *2* MDP induces autophagy in a NOD2-dependent manner, in APCs. Autophagy proteins ATG16L1, ATG5 and ATG7 are implicated in antigen presentation via MHC-II molecules. NOD2 and ATG16L1^{T300A} variants are associated to decreased antigen presentation of bacteria associated antigens. This could contribute to abnormal regulation of adaptive immunity and control of the commensal flora. ATG16L1 and IRGM deficiencies are correlated with increased duration of synaptic contact between T cells and dendritic cells, skewing T cell polarization towards Th17 pro-inflammatory phenotype. *3* NOD2 loss of function mutations lead to a decreased secretion of antibacterial peptides by Paneth cells located in the gut epithelium, inhibiting bacterial clearance thus contributing to a hyper inflammatory environment. Autophagy proteins ATG16L1 and ATG5 are involved in granule exocytosis by Paneth cells, in response to NOD2 stimulation, contributing to bacterial clearance in the gut. ATG16L1^{T300A} polymorphism is linked to reduced antimicrobial peptide secretion, particularly under ER-stress and/or viral infection. *4* ATG16L1 deficiency in macrophages is associated with an increased pro-inflammatory cytokine (IL-1ß and IL18) secretion dependent on inflammasome activation after PRR stimulation. Autophagy proteins ATG5, ATG7 and ATG16L1 are known to downregulate inflammasome activity. ATG16L1 deficient and ATG16L1^{T300A} mice are more prone to activate inflammasome, suggesting the importance of autophagy in inflammation response regulation in CD. An autophagy-independent role for ATG16L1 is the control of IL-8 secretion in response to NOD receptor stimulation. Abbreviations: *ATG* autophagy-related genes, *NOD* nucleotide oligomerization domain, *IRGM* immune related GTPase, *CD* Crohn's disease, *MDP* muramyldipeptide, *MHC-II* major histocompatibility complex class II, *IL* interleukin, *PRR* pattern recognition receptors

From this discovery, autophagy was a matter of great interest in the field of CD research. Interestingly, several studies showed common potential relations between ATG16L1 polymorphisms and NOD2 loss-of-function. First, autophagy is directly linked to the elimination of pathogens as it is involved in xenophagy (Fig. 1, frame 1). The first study showing a link between NOD receptors and autophagy was published

by Travassos and colleagues [88]. They first found that muramyldipeptide (MDP), ligand for NOD2, was able to induce autophagic activity. Moreover, during infection by invasive bacteria, NOD1 is recruited at the plasma membrane along with ATG16L1. This core-ATG protein favoured the elimination of *Shigella flexneri* after its capture at the plasma membrane, and was physically associated with both NOD1 and NOD2. Of much interest, the authors showed that the most common NOD2 mutant associated with CD was unable to lead to ATG16L1 recruitment at the plasma membrane and failed to induce autophagy. In addition, the T300A variant of ATG16L1 protein was inefficient to induce LC3 punctate structure in response to MDP while no difference was seen under rapamycin stimulation. This suggests a selective effect of the T300A mutant on xenophagy in regard to other specialized forms of autophagy. This very elegant study, proposing a rationale for a role of autophagy, in conjunction with NOD2 in the control of gut flora, was followed by another study published by Cooney and colleagues [19]. In conjunction with insufficient elimination of invasive bacteria after NOD stimulation, they propose that ATG16L1 mutations in CD impair the regulatory function of antigen presenting cells (APC; Fig. 1, frame 2). Their study on dendritic cells (DCs) cells showed that MDP stimulation induced autophagy, through a NOD2-dependent mechanism. This increase of LC3-decorated membrane load, needed the canonical core-machinery proteins ATG5 and ATG7, and promoted presentation by major histocompatibility complex (MHC) class II molecules of *Salmonella enterica*-associated antigens to T cells. They unambiguously show that the most common NOD2 and the ATG16L1^{T300A} variants, associated to CD, also led to impaired presentation in the same assay. Thus in addition to eliciting the efficacy of xenophagy, the authors propose that the ATG16L1-related autophagy induced by NOD2 also prime adaptive immune response to control gut pathogens. With respect to antigen presentation, autophagy could also regulate the activation of cognate T cells by APCs, via regulation of the stability of the immune synapse. Wildenberg and collaborators showed that DCs derived from patients carrying the *ATG16L1*^{T300A} allele induced more stable synapses with T cells during antigen presentation, favouring Th17 cell differentiation [94]. They confirm the possible involvement of ATG16L1 in this phenomenon and suggest IRGM as also implicated, by knock down experiments. Autophagy deregulation could thus impair the control of gut flora both by innate and adaptive immunity.

Another interesting point linking NOD2 with ATG16L1 in CD pathogenesis, is their described role in intestinal epithelium homeostasis and especially in Paneth cell function (Fig. 1, frame 3). Pr Virgin's team generated mice bearing a hypomorphic (*HM*) allele of *Atg16L1* by gene trap strategy [9]. As ATG16L1 complete deficiency is lethal in mice, this new model allowed studying *in vivo* the consequences of a diminished expression of this essential autophagy gene. The authors compared *Atg16L1*^{HM} mice with transgenic mice harbouring a conditional deletion of *Atg5* in cells expressing CRE recombinase, under the control of the Villin promoter, restricting autophagy deficiency to the intestinal epithelium. This work showed that both ATG5 and ATG16L1, and thus part of autophagic machinery, were integral to granule exocytosis by Paneth cells. Moreover, the adipocytokines leptin and adiponectin

are increased at the transcriptional level in Paneth cells from *Atg16L1*^{HM} mice. Interestingly, the production of these cytokines is increased in patients suffering from CD. The same study validated the abnormal granule production or cytoplasmic localization in biological samples from patients carrying the T300A risk allele. The impact of this most common variant found in CD on Paneth cell function was confirmed by Xavier's team, using a mouse model knock-in for *Atg16L1* locus, with the T300A allele [41]. Cadwell and colleagues published 2 years after their initial description of *Atg16L1*^{HM} mice, that when they re-derived breeders from embryos in an enhanced barrier animal facility, they did not find any Paneth cell defect in the progeny, contrary to what they had previously described [10]. Most interestingly, virus infection by CR6 strains of norovirus (NRV) leading to a persistent intestinal infection, and present in the former conventional animal facility, recapitulated previously observes granule abnormalities in Paneth cells. This virus plus susceptibility gene interaction necessary for Paneth cell abnormalities, did however not spontaneously induces colitis, pointing out a role for another environmental trigger, additional to the genetic background and viral infection. The authors thus provoked colitis by chemical-induced injury after dextran sodium sulphate (DSS) treatment. They described that *Atg16L1*^{HM} mice, infected by NRV CR6 strain exhibited aberrant response to DSS in the colon leading to atrophy of the mucosa. Inhibiting TNF-α and IFN-γ, which is quite relevant considering CD characteristic inflammation and its common treatment, reduced tissue damage. Other stimulus like ER-stress, seems to be able to trigger intestinal inflammation. Using mice deficient for *Xbp1* specifically in the intestinal epithelium, Blumberg's team showed that ER-stress induced autophagy in this tissue [1]. Invalidation of *Atg16L1* simultaneously with *Xpb1* deletion in intestinal epithelium led to enteritis originating from Paneth cells. Two reports investigated in details autophagic activity in Paneth cells from CD patients [15, 86]. They paradoxically found an increased autophagic activity in Paneth cells. An aberrant distribution of granules in the cytoplasm was nevertheless confirmed, associated to detectable crinophagy, mechanism targeting secretory granules toward autolysosomes. From these studies it seems that a deregulated or aberrantly targeted autophagy, rather than simply diminished autophagy, contributes to abnormal granule exocytosis in CD.

Aside impaired bacterial clearance resulting from defective xenophagy, adaptive immunity, and antibacterial peptide secretion, several studies investigated the potential role that autophagy deregulation could play on inflammatory mediators (Fig. 1, frame 4). As mentioned earlier, autophagy limits inflammasome activation. In the previously cited publication by Saitoh and colleagues [81], *Atg16L1* deficiency was shown to favour increased IL-1ß and IL-18 secretions by macrophages in response to lipopolysaccharide (LPS). Foetal liver chimeric mice grafted with hematopoietic cells deficient for ATG16L1, exhibited more severe DSS-induced colitis than wild type counterparts. This phenomenon was at least in part dependent on IL-1ß and IL-18 secretions.

The role of inflammasome in the development of CD is controversial, as IL-1ß secretion is also believed to be necessary for tissue repair. As a matter of fact, DSS-induced colitis, partly dependent on inflammasome activation, does not totally

recapitulate CD inflammation. Moreover, NLRP3 variants linked to the development of CD, are correlated to low mRNA levels [91] arguing against an increased inflammasome activity during CD. It remains however possible that IL-1ß secretion above homeostatic level contributes to CD inflammation when autophagy is impaired. Mihai Netea's group thus showed that PBMCs from patients carrying the *ATG16L1*^{T300A} allele, expressed higher levels of IL-1ß after NOD2 stimulation [70]. This increased production did not seem in that case correlated with pro-IL1ß processing by caspase 1. Lassen and colleagues proved that *Atg16L1*^{T300A} mice were more susceptible to IL-1ß-linked inflammation induced by bacteria. This suggests again a role for inflammasome activation in the regulation of response towards intestinal pathogens or commensal flora [41].

Interestingly, this latter work showed that the T300A allele modulated ATG16L1 expression by rendering it more susceptible to degradation by caspase 3. A study published the same year also describes enhanced degradation of ATG16L1^{T300A} protein variant by caspase 3 [58]. This loss of stability is responsible for enhanced pro-inflammatory cytokine secretion by macrophages and defective clearance of Y. enterocolitica in the intestine. This mechanism is caspase-dependent as suppression of the mutation-associated caspase cleavage site abolishes hyper inflammation.

Thus apart from its major role in regulating xenophagy, antigen presentation and inflammasome activity, ATG16L1 expression level seems in general linked to pro-inflammatory cytokine secretion. A recent study showed that this regulatory role could be partly played by autophagy-independent mechanisms. Sorbara and colleagues studied in details the interaction between NOD1 and NOD2 receptors with ATG16L1 [84]. They showed that an ATG16L1 protein incapable to induce autophagy was still able to down regulate cytokine production in response to NOD1 and NOD2 stimulations. They further show that the ATG16L1^{T300A} contributes to increase IL-8 signalling in response to *Shigella* infection, in an autophagy independent manner. This points out again a role for ATG proteins in regulating inflammation independently from canonical autophagy as proved for the regulation of STING-TBK1 association by ATG9a or of RLR store level by ATG5-ATG12 conjugates.

3 Autophagy Inflammation Associated to Metabolic Syndrome

Metabolic syndrome consists in a combination of clinical factors linked to a deregulated metabolism. It includes obesity, high insulin level, hypertension, and high cholesterol load. These parameters can lead to pathologies tightly linked to inflammation, like type II diabetes (TIID), atherosclerosis and other cardiovascular diseases. According to the central role autophagy plays on both regulation of metabolism and inflammation, great attention has been paid to this field.

Indeed, autophagy is known be downregulated under high energetic diet, increased in frequency with modern western style nutrition. Autophagy also decreases with age, when metabolic syndrome prevalence increases (Fig. 2, frame 1).

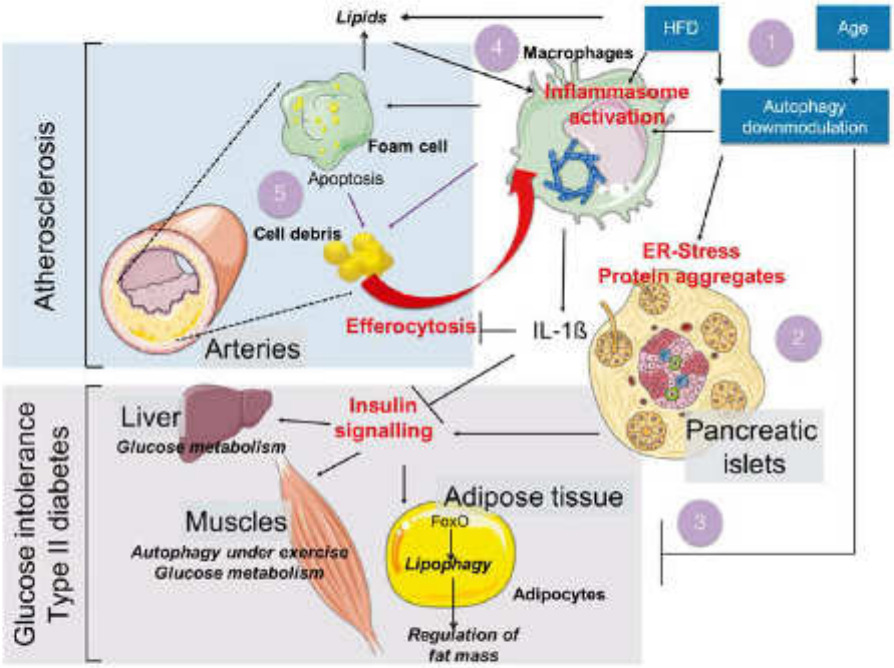

Fig. 2 Implications of autophagy in the metabolic syndrome. *1* Autophagy activity declines with age and can be downregulated by HFD. *2* Autophagy directly contributes to islet beta survival and function by limiting accumulation of protein aggregates and ER-stress. *3* Lipophagy, a form of autophagy involved in lipid droplets elimination, regulating fat mass and lipid release, could also be impaired by an autophagic activity decline. Moreover autophagic activity, specially induced during exercise, is important for glucose tolerance in muscles. *4* Inflammasome activity in macrophages is increased by HFD, including by the direct impact of lipid uptake, like fatty acids and cholesterol. This inflammatory activity is further increased by autophagy decline. IL-1ß released in this context can interfere with insulin signaling, contributing to glucose intolerance. *5* During atherosclerotic plaque formation, inflammation contributes to the recruitment of macrophages that can differentiate into foam cells. Autophagy impairment contributes to inflammation, recruitment of inflammatory cells and foam cell apoptosis. The debris generated by dead cells and the lipids released in the intima can further enhance inflammation. IL-1ß, over-produced by macrophages when autophagy is impaired, can also inhibit efferocytosis, which contributes to the accumulation of debris. Abbreviations: *HFD* high fat diet, *ER* endoplasmic reticulum, *IL* interleukin

These statements led to the hypothesis that autophagy impairment could participate in the development of pathologies associated to metabolic syndrome.

A pioneer study in the field of T1D showed that ß-cells from rat under high fat diet (HFD), exhibited abnormal ubiquitin protein aggregates [36]. Interestingly, these aggregates could be reproduced *in vitro* in a pancreatic cell line, under high glucose concentration treatment, and were shown to be dependent on oxidative stress. These aggregates, not related with aggresomes as their formation are actin and microtubule independent, are increased after 3-methyladenine treatment, an inhibitor of autophagy. Autophagy may thus be involved in their clearance (Fig. 2, frame 2).

This suggested protective role of autophagy was confirmed by another study in mice. C57BL/6 mice under HFD and db/db mouse, models for TIID, exhibited increased autophagic activity in pancreatic ß-cells [21]. This finding was reminiscent of a publication by Li and colleagues describing autophagosomes in ß-cells from Zucker diabetic fatty rats [46]. A later work described that ablation of autophagy genes specifically in ß-cells, compromised islet cell structure and survival [35]. This leads to a loss of glucose tolerance and insulin secretion, exacerbated under HFD. These results were confirmed by another study showing that autophagy deficiency in ß-cells led to ER-stress correlated with progression towards obesity in mice [73]. The progressive decreased activity of protective autophagy in TIID, could be linked to the accumulation of islet amyloid peptide (IAPP), as shown by Costes' group [78]. IAPP aggregates, co-expressed with insulin, are associated with obesity and block autophagic flux. The direct link *in vivo*, between increased IAPP accumulation and impaired autophagy was recently demonstrated in a mouse model with specific expression of IAPP in ß-cells, concomitant with *Atg7* deletion [39]. Autophagy thus allows the degradation of IAPP aggregates, preventing ß-cell apoptosis and diabetes. Interestingly, enhancement of autophagy by trehalose administration to HFD mice improved glucose tolerance, validating autophagy as a seducing target for treatment of TIID.

Autophagy is also important in the regulation of lipid metabolism (Fig. 2, frame 3). A mechanism called lipophagy, is particularly important to control the load of lipid droplets in the adipose tissue. Ciriolo's team showed that the forkhead homeobox type protein O1 (FoxO1) was activated under nutrient restriction in a murine adipocyte cell line [44]. FoxO transcription factors are related to the regulation of lysosomal processes in general, and autophagy in particular, at the transcriptional level. In this context, FoxO1 activation led to improved lysosomal degradation of lipid droplets via lipophagy. The drug metformin used as a treatment for TIID, and known to induce autophagy, led to the same effect. Thus autophagy regulates fat mass, lipid metabolism and the release of free fatty acids (FFA) by adipocytes.

The importance of autophagy on the onset of diabetes is not restricted to adipocytes and pancreatic islet cells. Mobilization of energy from muscles is also involved in TIID development and autophagy plays also here a regulatory role (Fig. 2, frame 3). Beth Levine's group demonstrated that preventing Beclin1-induced autophagy upon starvation and exercise, compromised glucose metabolism in skeletal and cardiac muscles, predisposing to glucose intolerance [28].

In addition to the increasing evidence about the protective role of autophagy on pancreatic ß-cell survival under stress, several studies highlighted a direct link between autophagy impairment and chronic inflammation characteristic of diabetes (Fig. 2, frame 4). IL-1ß is known to participate in insulin resistance, by directly inhibiting Akt signalling after insulin receptor stimulation, and by TNF-α, also known to limit insulin effect. The uptake of the saturated fatty acid palmitate, has been shown to induce NALP3 inflammasome activation in macrophages via NADPH oxidase activation and ROS generation [93]. Inflammasome activation in HFD regimen is here shown to induce insulin resistance *in vivo*. In homeostatic condition, the AMP-activated protein kinase (AMPK) activity can limit ROS generation by favouring ß-oxidation of

FFA. Strikingly, palmitate treatment in addition to LPS stimulation led to a decrease in AMPK activation, and inhibits autophagic activity. This decrease is suspected to impair mitophagy and to favour mitochondrial ROS release as mentioned above, participating in the overexpression of IL-1ß.

In contrast, an uncontrolled activation of autophagy could have deleterious effects on inflammation, in non-immune cells. The mouse beta cell line INS-1(823/13) treated with palmitate, over-expressed cathepsin B in an *Atg7*-dependent manner, cathepsin B being a lysosomal protease responsible for increased pro-inflammatory cytokine expression [45]. Inflammasome activation in that case may be favoured by excessive autophagy, leading to IL-1ß production that contributes to cell stress and limits insulin secretion in response to glucose stimulation. Thus autophagy must be tightly regulated to prevent excessive inflammasome activation and IL-1ß production.

The majority of the previously cited studies, focused on one cell type either immune or non-immune. A recent report showed that global decrease of autophagic activity modelled by $Atg7^{+/-}$ mice, led to low-grade inflammation associated to TIID after crossing with ob/ob mice [48]. This could explain why the risk to develop TIID increases with age when autophagic activity declines, and validate systemic autophagy modulation as a valuable therapeutic strategy.

Another manifestation of the metabolic syndrome tightly linked to inflammation is atherosclerosis (Fig. 2, frame 5). HFD can lead to the accumulation of lipids under the arterial epithelium, in a region called intima. This region then expands while the activation of the epithelium leads to cytokine secretion that attracts monocytes, which will further differentiate into macrophages. Native or oxidized lipids, low density lipoproteins (LDL), are then uptaken via scavenger receptors by macrophages. Macrophages then accumulate, perpetuating inflammation by cytokine or ROS release, which will further oxidize lipids. Macrophages can also differentiate into foam cells, containing elevated stores of lipids. These cells are prone to apoptosis and necrosis, generating debris that will also contribute to fuelling inflammation.

As endothelial cells, vascular smooth muscle cells (VSMC) are also directly sensitive to inflammatory cytokines. Jia and colleagues showed that TNF-α activated autophagy in VSMC isolated from atherosclerotic plaques [33]. In this context autophagy is suspected to participate in plaque instability by contributing to stress and cell death. Interestingly, autophagosome formation and LC3 processing are detected in cells of the intima of atherosclerotic plaques, including macrophages [52, 53]. To delineate the potential roles of autophagy activation in the plaque, two studies generated and described mouse models deficient for *Atg5* in macrophages, on a pro-atherosclerosis background (apolipoprotein null mice or LDL-receptor deficient mice; [47, 75]). As expected, ATG5-deficient macrophages secreted larger amounts of IL-1ß after LPS stimulation than wild-type counterparts. Of note co-incubation with cholesterol crystals, abundant components of the plaque also activated the inflammasome, probably via impairment of lysosomal degradation, which impacts autophagy. The inflammation induced by cytokines released from activated macrophages attracts immune cells and is accompanied by an increase in cell death.

Efferocytosis, a phagocytic process dedicated to the elimination of cell debris, is also frequently described as defective in atherosclerotic plaques. Inhibited

autophagy, in the work of Liao and collaborators, leads to sensitization of macrophages to cell death after treatment with an oxisterol found in plaques and known to induce ER-stress. At the same time oxidative stress is increased in macrophages deficient for autophagy, rendering them less sensitive to clearance by surrounding phagocytes.

Autophagy is also important for lipid degradation by lysosomes in foam cells and for the subsequent cholesterol efflux [65]. In this context autophagy is induced in macrophages by the regulation of the ataxia telangiectasia mutated (Atm)-mammalian target of rapamycin pathway [42]. In line with this observation, impaired autophagy in advanced plaques, could contribute to foam cell apoptosis, lipid release, and cell debris accumulation, again contributing to inflammation. Autophagy can thus protect cells from death or contribute to their elimination although the latter mechanism is not totally understood. Autophagy in phagocytes also contributes to efferocytosis and defects in this specialized LC3-assisted phagocytosis (LAP) could also be involved in plaque formation [54].

Thus autophagy appears as an induced protective mechanism against plaque formation. Its progressive down regulation may participate to plaque evolution although the causes are not totally understood. Progressive inhibition of autophagy with age by increase of MiR-216a expression in endothelial cells may be a causal factor [57]. Aside from aging, and MiR expression, complex genetic background could participate in atherosclerosis susceptibility. A better understanding of the role of autophagy in metabolic disorders in general, partly linked to life-style and nutrition, could improve the existing treatments by limiting chronic inflammation.

4 Autophagy and Autoimmune Chronic Inflammation

Autoinflammatory diseases are chronic inflammations, clearly involving immune reactions toward autoantigens, *i.e.* molecules encoded by the self-genome. These disorders, as other auto inflammatory conditions, are mostly linked to a combination between environmental factors and genetic background. Both innate and adaptive immunity deregulations are prone to trigger autoimmunity. Pro-inflammatory background is eager to favour autoimmunity occurrence, and here again autophagy is central.

As for CD, GWAS designated new candidate genes for the development of autoimmunity (Fig. 3, frame 1). Among them several were linked to the development of systemic lupus erythematosus (SLE). This systemic autoimmune disease is characterized by the production of autoantibodies directed against nuclear auto antigens. Antibody deposits lead to chronic inflammation in several tissues like skin, kidney, cardiovascular system, and nervous system. In 2008, SNPs in *ATG5* locus were linked to the development of SLE [27]. Other studies confirmed this potential association in Asian population [99] while other failed to identify *ATG5* polymorphisms in a Finnish cohort of patients [32].

The functional relevance of SNPs identified in *ATG5* locus is not proven. One study demonstrated that one allelic variant more frequent in SLE patients was

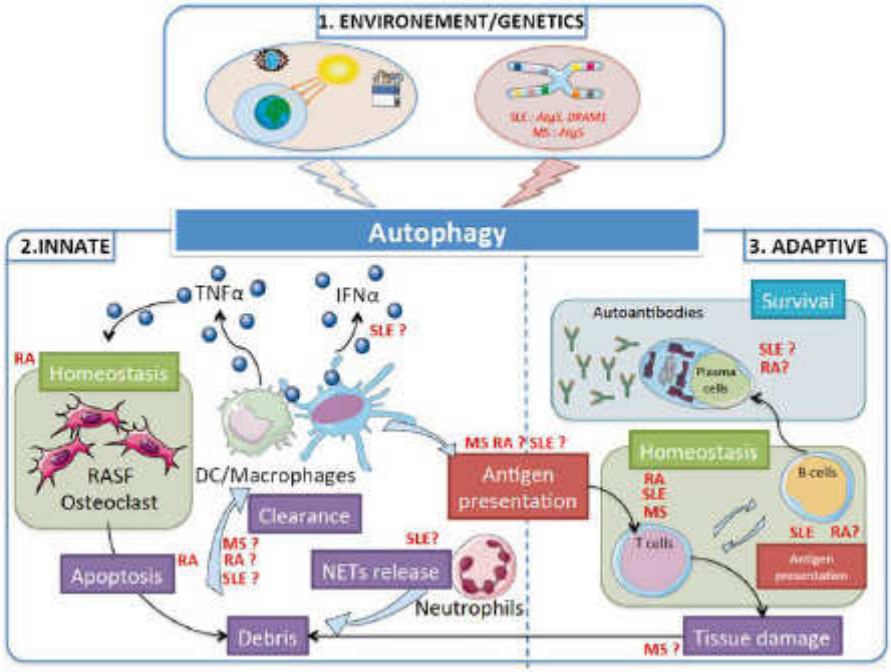

Fig. 3 Mechanisms linking autophagy deregulation to autoimmune disorders. *1* Environmental factors and genetic background could contribute to the deregulation of autophagy. Polymorphisms of unknown functional relevance have been linked to SLE and MS susceptibility. Autophagy deregulation can affect both innate and adaptive immune responses. *2* Autophagy could be involved in the deregulation of innate immunity. Autophagy regulates IFN-I production in response to nucleic acid-containing antigens, which is relevant to SLE. Autophagy is induced in RASF upon TNF-α stimulation. TNF-α is overexpressed in RA. Autophagy in response to TNF-α contributes to survival of RASF and activates osteoclastogenesis, leading to bone resorption. Autophagy contributes to clearance of dead cells by macrophages. The latter mechanism is defective in SLE and its impairment could also contribute to fuel inflammation in RA and MS. Excessive NETs release by neutrophils, mechanism dependent on autophagy, could also lead to the accumulation of debris containing nucleic acids, eliciting inflammation in SLE. Autophagy impacts antigen presentation by macrophages and DCs and could participate in autoantigen presentation in RA and SLE. This has been shown for the EAE model. Autophagy is also involved in the presentation of citrullinated epitopes, frequent in RA, to T cells. *3* Apart from antigen presentation, defects of autophagy, intrinsic to the adaptive immune system, could contribute to autoimmune disorders. Autophagy is implicated in T lymphocyte homeostasis and in the survival of memory B cells and plasma cells. This could contribute to abnormal autoreactive T cell survival in RA, SLE and in MS leading to tissue damage. In RA and SLE, it could contribute to the chronic secretion of pathogenic autoantibodies. Abbreviations: *Atg* autophagy-related gene, *DRAM1* DNA damaged-related autophagy modulator 1, *IFN* interferon, *SLE* systemic lupus erythematosus, *MS* multiple sclerosis, *RA* rheumatoid arthritis, *IFN* interferon, *TNF* tumour necrosis factor, *RA* rheumatoid arthritis, *RASF* RA synovial fibroblasts, *NET* neutrophil extracellular trap, *DC* dendritic cells, *EAE* experimental autoimmune encephalomyelitis

associated with increased *ATG5* mRNA expression [99]. Interestingly, another study focusing on asthma, also described SNPs in *ATG5* locus associated with the pathology, and with an increased activity of the promoter [51]. Correlatively, *ATG5* mRNA expression is increased in acute asthma. Although the direct link between *ATG5* allelic variants and its expression in SLE is not established, other genes regulating autophagy have been identified in GWAS such as DNA-damage regulated autophagy modulator 1 [95], which is involved in autophagy induction upon genetic stress via p53 activation.

Genetic links with other autoimmune diseases are less clear. Polymorphisms on *ATG5* were identified in rheumatoid arthritis (RA) context but could not be definitely confirmed after stringent statistical correction [64]. RA is characterized by autoantibody secretion responsible for systemic manifestations and T cell-related inflammation, directly linked to cartilage destruction. Genetic predisposition in relation to autophagy has also been suggested for multiple sclerosis (MS). This organ-specific pathology results from damages in the nervous system by inflammation-induced demyelination. T cells are the main pathogenic actors in this context. SNPs in the putative *ATG5* promoter region have been recently described as associated with MS [55] while another study failed to prove any association with the disease [11]. As for lupus, even if direct genetic predisposition linked to *ATGs* cannot be formally proved for RA and MS, indirect causes could impair autophagic activity, and favour chronic inflammation in these contexts.

Systemic pathologies like SLE and RA are linked to aberrant production of, and/or reaction towards, type I IFN and TNF-α respectively (Fig. 3, frame 2). Given the important relationship between ATG5/ATG12 and IFN-I secretion, by regulation of RLR and TLR availability for their ligands, it is possible that impairment of autophagic activity contributes to increased IFN-I production. Plasmacytoid dendritic cells (pDC) are important producers of IFN-α, especially via recognition by the intracellular sensor TLR9. Interestingly, a form of LAP has been linked in these cells to translocation of antibody-associated DNA complexes after recognition via Fc receptors [29]. Autophagy machinery in this context was shown to contribute to translocation of endocytosed DNA to TLR9 positive compartments, inducing IFN-α secretion. A deregulation in this traffic route could be relevant in SLE as insufficient clearance of nuclear debris, associated to antibodies, are thought to trigger and/or to sustain inflammation. Aside from insufficient clearance of debris resulting from apoptotic cells, DNA can also be released from activated neutrophils in neutrophil-extracellular traps (NETs). When produced in excess, they could contribute to provide TLR9 ligands phagocytosed by pDCs [40]. Interestingly, autophagy has been shown to participate in NET release [76]. Future studies should be done on lupus animal models or with SLE patient's samples to assess a potential link between deregulated autophagy and IFN-I secretion.

In contrast more experimental results have been obtained regarding the interplays between TNF-α, autophagy and RA. TNF-α is an inducer of autophagy in several cell types as vascular smooth muscle cells [33], skeletal muscle cells [38], epithelial cells but also in immune cells like macrophages [5]. TNF-α has been shown to induce autophagy in RA synovial fibroblasts (RASF) isolated from patients [18]. These cells

are central to the development of cartilage inflammation thanks to the inflammatory cytokines and growth factors they secrete. These results are in line with other studies [37, 83], which showed that autophagy is induced under ER-stress and further increased by TNF-α. Autophagy protects fibroblasts from cell death, in concert with CCAAT/-enhancer-binding protein homologous protein under-expression, and probably contributes to their abnormal survival and secretion of growth factors. The microbial product LPS in complexes with damage associated molecular pattern HMGB1, are known to trigger experimental arthritis in mice. In the human pathology, these complexes could result from cell debris on a non-sterile inflammation site. LPS-HMGB1 complexes favour the differentiation and survival of RASF, concomitant with autophagy activation [72], again pointing out a protective role of autophagy on RASF and thus on the maintenance of inflammation. Autophagic vacuoles and expression of Beclin-1 and ATG7 are also increased in osteoclasts from RA patients [49]. Autophagy in this work was shown to help osteoclastogenesis and contributes to bone-resorption in a TNFα-dependent manner.

SLE and RA have in common the chronic generation of cellular debris at the site of inflammation. Adequate clearance of cell remnants is thought to be central to the prevention of autoimmunity. Numerous mouse models deficient for apoptotic cell clearance are prone to lupus-like pathologies. A specialized form of LAP, involving LC3A, has been shown to be implicated in the elimination of dead cells by macrophages [54]. Invalidation of this pathway leads to increased pro-inflammatory cytokine production by macrophages. The *in vivo* relevance of this observation and the potential link with human SLE are still to be demonstrated.

The existence of a pro-inflammatory background contributes to break the tolerance against auto antigens. A chronic inflammatory microenvironment is eager to activate APCs that become abnormally able to prime T cells against self-peptides. Autophagy plays an important role in antigen presentation, both by MHC class I and II molecules. Autophagy has been shown to be necessary for the presentation of endogenous self-peptides by thymic epithelial cells (TECs). Mice with autophagy-deficient thymus [60] or conditionally deleted for *Atg5* specifically in TECs [2], exhibit abnormal central selection of T cells leading to an autoimmune phenotype consisting on colitis. Defects in central tolerance are not formally proved in the majority of autoimmune diseases. It remains however possible that defects in autophagy activity in the thymus, associated for example with age (when inflammatory diseases become more frequent), contributes to skew T cell repertoire towards autoimmunity. Moreover, defects in Treg generation and survival are associated with colitis, an autoimmune phenotype observed in the study published by Nedjic and colleagues [60]. It is thus possible that autophagy defects in the thymus contribute to abnormal peripheral tolerance.

A role for autophagy in autoantigen presentation in the periphery is also plausible. Indeed, autophagy contributes to the presentation of cytosolic epitopes to CD4 T cells, including self-epitopes, which could be quite relevant for autoantigen presentation. A recent study showed that specific deletion of autophagy in DCs limits the development of experimental autoimmune encephalomyelitis (EAE), a murine model for human MS [7]. The improvement of the clinical score was correlated with

a decrease in CD4 T cell priming. Although not yet proven in the case of RA, autophagy could contribute to autoepitope presentation. Unanues' group showed that autophagy in APCs, i.e. DCs, macrophages and B lymphocytes, contributed to the presentation of citrullinated epitopes [31]. Interestingly citrullinated peptides are common antigens in RA, and aberrant autophagy could contribute to generate such autoantigens. Autophagic activity has been shown to be upregulated in B cells from SLE patients and mouse models for lupus [16]. Increased autophagy could then contributes to autoantigen presentation, including citrullinated epitopes, but to date, no experimental proof has been provided.

The initial peripheral break of tolerance in autoimmune diseases leads to abnormal autoreactive lymphocyte survival (Fig. 3, frame 3). Here again, deregulated autophagy can contribute to the chronicity of autoimmune inflammation. Autophagy plays an important role in T lymphocyte survival and polarization. The first study suggesting a link between deregulated autophagy in lymphocytes was performed in T cells from MS patients. ATG5 expression was found increased in T cells isolated from EAE mice and MS patients [4]. A work performed in our laboratory identified autophagy deregulation in T cells from both mouse models for lupus and SLE patients [24]. The autophagic vacuole load in T cells was mainly observable under T cell receptor (TCR) related stimulation in mice and was increasing with age, contrary to control mice. An increase in autophagic compartments in SLE T cells was confirmed by three other studies [3, 12, 16]. Interestingly the study by Alessandri and colleagues showed an increase in the autophagosome-associated marker LC3, especially in naive CD4 T cells suggesting a predisposed deregulation. Our study including induction of systemic acute inflammation in normal mice showed that the activation of T cells in this context, was not sufficient to increase autophagy. Pierdomonici's group concludes that accumulation of autophagosomes is due to a blockade of autophagy rather than increased autophagy induction. We cannot formally exclude this hypothesis as our observation could results from disequilibrium between induction and degradation of autophagic vacuoles. However in our setting, the blockade was not total as LC3 was still accumulated after treatment with lysosomal protease inhibitors. Moreover Alessandri and colleagues use starvation or treatment with autologous serum from SLE patients, as triggers of autophagy. Interestingly, they show that SLE serum can induce autophagy in normal T cells, reminiscent of another study identifying the pro-autophagic impact of SLE serum on a neuron cell line [87], but not in SLE T cells. It is possible however that SLE T cells cannot further increase autophagy under metabolic demand or when re-exposed to their already stimulating environment. It is also possible that other pathways like the TCR pathway, relevant for auto reactive T cell activation, can contribute to elevate LC3 levels. In any case, further investigation is needed to discriminate at which level the deregulation occurs, and if an increase in autophagosome generation or a decrease in degradation, or both, could be involved. Furthermore, metabolic versus antigen-induced autophagic stimulations should be distinguished as their regulations and outcomes may be different.

The regulation of autophagy in T cells from RA has also been recently studied. Weyand's team described a deficiency in glucose metabolism in CD4 T cells from

RA, in response to TCR stimulation [96]. This was associated with insufficient 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) induction. This impairment in energy mobilization could explain part of the apoptosis-prone phenotype of RA T cells. Interestingly, autophagy was also impaired by PFKFB3 deficiency, contributing to the difficulty to mobilize energy during activation.

Thus autophagy could play dual roles in autoreactive T cells, contributing to their abnormal survival when increased and sensitizing to apoptosis when impaired. In the context of MS, inhibiting autophagy in T cells from autoimmune diseases could be a therapeutic option as shown in the study by Kovacs and colleagues. They demonstrate that mice with specific deletion of Beclin1 in T cells are less sensitive to EAE induction. This was correlated with increased cell death, especially for Th1 cells, pointing out an interesting regulatory role of autophagy on Th cell polarization. The induction of apoptosis in the absence of autophagy could be related, in this experimental model to increased stores of pro-caspases.

In RA, and SLE, autoantibody secretion is part of the pathology. Deregulation of B lymphocyte homeostasis is thus one typical feature of these systemic autoimmune diseases. Clarke and colleagues were the first to describe autophagy increase in B cells from lupus prone mouse model NZB/W, and from SLE patients [16]. Two major studies showed a role for autophagy in memory B cell and plasma cell survival [14, 68]. An increase in autophagy could contribute to the abnormal survival of autoreactive B lymphocytes and autoantibody secreting plasma cells. It thus appears that modulation of autophagy in lymphocytes could be a beneficial strategy to limit auto reactive lymphocyte survival. Moreover, autophagy is suspected to play a role in the relocalization of DNA containing antigenic complexes toward TLR9 positive endosomes [13]. Autophagy machinery could thus contribute to B cell hyperactivity against nuclear antigens. Studies on mouse models with autoimmune-prone backgrounds, also deficient for autophagy in lymphocytes, could help decipher the role of ATGs in the development of the pathology.

Of much interest, therapies like rapamycin, hydroxychloroquine or P140 peptide [66], are known to modulate autophagic activity. It would be interesting to investigate if such therapeutic effects are actually linked to modulation of autophagy in immune cells like APCs or lymphocytes.

5 Conclusions

Autophagy is a physiological response, at the crossroads between energy sensing, and reaction to stress induced by tissue damage and/or by infection. This mechanism is thus a master integrator of both innate and adaptive immunity to the surrounding environment, by regulation of inflammation. Both genetic and environmental factors could contribute to the deregulation of autophagy. Allelic variations on autophagy genes seem to be strongly related to the susceptibility to develop autoinflammatory diseases like CD. Other polymorphisms on *ATGs* suggest a role for autophagy deregulation in autoimmune diseases, like SLE, RA and MS, although the functional relevance of

these variants remains to be fully determined. Environmental factors like infection, or changes in metabolic equilibrium linked to diet, or genetically programmed like aging, can contribute to provide a low-grade inflammatory environment, prone to trigger chronic inflammation. Autophagy involvement in preventing inflammation linked to these three causes, linked to TIID and atherosclerosis, is now clearly demonstrated. Apart from RA, MS, SLE and CD, other inflammatory pathologies could also imply autophagy deregulation. Interestingly, therapeutic molecules modulating the autophagy process have shown efficacy in autoinflammatory diseases like metformin for TIID, rapamycin, hydroxychloroquine and P140 peptide, in autoimmune diseases. A better understanding about the precise roles of autophagy in chronic inflammation will help design new molecules, and new therapeutic approaches, to treat these complex diseases.

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3 Publication 4

Autophagy in T and B cell homeostasis: recycling for sustainable growth

(Article in French)

Johan Arnold, **Diane Murera**, Florent Arbogast, Sylviane Muller, Frédéric Gros

Abstract

Macroautophagy often abbreviated by "autophagy" is an intracellular degradation mechanism linked to lysosomal activity. Autophagy is conserved from yeast to mammals and plays a role in the response to energetic stress and in organelle homeostasis. Autophagy is also involved in the regulation of immunity, in particular in the adaptive immune response, which involves B and T lymphocytes. It was indeed shown that autophagy impacts the development of B and T cells as well as the education of T cells in the thymus. Autophagy also modulates activation, survival and polarization of T cells. It plays a role in antigen presentation by B cells, and in their TLR-mediated activation, and thus likely in their initial activation. Finally, autophagy is required for the survival of memory lymphocytes and effector cells like antibody-producing plasma cells. Interestingly, autophagy is deregulated in several autoimmune pathologies. The modulation of this phenomenon could possibly lead to new treatments aiming at limiting lymphocyte activation driving these pathologies.

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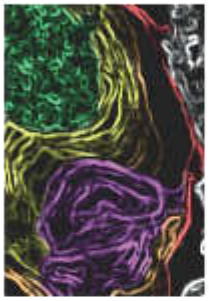
L'autophagie et l'homéostasie des lymphocytes T et B

Bien recycler pour un développement durable

Johan Arnold[1], Diane Murera[1], Florent Arbogast[1], Sylviane Muller[1,2], Frédéric Gros[1,2]

> La macroautophagie, souvent abrégée par « autophagie », est un processus de dégradation intracellulaire lié à l'activité lysosomale. Ce mécanisme, conservé depuis la levure jusqu'aux mammifères, joue un rôle central dans la réponse au stress énergétique et dans l'homéostasie des organites intracellulaires. L'autophagie exerce également des fonctions majeures dans la régulation du système immunitaire, en particulier dans l'immunité adaptative constituée par les lymphocytes T (LT) et B (LB). Il a ainsi été montré que l'autophagie était impliquée dans le développement des LT et LB, ainsi que dans l'éducation des LT dans le thymus. L'autophagie module notamment l'activation, la survie et la polarisation des LT. Elle joue un rôle éminent dans la présentation antigénique par les LB ainsi que dans le trafic du récepteur à l'antigène après son engagement. Ainsi, l'autophagie pourrait être impliquée dans l'activation initiale des LB. Enfin, elle est nécessaire à la survie des lymphocytes mémoire et d'effecteurs tels que les plasmocytes producteurs d'anticorps. L'autophagie des lymphocytes est dérégulée lors de certaines pathologies auto-immunes. La modulation de ce processus cellulaire pourrait permettre à terme d'envisager de nouveaux traitements afin de limiter l'activation des lymphocytes au cours de ces pathologies. <



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L'autophagie est un mécanisme catabolique complexe issu de la formation en amont dans le cytoplasme de vésicules à double membrane qui fusionnent en aval avec des lysosomes (*figure 1*) [1] (➔).

(➔) Voir la Nouvelle de P. Codogno, m/s n° 8-9, août-septembre 2004, page 734

L'autophagie induite par un stress énergétique favorise la survie des cellules. Une activité autophagique basale permet en outre l'élimination des gros agrégats protéiques et des organites altérés [1]. Ce rôle est particulièrement important dans les cellules à longue durée de vie comme les neurones. La machinerie autophagique est composée d'une trentaine de gènes appelés *ATG* pour *autophagy-related genes* [2]. Cette appellation souligne le fait que les protéines ATG peuvent intervenir dans d'autres processus que l'autophagie, notamment dans la régulation de la mort cellulaire, l'exocytose ou certaines formes d'endocytose [3].

L'autophagie, basale ou induite, joue des rôles spécialisés en fonction des compartiments physiologiques considérés. En particulier, l'implication de l'autophagie dans l'immunité a été dévoilée au cours de la dernière décennie [4]. La dégradation de composants intracellulaires par autophagie permet en effet l'élimination de microorganismes viraux ou bactériens par un mécanisme appelé xénophagie[1] [5, 6] (➔).

(➔) Voir la Synthèse de P.E. Joubert et al., m/s n° 1, janvier 2011, page 4

L'autophagie permet également de réguler l'inflammation en limitant la sécrétion de cytokines pro-inflammatoires. Ce lien entre autophagie et pathologies inflammatoires humaines est notamment illustré au cours de la maladie de Crohn[2]. En effet, des études génétiques ont révélé une association entre des polymorphismes affectant un des gènes *ATG*, *ATG16L1*, et le développement de cette maladie inflammatoire chronique [7, 8] (➔).

(➔) Voir la Nouvelle de A.L. Glasser et al., m/s n° 4, avril 2009, page 349

[1] La xénophagie, ou dégradation par la voie autophagique de microorganismes intracellulaires, désigne la capacité à capturer les pathogènes dans les autophagosomes puis à les dégrader avec les autolysosomes.

[2] Maladie chronique inflammatoire de l'intestin.

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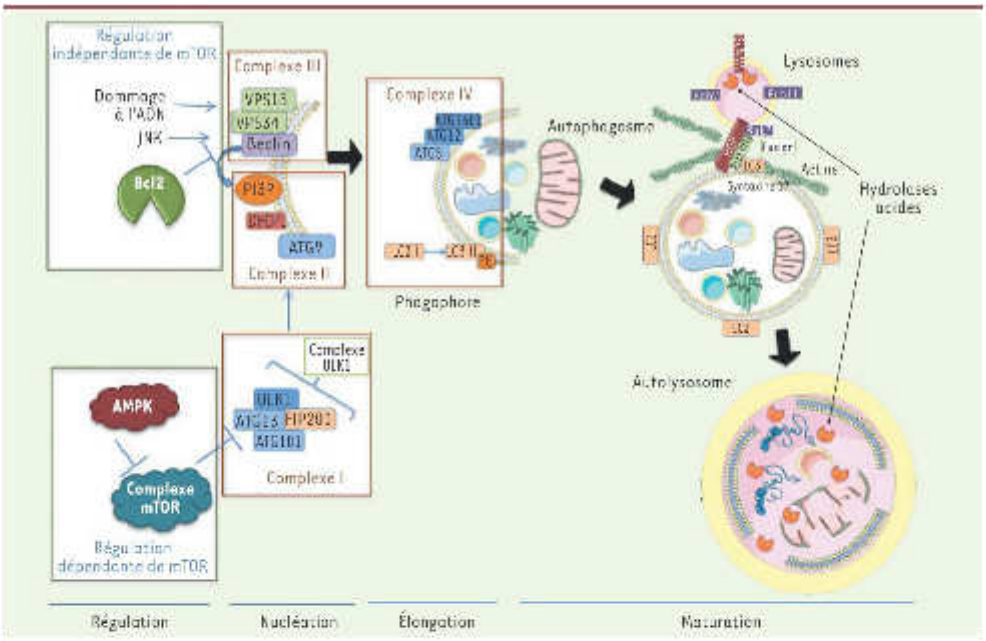

Figure 1. *La machinerie moléculaire de l'autophagie.* L'autophagie peut être induite par des signaux liés à un stress énergétique (voie dépendant de mTOR) mais également par d'autres stimulus (voies indépendantes de mTOR) comme les dommages à l'ADN, la signalisation via la voie JNK (*c-Jun N-terminal kinase*) ou encore l'action de la protéine Bcl2 (*B-cell lymphoma 2*). L'autophagie se déroule en trois grandes étapes : le recrutement de la membrane, grâce au complexe ATG9c (*autophagy related 9A*) qui permet d'initier la formation d'un phagophore (vésicule initialement formée dans le contexte de l'autophagie) avec le concours des complexes ULK1 (*unc-51-like kinase 1*) et Beclin1 (correspondant à la nucléation). L'étape d'élongation, faisant intervenir les complexes ATG5–ATG12/ATG16L1 et LC3 (*microtubule-associated protein-light chain 3*), permet alors de former une vésicule close à double membrane (l'autophagosome) qui séquestre le matériel cytoplasmique. L'étape de maturation, au cours de laquelle l'autophagosome fusionne avec les lysosomes contenant des hydrolases acides, grâce à l'action des protéines HOPS (*homotypic fusion and protein sorting*), PLEKHM1 (*pleckstrin homology domain containing protein family member 1*) et du cytosquelette, mène enfin à la dégradation du contenu cytoplasmique de la vésicule. Du contenu cytosolique, des mitochondries endommagées, du réticulum endoplasmique et des gros agrégats protéiques peuvent être pris en charge par la machinerie autophagique. Le complexe ULK1, nécessaire à l'initiation de l'autophagie, contient les kinases ULK1, ATG101, FIP200 (*FAK family kinase-interacting protein of 200 kDa*) et ATG13. Ce complexe sert au recrutement de la membrane isolée, avec l'aide de la protéine ATG9a, permettant la mise en place du phagophore. Ces protéines régulent un autre complexe majeur impliqué dans la formation du phagophore, à savoir le complexe Beclin1/VPS34 (*vacuolar protein sorting 34*)/VPS15. La machinerie d'élongation est composée de deux systèmes de conjugaison, à savoir les systèmes ATG12 et LC3 (*microtubule-associated protein-light chain 3*). Ils sont incorporés dans les membranes de l'autophagosome sous la forme d'un complexe ATG12–ATG5/ATG16L1 et sous forme lipidée pour LC3 après incorporation d'une phosphatidylethanolamine (LC3-PE ou LC3-II). La maturation est le dernière étape de formation de l'autophagosome. L'accrochage de ces deux vésicules, autophagosome et lysosome, est réalisé entre autres par des protéines SNARE (*soluble N-ethylmaleimide-sensitive factor attachment protein receptor*)/Rab (*Ras-related in brain*) et les composants du cytosquelette tels que l'actine. La fusion en elle-même est réalisée par l'interaction du complexe PLEKHM1/HOPS avec la protéine LC3. Ce complexe qui interagit avec Rab7, forme un ensemble qui permet la fusion entre l'autophagosome et les lysosomes. Cette fusion crée une vésicule appelée autolysosome dans laquelle sera dégradé le contenu cytoplasmique séquestré. AMPK : AMP [adénosine monophosphate]-activated protein kinase ; PI3P : phosphatidylinositol-3-phosphate.

Dans ce cas, un déficit d'activité autophagique favorise l'inflammation. L'autophagie est également impliquée dans la présentation des antigènes par le complexe majeur d'histocompatibilité de classe II (CMH-II) [9]. Par son rôle dans les processus inflammatoires et la présentation antigénique, elle participe ainsi à l'activation de l'immunité adaptative. L'autophagie intrinsèque des lymphocytes joue également un rôle important dans la biologie de ces cellules.

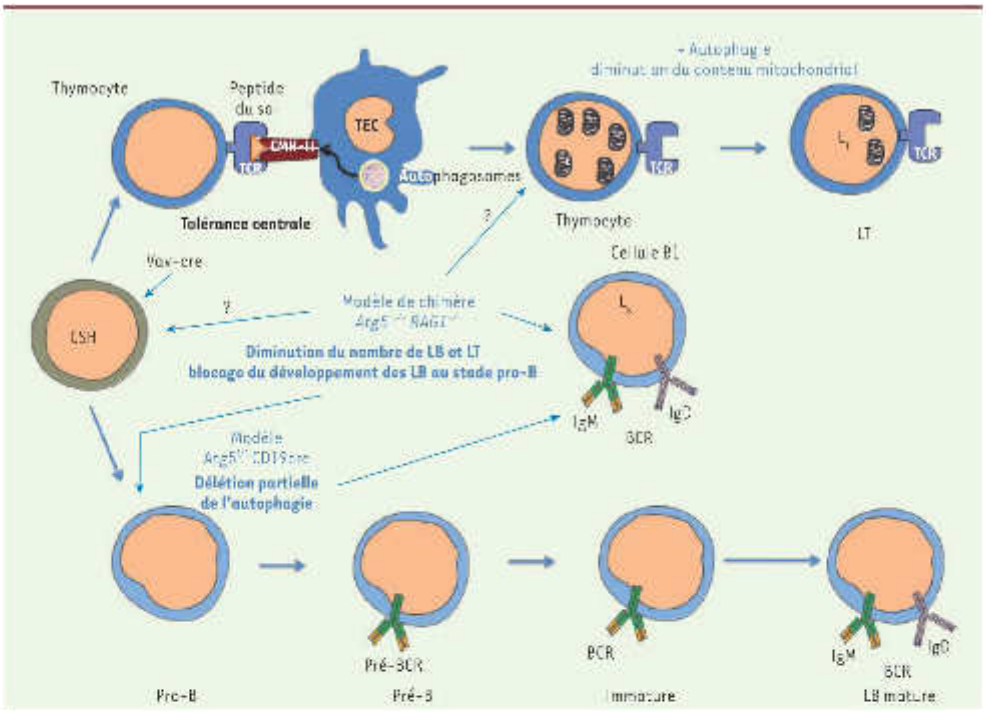

Figure 2. *Implication de l'autophagie dans le développement des lymphocytes T et B.* L'autophagie est nécessaire à la survie et à la différenciation des cellules souches hématopoïétiques (CSH) [16]. Le transfert de cellules de foie fœtal de souris déficientes pour *Atg5* chez des souris dépourvues de lymphocytes provoque, outre une forte perte de thymocytes, une forte baisse du nombre de LT et LB en périphérie [10, 14]. Les cellules épithéliales thymiques (TEC) présentent un niveau élevé d'autophagie basale si on le compare à celui d'autres tissus. Cette activité autophagique permet la présentation de peptides du soi intracellulaires sur le CMH de classe II et intervient dans l'éducation des LT CD4+ [12, 29, 31]. Les LT déficients en autophagie depuis le développement thymique maintiennent une quantité de mitochondries importante lors de leur passage de la zone corticale vers la zone médullaire, les sensibilisant à l'apoptose [10, 12]. Les LB de souris chimères, générées à partir de cellules de foie fœtal déficientes pour *Atg5*, subissent un blocage développemental à partir du stade pro-B [14]. En revanche, le modèle murin de délétion d'*Atg5* sous le contrôle du promoteur CD19 exprimé à partir du stade pré-B ne montre pas un tel défaut. La délétion partielle pourrait expliquer ce phénomène. Dans ces souris, aucun défaut majeur dans la proportion des différentes populations de LB en périphérie n'est observé, à l'exception d'une diminution de la population de cellules B-1a du péritoine. BCR : *B cell receptor* ; TCR : *T cell receptor* ; CMH-II : complexe majeur d'histocompatibilité de classe II ; Ig : immunoglobuline ; LB : lymphocyte B ; LT : lymphocyte T.

Autophagie et développement des lymphocytes

La première étude s'intéressant au rôle de l'autophagie dans les lymphocytes a été publiée par Heather Pua [10], à partir de modèles murins de délétion du gène *Atg5*. Cette délétion étant létale à la naissance, lorsqu'elle est systémique, les auteurs ont utilisé des souris chimériques établies à partir de cellules de foie fœtal, isolées de souris déficientes pour le gène *Atg5*, transférées chez des animaux *Rag1*$^{-/-}$ dépourvus de LT et LB. Une baisse importante du nombre de LT et LB en périphérie a été observée (*Figure 2*). Dans le cas des LT, il n'apparaît pas de changement dans la répartition des sous-populations de thymocytes, mais une baisse de leur nombre. D'autres études se sont intéressées au rôle de l'autophagie des LT grâce à des modèles de délétion plus tardifs [11, 12]. Ainsi, en utilisant le promoteur proximal du gène *lck* (*lymphocyte-specific protein tyrosine kinase*), exprimé tôt lors du développement thymique, ce qui permet d'invalider l'autophagie seulement à partir de ce stade, les auteurs ont observé une baisse de cellularité au niveau du thymus mais également en

périphérie. L'autophagie joue donc un rôle dans le développement des thymocytes et aussi lors de leur migration vers la périphérie. Elle favorise l'élimination des mitochondries endommagées. De manière intéressante, les thymocytes, avant de migrer en périphérie, réduisent leur nombre de mitochondries. L'absence d'élimination des mitochondries par les thymocytes déficients en ATG5 conduirait à une accumulation d'espèces réactives de l'oxygène (ROS), ce qui favoriserait l'apoptose des cellules en périphérie. Récemment, il a été montré que, contrairement aux lymphocytes T impliqués dans l'immunité adaptative, les cellules invariantes NKT[2] requéraient une autophagie compétente dès le développement thymique [13], ce qui souligne l'existence d'une transition de métabolisme très précoce pour ces lymphocytes de l'immunité innée.

En utilisant le même modèle de souris chimères qui avait été développé par Pua et al., l'équipe de Virginia s'est intéressée en détail au rôle de l'autophagie dans le développement des LB [14, 15] (➜).

(➜) Voir la Synthèse de M. Hamze et al., m/s n° 10, octobre 2014, page 874

Ces auteurs ont ainsi relevé un défaut de développement dans la moelle osseuse des souris chimères lors de la transition au stade pro-B ou stade pré-B, pouvant expliquer la baisse du nombre de LB en périphérie déjà observée par Pua et ses collègues. Les auteurs ont également généré une souris dont les LB sont spécifiquement déficients en autophagie dès le stade pro-B. De manière étonnante, ils ne retrouvent pas de blocage développemental. Le nombre de LB en périphérie est alors normal, à l'exception des cellules B-1a[3] du péritoine qui sont fortement impactées par la perte de l'autophagie. Il est possible que, dans ce cas, la délétion conditionnelle d'*Atg5* ne soit pas totale au stade pro-B et permette la survie des cellules au fil de leur développement. En effet, le promoteur (*cd19*) utilisé dans ce travail conduit l'expression de la recombinase cre. Son activité n'induit, dans beaucoup d'études, qu'une excision partielle du gène cible au sein des LB. Que ce soit pour les LB ou les LT, les défauts développementaux observés dans les souris chimères pourraient refléter des anomalies touchant des stades antérieurs à la séparation des lignées B et T. En effet, dans les souris chimères, ATG5 est absent des précurseurs hématopoïétiques, et ce dès le début de la vie embryonnaire. Il faut donc considérer que l'autophagie est nécessaire à la survie des cellules souches hématopoïétiques [16]. Dans les souris dont l'autophagie est invalidée au début de l'hématopoïèse (souris Vav-cre), on observe, entre autres défauts, une baisse drastique du nombre de LB et LT. Ainsi, même si l'autophagie semble clairement impliquée dans le développement des LT dans le thymus, son rôle dans la lignée B reste confus. Des modèles de délétion à différents stades de développement des LB, ou l'usage de modèles pleinement déficients en autophagie au stade pro-B pourront éclaircir l'implication de ce processus au cours du développement des LB.

[2] Cellules invariantes NKT : iNKT (pour *invariant natural killer T*), une population particulière de lymphocytes intervenant dans la réponse innée.

[3] Les lymphocytes B-1a sont absents du sang périphérique. Ils sont en effet majoritairement dans les cavités pleurale et péritonéale et sont responsables de la production des auto-anticorps naturels.

Autophagie et éducation des lymphocytes T

Les cellules épithéliales du thymus (TEC) et les cellules dendritiques de la médullo-thymique jouent un rôle prépondérant dans l'éducation des LT. Ces cellules sont impliquées dans la présentation aux LT de peptides du soi sur les molécules du CMH. Lors de la sélection positive, seules les cellules T possédant un récepteur (TCR) peu affin pour le complexe CMH/peptide survivent, assurant la restriction des TCR pour le CMH de l'individu. Lors de la sélection négative, les LT possédant un TCR qui reconnaît les complexes CMH/peptides du soi sont alors éliminés, limitant ainsi l'autoréactivité des lymphocytes. La nature des peptides présentés par le thymus est donc primordiale pour assurer la tolérance au soi. De manière intéressante, les TEC présentent une forte activité autophagique spontanée (*Figure 2*) [17]. L'autophagie permet la présentation de peptides d'origine intracellulaire par les molécules du CMH de classe II. Partant de ce constat, une première étude réalisée par Klein et ses collaborateurs a montré que la greffe d'un thymus totalement déficient en ATG5 compromettait l'éducation des LT lors du processus de sélection positive [17]. Ce défaut n'affecte que les LT CD4+ qui sont restreints au CMH-I, ce qui souligne le rôle particulier de l'autophagie dans la présentation par cette classe de CMH. À noter que les souris possédant un thymus déficient pour ATG5 développent des colites d'origine auto-immune. Une étude autrichienne a néanmoins remis en cause les travaux de L. Klein. Elle avance que les défauts observés initialement pouvaient être dus à des effets non-autophagiques d'ATG5, ou encore à une conséquence de l'absence d'autophagie dans des sous-types cellulaires autres que les cellules épithéliales [18]. Les résultats obtenus avec un modèle murin présentant une délétion conditionnelle en ATG7 dans l'épithélium thymique a conduit les auteurs à conclure que l'autophagie ne participait pas à l'éducation thymique. On peut cependant penser que dans ce modèle déficient en ATG7, une activité autophagique résiduelle permettrait l'éducation des LT. L'équipe de Klein a montré peu de temps après, grâce à un nouveau modèle de délétion conditionnelle, que l'autophagie des cellules épithéliales thymiques exprimant AIRE (*autoimmune regulator*) [19] (➜), participait à la sélection négative via la présentation par le CMH de classe II [20]. Le rôle de l'autophagie du stroma thymique dans l'éducation des LT semble conforté par une étude récente. En effet, Schuster et ses collègues ont montré que le gène *Clec16A*, orthologue murin d'un

(➜) Voir la Synthèse de N. Lopes et al., m/s n°8-9, août-septembre 2015, page 742

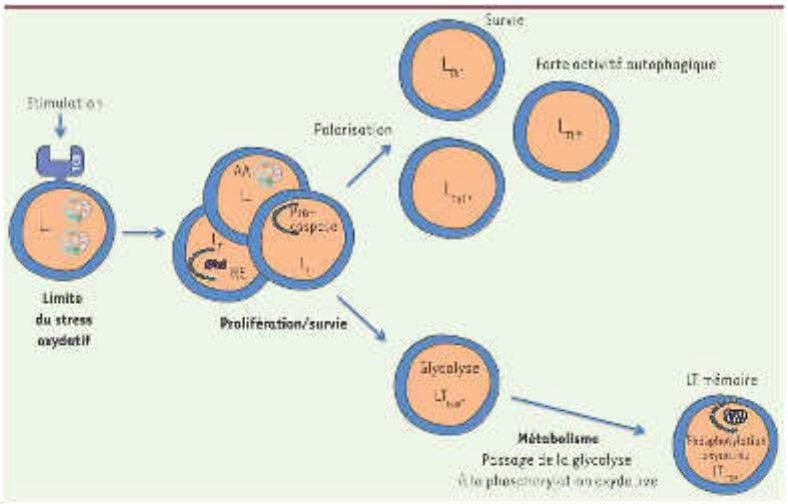

Figure 3. *Implication de l'autophagie dans la différenciation des lymphocytes T.* L'autophagie est induite dans les LT lors de l'activation par le TCR (*récepteur des cellules T*) [10]. L'autophagie sélectionne les cargos à dégrader. On peut donc penser qu'en absence d'autophagie les défauts de prolifération et de survie observés en réponse à une stimulation du TCR, sont en partie dus à l'expansion du réticulum endoplasmique (RE) [22, 23] et de la masse mitochondriale [11, 12]. L'autophagie permet également de soutenir la demande métabolique en générant des acides aminés (AA) [24]. Enfin, elle permet de dégrader les pro-caspases [25]. Les cellules Th (*T helper*) peuvent se différencier en plusieurs sous-types. Les premières études sur l'implication de l'autophagie dans la différenciation des LT auxiliaires ont montré que les cellules Th2 ont un taux d'autophagie plus élevé que les cellules Th1. Les sous-populations de lymphocytes Th1 et Th2 montrent une moins bonne survie que les Th17 après stimulation en absence d'autophagie. L'autophagie joue un rôle important dans l'évolution du métabolisme des LT CD8+ effecteurs lors de leur transition en cellules mémoire [36, 37]. L'autophagie, dans ce cas, paraît nécessaire au maintien du métabolisme lié à l'oxydation des acides gras, probablement de par le contrôle du bon fonctionnement de l'activité mitochondriale. LT : lymphocyte T ; TCR : T cell receptor.

gène humain de prédisposition à plusieurs maladies auto-immunes, régulait l'activité autophagique du thymus [21]. La diminution de l'expression de *Clec16A* chez la souris réduit l'activité autophagique de l'épithélium thymique. Chez la souris NOD (*non-obese diabetic*) qui développe un diabète auto-immun, une baisse de la pathogénicité des LT est observée après inhibition de l'expression de *Clec16A*. La modulation qualitative des peptides présentés lors de l'éducation thymique par l'autophagie permettrait donc de limiter la réponse auto-immune. Ces deux derniers résultats s'accordent pour établir un rôle primordial de l'autophagie du thymus dans la prévention de l'auto-immunité.

Le rôle de l'autophagie dans la survie et l'activation des lymphocytes

Les modèles murins de délétion spécifique de l'autophagie des LT dès la différenciation thymique ont donc permis de mettre en évidence des anomalies liées à la charge en mitochondries. De fait, en absence d'autophagie, les LT matures présentent des défauts de survie à l'état basal, étant plus sensibles à l'apoptose (*Figure 3*) [10-12, 22, 23]. Après stimulation de leur TCR *in vitro*, ils présentent également une survie et une prolifération défectueuses. De fait, une accumulation du marqueur *light chain* 3-II (LC3-II), présent à la membrane des autophagosomes, est observée dans les LT dont le TCR a été stimulé par des anticorps, ou après traitements pharmacologiques qui activent les voies de signalisation liées à ce récepteur [10]. Plusieurs éléments justifient le rôle de l'autophagie dans l'activation des LT. Tout d'abord, elle permet à la cellule de répondre à la demande d'énergie qui résulte de la stimulation du TCR [24]. En réduisant le nombre de mitochondries non fonctionnelles, elle prévient de plus l'accumulation de ROS qui favorise l'apoptose [11, 12]. L'autophagie régule également le stress du réticulum endoplasmique (RE) en intervenant dans la dégradation sélective de ses membranes [22, 23]. Enfin, elle permet le catabolisme de procaspases désensibilisant ainsi les cellules à l'apoptose [25]. Un rôle de l'autophagie dans la polarisation des cellules T auxiliaires (Th) a également été rapporté [22, 23]. En effet, elle apparaît plus importante pour la survie des cellules Th1, Th2 et Th0 que pour celle des cellules Th17 [26] avec une plus forte induction dans les cellules Th2 que dans les cellules Th1 [26]. Dans tous les cas, des variations de métabolisme et de sensibilité à l'apoptose entre les différents types de cellules Th pourraient être à l'origine de ce besoin différentiel en autophagie.

Comme pour les LT, les premières études s'intéressant à l'autophagie dans les LB ont montré une induction de la formation d'autophagosomes après stimulation de leur

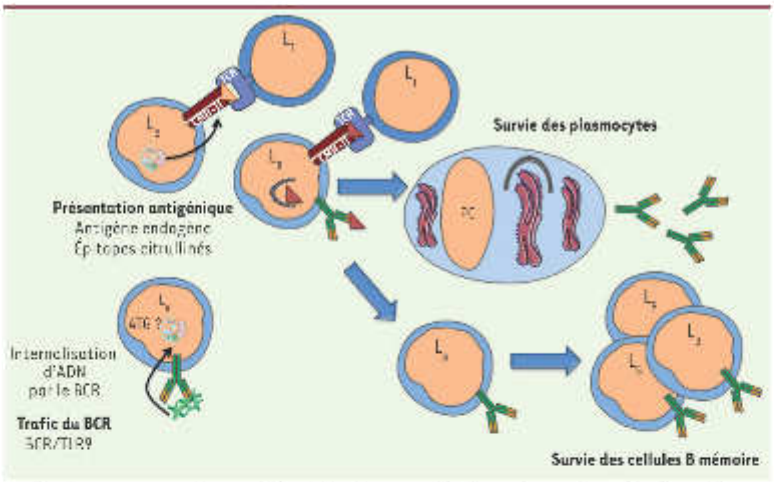

Figure 4. *Implication de l'autophagie dans les lymphocytes B.* Le BCR (*B cell receptor*) internalisé colocalise avec des structures à morphologie autophagique, dans ce cas l'autophagie ou sa machinerie est requise pour la translocation de l'antigène vers des compartiments contenant le TLR9 (*toll like receptor 9*) [34]. De plus, l'autophagie dans les LB est nécessaire à la présentation d'épitopes endogènes [31] ou citrullinés provenant d'antigènes internalisés par le BCR [29]. L'activité autophagique augmente pendant la différenciation plasmocytaire. L'autophagie est nécessaire à la survie des plasmocytes [39], en régulant la quantité de réticulum endoplasmique (RE) [31]. L'autophagie peut également influencer la survie des LB mémoire et permettre la génération d'une réponse secondaire efficace [32]. L'autophagie n'est pas requise lors de l'initiation de la génération des LB mémoire mais pour leur maintien à long terme [31]. ATG (*autophagy related genes*) ; CMH-II : complexe majeur d'histocompatibilité de classe II ; PC : plasmocyte.

récepteur à l'antigène, le BCR (*B cell receptor*) [27]. Cette activation a cependant été remise en question dans une étude plus récente [28] dont les résultats on été confirmés [29]. Quoi qu'il en soit, les données obtenues dans les modèles d'étude *in vivo* chez la souris ont conduit à une même conclusion : l'autophagie est peu impliquée dans la survie des LB en périphérie (à l'exception des cellules B-1a du péritoine) et dans leur activation initiale en réponse à la stimulation de leur récepteur (*figure 4*) [14, 30, 31]. Il est en revanche probable qu'elle contribue à l'activation des LB dépendant des LT au cours de la présentation antigénique. L'autophagie favorise en effet la présentation d'antigènes cytosoliques du soi [32] ou d'antigènes viraux intracellulaires par le CMH-II [33]. Si aucune étude n'a montré la pertinence de ces observations *in vivo*, il apparaît néanmoins possible que l'autophagie, par la présentation antigénique aux LT CD4$^+$ spécifiques d'antigènes intracellulaires, contribue à l'activation de LB autoréactifs ou spécifiques d'antigènes viraux. Cependant, des études récentes ont montré qu'elle n'était pas nécessaire à la présentation antigénique *in vivo*. En effet, la formation des centres germinatifs, après immunisation par des antigènes modèles ou lors d'une infection par le virus de la grippe, apparaît normale dans des souris dont les LB sont déficients en autophagie [30, 31]. Il faut cependant noter que dans le modèle d'infection par le virus de la grippe, l'antigène présenté ne peut être totalement qualifié d'endogène ou d'exogène, les antigènes viraux pouvant provenir soit de particules virales capturées par le BCR, soit du cytosol de la cellule infectée. La notion selon laquelle l'autophagie n'exercerait aucun rôle dans les étapes initiales de l'activation des LB nécessite donc de nouvelles études pour déterminer son éventuel rôle sélectif dans la présentation d'antigènes endogènes et exogènes.

L'absence d'implication de l'autophagie paraîtrait cependant surprenante. En effet, la machinerie autophagique semble reliée au trafic intracellulaire du BCR qui, après internalisation, peut être colocalisé avec des structures à morphologie autophagosomale [34] et avec des molécules de la machinerie autophagique [29]. Cette convergence entre le trafic du BCR et les protéines impliquées dans l'autophagie permet sa translocation vers des compartiments cellulaires où est exprimé le récepteur de type Toll (*toll-like receptor*) TLR-9, un récepteur de l'immunité innée spécifique de l'ADN. Son interaction avec des antigènes contenant de l'ADN permettrait ainsi d'abaisser le seuil d'activation des LB après leur reconnaissance par le BCR. L'autophagie participe également à la citrullination[1] des antigènes qui ont été internalisés par le BCR [29] ; les antigènes reconnus par le BCR étant transportés par la machinerie autophagique vers des compartiments cellulaires contenant la peptidyl arginine déiminase, l'enzyme responsable de la citrullination. Un rôle majeur de protéines de l'autophagie a également été mis en évidence dans certaines formes d'endocytose, comme la phagocytose assistée par LC3 (*microtubule-associated protein light chain-3 [LC3]-associated*

[1] La citrullination ou déimination est une conversion d'arginine présente dans une protéine en citrulline. Les peptidyl arginine déiminases (PAD) remplace le groupement (=NH) de l'arginine par une cétone (=O).

phagocytosis) (LAP) dans laquelle la protéine autophagique LC3 est associée aux membranes des phagosomes. Elles pourraient participer à la présentation d'antigènes d'origine extracellulaire [45].

L'autophagie et la fonction effectrice des lymphocytes

L'importance de l'autophagie dans la survie à long terme des lymphocytes a été mise à jour par deux études récentes. Les équipes de Simon et d'Ahmed ont en effet montré que l'autophagie était indispensable au maintien des cellules T CD8⁺ mémoire (*Figure 3*) [36, 37]. Afin de résoudre les problèmes rencontrés dans les modèles que nous avons décrits plus haut pour la survie basale des LT déficients en autophagie, Ahmed *et al.* ont utilisé un modèle permettant l'inactivation de l'autophagie au seul stade effecteur des cellules T CD8⁺. De manière étonnante, dans ce modèle, l'autophagie n'a qu'un faible impact lors d'une première stimulation antigénique *in vivo* dans les premières heures d'activation en réponse à la signalisation du TCR, suggérant que l'accumulation du marqueur LC3-II qui avait été observée dans les précédentes études était en fait due à un blocage de la dégradation des autophagosomes plutôt qu'à une activation de l'autophagie. Des études sont donc encore nécessaires pour comprendre comment l'activation du TCR peut moduler la balance entre génération d'autophagosomes et dégradation lysosomale. En revanche, dans ces études *in vivo*, une induction nette de l'activité autophagique a été observée au moment de la transition des cellules vers le phénotype mémoire [37]. Il est ainsi possible que l'autophagie soit particulièrement importante lorsque les LT passent d'un métabolisme, dans les premières heures d'activation, qui ne sollicite pas la machinerie autophagique mais repose sur la glycolyse, à d'autres sources d'énergie. Ainsi, dans des cellules à longue durée de vie comme les cellules mémoires, l'autophagie permettrait le maintien d'une activité optimale de la phosphorylation oxydative, source principale d'énergie à ce stade de différenciation. Cette hypothèse est soutenue par une étude récente montrant l'implication des ROS dans la survie limitée de lymphocytes T CD8⁺ mémoires déficients pour l'autophagie [38]. En effet, l'autophagie permet le maintien d'une population de mitochondries fonctionnelles. Elle limiterait également l'accumulation d'agrégats protéiques et favoriserait de ce fait la survie à long terme des cellules mémoire.

L'autophagie est également importante dans la différenciation des LB en cellules effectrices. En effet, Pengo et ses collaborateurs ont montré que, bien qu'elle ne soit pas requise pour l'activation initiale des LB, l'autophagie permet la survie des plasmocytes (*Figure 4*) [38, 39]. Dans ces cellules, l'activité autophagique permet de limiter la quantité de réticulum endoplasmique, et donc le stress qui est lié à son accumulation dans la cellule. L'inactivation de l'autophagie dans les LB conduit à une diminution des sécrétions d'immunoglobulines IgM et IgG après immunisation ou infection. De même, la survie à long terme des plasmocytes à longue durée de vie dans la moelle osseuse nécessite une activité autophagique. L'autophagie semble également importante pour la survie des LB mémoires. Ainsi, lors d'une immunisation ou d'une infection par le virus de la grippe, l'autophagie est nécessaire pour obtenir une réponse secondaire [38]. L'activité autophagique s'avère donc indispensable, non pour la différenciation des cellules mémoires, mais pour leur survie [40]. Cependant, le mécanisme par lequel l'autophagie favorise la survie des LB mémoire n'est pas encore élucidé, mais il est possible qu'il soit similaire à celui observé dans le cas des LT.

Autophagie des lymphocytes et physiopathologie

L'importance de l'autophagie dans l'homéostasie lymphocytaire suggère qu'une dérégulation de ce processus pourrait entraîner des dysfonctionnements majeurs de l'immunité. Des facteurs génétiques ou environnementaux modifiant l'activité autophagique pourraient ainsi être impliqués dans certaines pathologies. La première étude s'intéressant à l'activité autophagique des lymphocytes lors de dysfonctionnements immunitaires décrit une augmentation de l'expression de la protéine ATG5 dans les LT qui infiltrent les lésions observées dans la sclérose en plaques (SEP) [41]. L'absence d'autophagie dans les LT diminue ainsi les symptômes de la pathologie dans un modèle murin de SEP [25], l'amélioration clinique étant liée à une baisse du nombre de lymphocytes de type Th1. Dans une pathologie auto-immune systémique – le lupus érythémateux disséminé (LED)[3] –, l'identification de polymorphismes génétiques dans la région du gène *ATG5*, corrélés avec le développement de la pathologie, a conduit à s'interroger sur l'implication de l'autophagie [42]. Même si la signification fonctionnelle de ces polymorphismes est encore peu documentée, des auteurs ont proposé l'existence d'une corrélation entre une des variantes de la maladie et une plus forte expression transcriptionnelle d'*ATG5* [43]. Un des polymorphismes d'*ATG5* est également associé au développement de l'asthme, une autre pathologie caractérisée par une hyperactivation du système immunitaire [44]. Il est intéressant de noter que des molécules qui impactent l'activité autophagique sont utilisées pour le traitement du LED. C'est le cas de l'hydroxychloroquine, utilisée en routine, ou la rapamycine et le peptide P140 [45, 46] qui sont en essais cliniques. De plus, l'autophagie est dérégulée dans les LT, dans des modèles murins de lupus, et chez les patients atteints de LED [28, 47-49]. Les LT de ces patients présentent une charge en autophagosomes plus importante que des LT de sujets sains [47]. Cependant, l'origine et la signification physiopathologique de cette dérégulation ne sont pas connues, mais

[3] Le LED est caractérisé par la production d'autoanticorps dirigés contre différents constituants nucléaires, qui se déposent sous forme de complexes immuns et engendrent une inflammation dans de nombreux tissus comme les reins, la peau, les vaisseaux sanguins et le système nerveux central.

Il est possible que l'activation de l'autophagie favorise l'émergence et la survie de LT autoréactifs, qui associés aux LB induirant leur différenciation en cellules productrices d'autoanticorps. Il est également possible que l'accumulation d'autophagosomes soit liée à un blocage partiel de leur dégradation par les lysosomes. Dans ce cas, le déficit en activité autophagique sensibiliserait les LT à l'apoptose. Cette propension à l'apoptose des LT est caractéristique du LED *in vitro*. Elle pourrait résulter de l'activation chronique subie par ces cellules *in vivo* et favoriser l'accumulation d'autoantigènes dans la circulation et dans les tissus. Un déficit de l'autophagie a également été observé dans les LT de patients atteints de polyarthrite rhumatoïde (PR), une autre pathologie auto-immune systémique. Dans ce cas, la baisse de l'activité autophagique accroît la mort cellulaire des LT [50]. Il apparaît important de déterminer l'impact exact de la dérégulation de l'autophagie des LT lors des pathologies auto-immunes afin de mieux comprendre ses conséquences.

De manière intéressante, une étude suggère qu'une modification de l'activité autophagique des LB favorise l'auto-immunité. Un accroissement de l'activité autophagique dans les LB est en effet observé au cours du lupus [28], ce qui favoriserait la survie des LB autoréactifs mais également des plasmocytes, producteurs des autoanticorps. L'activité autophagique des LB pourrait également participer à la physiopathologie de la PR en favorisant la présentation d'épitopes citrullinés aux LT, ce qui entraînerait la production d'anticorps dirigés contre des structures déiminées [29]. De tels anticorps sont fréquemment retrouvés au cours de cette pathologie.

Conclusion

Les études récentes ont montré un impact crucial de l'activité autophagique dans toutes les phases de la vie des lymphocytes, du développement aux phases effectrices. Les modèles d'étude murins n'ont pas toujours permis de définir clairement ces rôles et d'autres études sont nécessaires afin d'affiner nos connaissances. Il reste ainsi à définir le rôle précis de l'autophagie dans le développement des LB. De plus, les causes de la dérégulation de l'autophagie des lymphocytes observée dans plusieurs pathologies comme le lupus ne sont pas encore expliquées. L'origine de ces anomalies pourra être mieux appréhendée lorsque les mécanismes de modulation de l'autophagie par la signalisation des récepteurs à l'antigène, des récepteurs de l'immunité innée et des cytokines seront mieux documentés. Enfin, le rôle, peut-être non strictement lié, de l'autophagie dans le trafic intracellulaire permettant l'activation des LB, et important dans la présentation antigénique devra faire l'objet de nouvelles recherches. Mieux comprendre l'impact d'une modification ciblée de l'autophagie des lymphocytes pourrait permettre d'éliminer spécifiquement les cellules effectrices et mémoire, afin de limiter leur impact délétère lors des pathologies auto-immunes. À l'inverse, activer l'autophagie des lymphocytes lors des vaccinations pourrait permettre d'améliorer leur efficacité, qui repose largement sur la génération et la survie des cellules mémoire. ◊

SUMMARY

Autophagy in T and B cell homeostasis: recycling for sustainable growth

Macroautophagy often abbreviated by "autophagy" is an intracellular degradation mechanism linked to lysosomal activity. Autophagy is conserved from yeast to mammals and plays a role in the response to energetic stress and in organelle homeostasis. Autophagy is also involved in the regulation of immunity, in particular in the adaptive immune response, which involves B and T lymphocytes. It was indeed shown that autophagy impacts the development of B and T cells as well as the education of T cells in the thymus. Autophagy also modulates activation, survival and polarization of T cells. It plays a role in antigen presentation by B cells, and in their TLR-mediated activation, and thus likely in their initial activation. Finally, autophagy is required for the survival of memory lymphocytes and effector cells like antibody-producing plasma cells. Interestingly, autophagy is deregulated in several autoimmune pathologies. The modulation of this phenomenon could possibly lead to new treatments aiming at limiting lymphocyte activation driving these pathologies. ◊

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Etude de l'autophagie des lymphocytes dans les réponses normales et autoimmunes

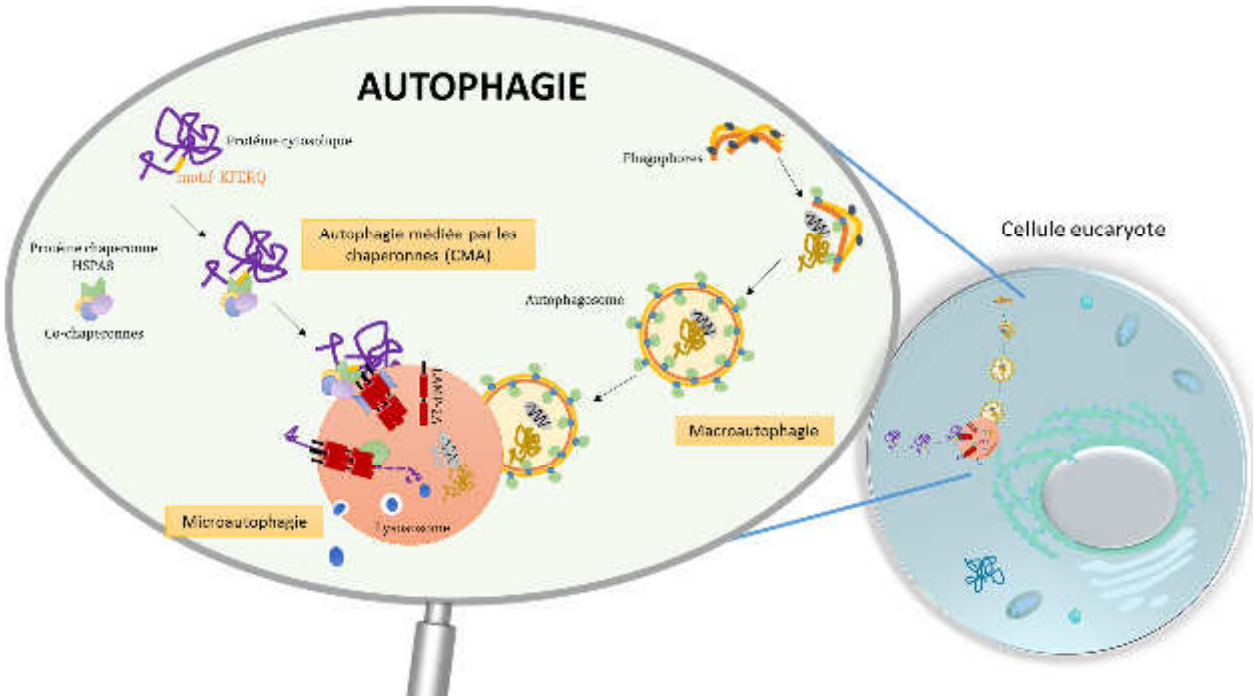
Le terme « autophagie » vient du grec et signifie se manger soi-même. Il a été introduit dans les années 1960 par Christian de Duve, un chercheur belge à l'origine de la découverte des lysosomes. L'autophagie désigne en effet la digestion du contenu intracellulaire en contradiction avec « l'hétérophagie » qui implique une dégradation de matériel extracellulaire via le lysosome.

Depuis les années 60 la recherche sur l'autophagie n'a pas cessé de croître et a permis d'identifier l'implication de ce processus dans des fonctions cellulaires diverses et variées. Les premières investigations ont surtout montré que l'autophagie joue un rôle important dans le métabolisme mais également dans le maintien de l'homéostasie cellulaire. Au fur des années il est devenu de plus en plus évident que l'autophagie est effectivement un mécanisme de survie étant donné qu'il contribue à l'élimination de protéines agrégées et d'organites dysfonctionnelles, protégeant ainsi la cellule de la toxicité cellulaire que de tels matériaux pourraient engendrer. De plus dans des conditions de déficit nutritionnel l'autophagie permet dans un processus catabolique, la dégradation de macromolécules cytoplasmiques dans le but de générer de nouveaux métabolites.

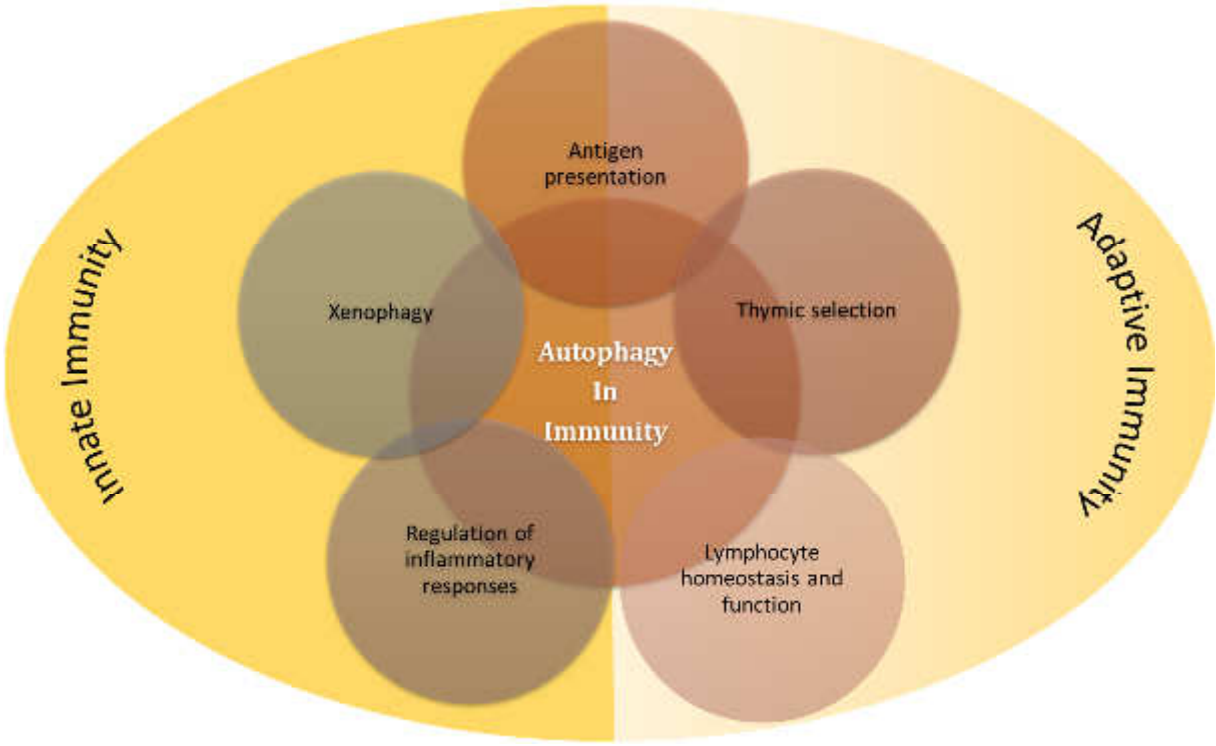
En ce qui concerne la mécanistique de ce processus, on distingue trois types d'autophagie : la microautophagie, l'autophagie médié par les chaperonnes (CMA) et la macroautophagie. Les deux premiers types d'autophagie ont été identifié comme étant impliqué dans l'élimination de petits constituants cytosolique par invagination direct du lysosome pour la microautophagie et par la reconnaissance spécifique de protéines via un motif peptidique KFERQ par un complexe protéique contenant la chaperonne HSPA8 pour la CMA respectivement. Néanmoins, la macroautophagie est le mécanisme le plus étudié. De ce fait dans la littérature il couramment désigné plus simplement par le terme « autophagie ».

Des trois types d'autophagies, la macroautophagie est également celle qui est la plus facile à identifier étant donné que son initiation conduit à la formation d'une vésicule à double membrane appelé autophagosome. En effet la dissection des mécanismes moléculaires a abouti à l'identification des différentes étapes nécessaires à la mise en place de ce processus mais également à l'isoler les protéines impliquées. Ainsi il a pu être montrer que l'autophagie

débute par l'initiation suivi par la nucléation conduisant à la formation de structures pré-autophagiques appelées phagophores. Cette étape est suivie par l'élongation des phagophores aboutissant à la formation de l'autophagosome puis leur maturation et leur fusion avec les lysosomes. Les protéases lysosomales sont responsables de la dégradation du contenu cytsosolique qui a été capturé au préalable pendant la phase d'élongation. L'étape finale correspond à la libération du matériel dégradé dans le cytosol.



L'autophagie joue un rôle indéniable et essentiel dans l'homéostasie cellulaire. Néanmoins, l'étude de ce processus dans différents tissus et conditions a permis la mise en évidence de variations dans les fonctions selon les systèmes étudiés. Ainsi dans l'immunité innée et adaptative, l'autophagie a émergé comme un acteur clé dans divers mécanismes tels que l'élimination de pathogènes, la régulation de l'inflammation et la présentation antigénique.



La clairance d'agents pathogènes (virus, bactéries, parasites) par le biais de l'autophagie est appelé xénophagy et appartient aux formes sélectives de l'autophagie. Ce processus a par exemple été montré comme étant impliqué dans la dégradation de *Streptococcus pyogenes* également connu sous le nom streptocoque du groupe A (GAS). L'infection de cellules HeLa par du GAS conduit à l'endocytose des bactéries qui se retrouve dans le cytosol dans des compartiments LC3 positif. Nakagawa et ses collègues ont nommé ces compartiments GcAVs, pour « GAS-containing LC3-positive autophagosome like vacuoles ». Ainsi ils ont pu montrer que les GcAVs fusionnent avec les lysosomes environ 4 heures après l'infection ce qui est corrélé avec une diminution intracellulaire de GAS suggérant l'implication de l'autophagie dans la clairance de Streptococcus. De plus, dans des cellules souches embryonnaires (cellules ES) et des MEFS déficients pour ATG5 infectées par GAS il n'y a pas de détection de ces GcAVs et l'inhibition de l'autophagie par les protéases lysosomales ont donné lieu à une augmentation de GAS intracellulaire (Nakagawa et al., 2004).

Dans l'ensemble, l'autophagie est capable de piéger l'agent pathogène soit directement dans le cytosol ou dans les vacuoles et d'induire leur dégradation ou d'inhiber la croissance bactérienne.

Cependant, d'autres agents pathogènes utilisent ce mécanisme pour leur avantage et détournent la machinerie autophagique afin de permettre leur maintien dans la cellule.

Staphylococcus aureus et *Serratia marcescens,* font parties de la catégorie de bactéries qui ont la capacité d'induire la formation d'un autophagosome mais inhibent la fusion avec les lysosomes, ce qui leur permet d'utiliser ces membranes autophagiques pour leur réplication. *S. aureus* est en effet capable de détourner cette voie, d'abord en se répliquant à l'intérieur des phagosomes pour ensuite induire une mort cellulaire dépendante de l'autophagie, comme ont pu le montrer Schnaith et collèques dans des MEFs ATG5 - / - (rieviewed dans Huang et Brumell, 2014) (Schnaith et al., 2007).

Pour monter une réponse appropriée contre les agressions extérieures, les eucaryotes supérieurs exigent la diaphonie entre l'immunité innée et adaptative. Alors que le système immunitaire inné reconnaît les PAMP via les PRR et initie le contrôle des pathogènes, la clairance totale et la protection à long terme nécessite des lymphocytes. Ces acteurs, les lymphocytes B et T, expriment des récepteurs d'antigènes variables, le récepteur des cellule B (BCR) et un récepteur des lymphocyte T (TCR) respectivement, qui leur confèrent la capacité de reconnaître pratiquement n'importe quel motif antigénique. Il a été suggéré que l'autophagie fait partie intégrante de l'immunité adaptative.

Étant donné que les peptides présentés par le CMH I sont le résultat du traitement du protéasome, il apparaît surprenant d'attendre l'implication de l'autophagie dans ce processus. En effet, seules quelques études suggèrent un lien direct entre l'autophagie et la présentation antigénique via le MHC I. Le groupe de Michel Dujardin fait partie de ceux qui ont décrit une présentation de protéines endogènes via CMH I dépendant de l'autophagie en utilisant un antigène de l'herpès simplex 1 (HSV1) comme modèle d'infection chez les macrophages. Ils suggèrent en effet une voie vacuolaire, en contraste à la voie classique protéasomale, pour la présentation de l'antigène endogène (English et al., 2009). D'autre part Li et ses collègues ont observé une augmentation de la présentation par le CMH I dans des macrophages déficients pour l'autophagie ainsi que la diminution des molécules du CMH I à la surface cellulaire après le traitement de la rapamycine, ce qui suggère une régulation négative de la présentation par le CMH I par la machinerie autophagique.

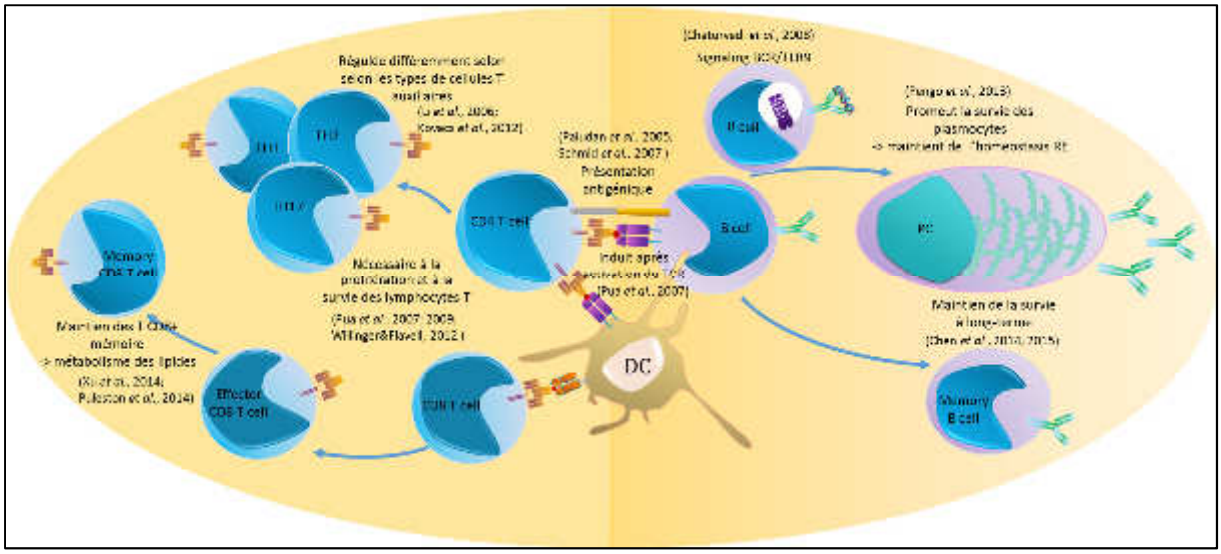
L'implication de l'autophagie dans la présentation du CMH II est par contre mieux documentée

que pour le MHC I. Le traitement de l'antigène dans le cas de présentation par le CMH II est en effet dépendant de la voie endosomal/lysosomale, ce qui rend le lien avec autophagie plus évident. Malgré que les molécules du CMH II soient principalement dédiées à la première présentation d'antigènes exogènes, elles se sont aussi révélés présenter des peptides d'origine endogène. Pareil que pour la présentation croisée via CMH I dans les DCs, autophagie semble être fortement impliqué dans ce processus. La première preuve de la présentation d'antigène endogène par le MHC II aux cellules T CD4+ a été donnée par Jaraquemada et ses collègues dans les années 1990 grâce à leurs études sur le traitement de l'antigène dans des cellules infectées par la vaccine (Jaraquemada et al., 1990). L'implication de l'autophagie dans ce processus a cependant été vraiment démontré quelques années plus tard par Paludan et ses collègues. Ils ont observé dans une lignée de cellules lymphoblastoïdes transformées par EBV, que la protéine virale du virus d'Epstein Barr 1 (EBNA 1), provoque l'inhibition de l'acidification des lysosomes, conduisant ainsi à l'accumulation d'autophagosomes. En outre l'inhibition de l'autophagie tant par des inhibiteurs pharmacologiques (3-MA) et que par la sous-expression d'un gène essentiel de l'autophagie (ATG12) a donné lieu à une baisse de l'activation des lymphocytes T CD4 par des cellules B transformées par EBV. En revanche l'inhibition du protéasome n'a eu aucun impact significatif sur la présentation de l'antigène et l'activation des cellules CD8 T n'a également pas été affectée. Ces résultats ont abouti à la conclusion que la présentation d'antigènes endogènes par le CMH II est en effet possible et qu'elle nécessite l'autophagie pour le traitement des antigènes (Paludan et al., 2005).

Comme mentionné dans les sections précédentes, l'autophagie joue un rôle important dans le renouvellement des protéines et organites. En tant que tel il est activé à un niveau de base dans la plupart des cellules, y compris dans les lymphocytes et est fortement impliquée dans le maintien de leur homéostasie. Les lymphocytes dépendent de l'autophagie à divers niveaux. Les cellules T ont été décrites comme dépendente de l'autophagie pour leur développement, ainsi que pour leur survie après l'activation et la polarisation, tandis que les cellules B semblent avoir besoin de ce processus pour leur développement et pour leur maintien après leur différenciation en plasmocytes.

Les informations sur l'implication de l'autophagie dans la biologie des lymphocytes ont été obtenus principalement en utilisant des modèles de souris avec une délétion conditionnel de ce dernier soit dans les cellules T ou B, ou les deux, ou en utilisant des souris chimériques. En

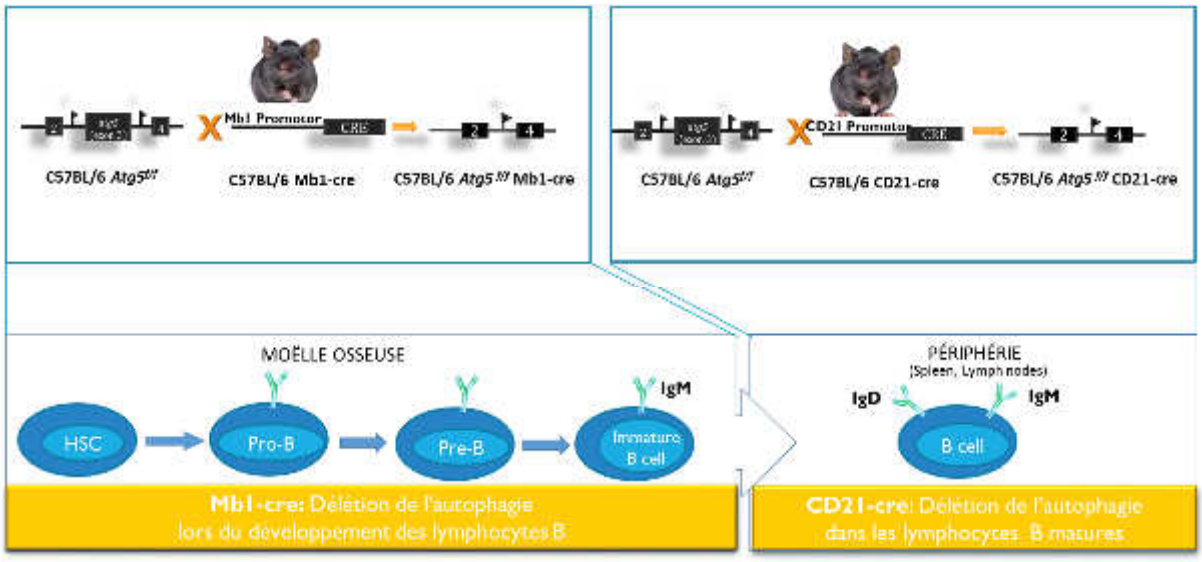
effet, la délétion totale de l'autophagie n'a pas d'impact sur le développement embryonnaire, mais est létale après la naissance (Kuma et al., 2004). Ainsi, les informations que nous possédons aujourd'hui sur le rôle de l'autophagie *in vivo* proviennent de souris modèles ayant subi une délétion de l'autophagie spécifique d'un tissu ou d'un type cellulaire.



Ces modèles de délétions ont permis de répondre à un certain nombre de questions sur le rôle de ce processus dans la biologie des lymphocytes. Mais jusqu'à très récemment l'invalidation de l'autophagie dans la plupart des modèles apparaissait au début du développement lymphocytaire. Or, il s'est avéré que l'autophagie joue un rôle central dans la génération de cellules progénitrices hématopoïétiques. Dans ces conditions, il est possible que les observations faites jusqu'à présent sur la survie des lymphocytes soient liés à un défaut précoce dans le développement. Ainsi, nous avons été particulièrement intéressés par l'étude du rôle de l'autophagie dans les lymphocytes matures *in vivo*. En effet, seules quelques recherches se sont Intéressaient aux réponses immunitaires *in vivo* en l'absence d'autophagie dans ces cellules.

Nos premières investigations ont conduit à la génération de deux modèles de souris avec une délétion spécifique dans des cellules B immatures et matures (Atg5 f/- Mb1-cre et Atg5 f/- CD21-cre). Nous avons pu montrer que l'autophagie est dispensable dans le développement précoce des cellules B. Nous avons également pu observer que les souris déficientes pour l'autophagie dans les cellules B périphériques (Atg5 f/- CD21-cre) étaient encore capables de générer une réponse humorale antigène spécifique à court-terme. Cependant lors de l'étude

des réponses humorales à long-terme dans C57BL/6 lpr/ lpr Atg5 f/- CD21-cre possédant un fond génétique autoimmun, nous avons pu remarquer que la réponse IgG anti-ADN double brin été détériorée. Ainsi, il apparaît qu'en l'absence d'autophagie, la survie des plasmocytes à long terme est compromise et conduit à une diminution de la sécrétion d'anticorps, en particulier dans un contexte auto-immun.



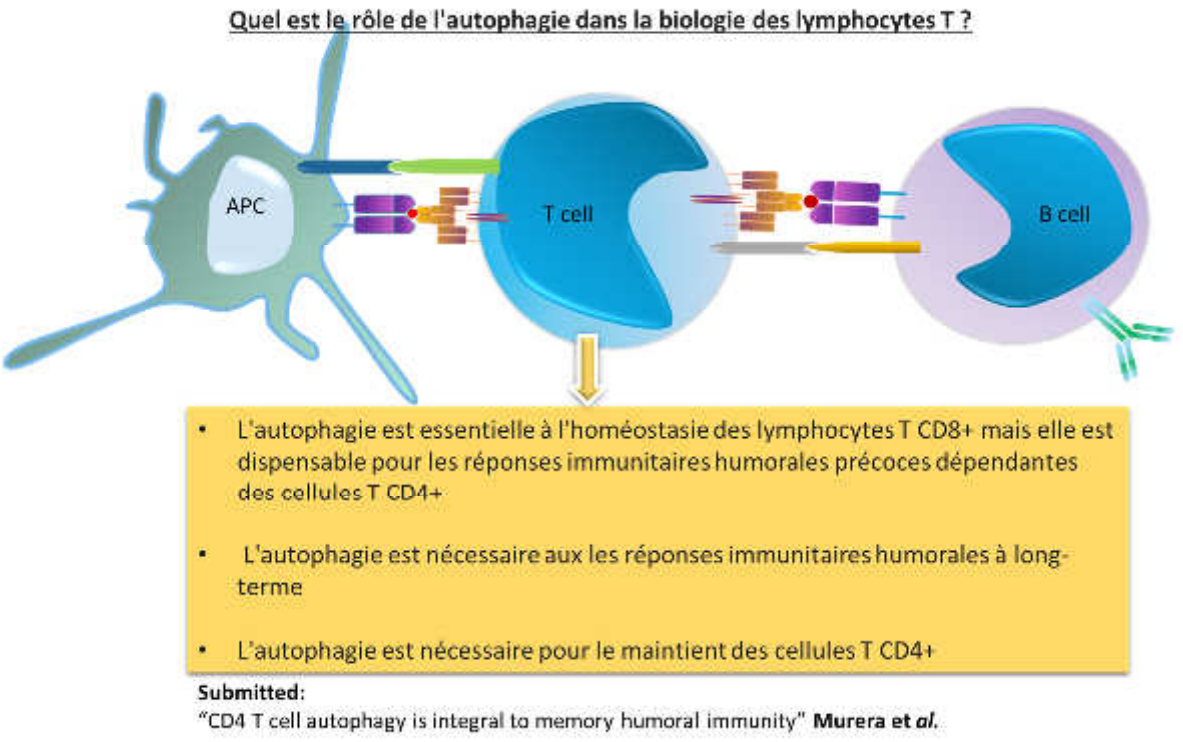
Mon projet de doctorat a été particulièrement axé sur l'étude du rôle de l'autophagie dans les cellules T avec deux principaux objectifs :

1) Nous avons d'abord voulu établir le rôle de l'autophagie dans les réponses humorales T dépendantes, afin de mieux comprendre comment, quand et pourquoi ce processus est important pour ces cellules.
2) Deuxièmement, nous avons voulu délimiter par quelle(s) voie(s) de signalisation l'autophagie est induite dans les cellules T, à la fois dans un contexte normal et un contexte lupique.

Pour traiter le premier point, nous avons généré des souris transgéniques déficientes pour l'autophagie uniquement dans les cellules T matures. Nous avons évalué la fonction des cellules T de ces souris *ex vivo* et avons pu observer une survie et une prolifération normale des lymphocytes T CD4+ après activation. Nous avons immunisé nos modèles de souris avec un antigène T dépendant, l'Ovalbumine, et nous avons remarqué que la réponse immunitaire primaire et même secondaire était normale en amplitude, mais que la réponse humorale à long terme elle été altérée. Ceci nous a amené à la conclusion que la réponse mémoire

pourrait être affectée par l'absence d'autophagie dans les lymphocytes T. Afin d'évaluer si le besoin de l'autophagie était intrinsèque aux cellules T CD4+ mémoires, nous avons transféré des cellules T CD4+ incompétentes pour l'autophagie dans des souris receveuses à partir de souris hôtes immunisées, qui ont ensuite été immunisées avec le même antigène.

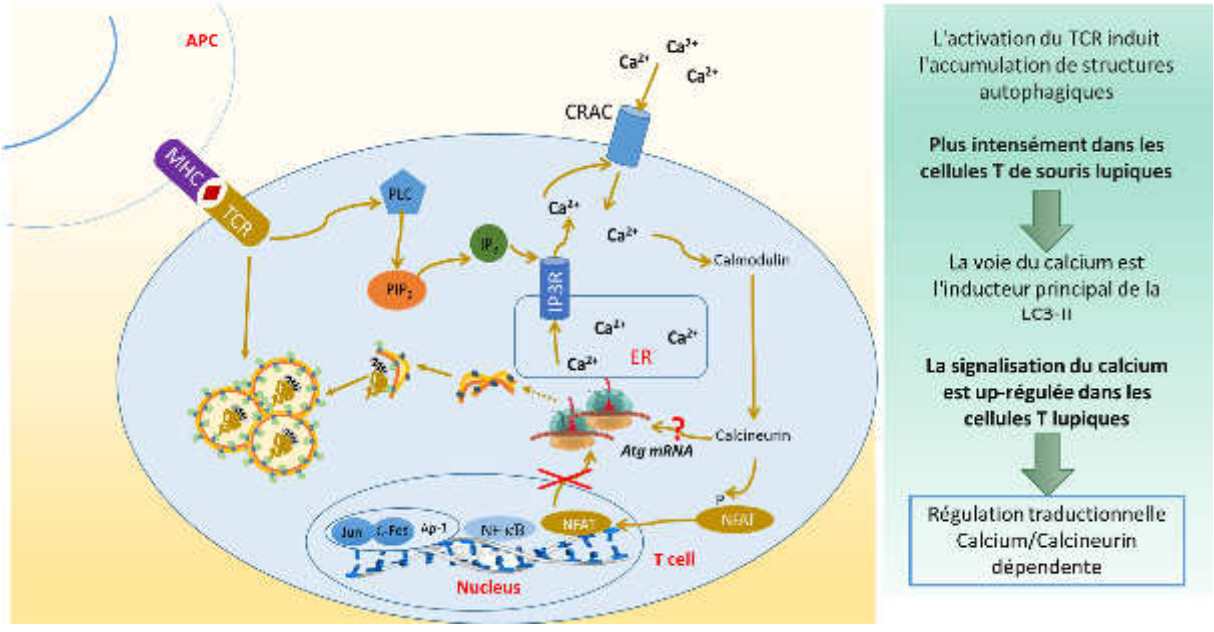
Par rapport aux contrôles, leur réponse humorale mémoire était en effet plus faible. Nous avons par ailleurs montré que l'absence d'autophagie a un impact sur la survie des lymphocytes T CD4 de mémoires, en induisant une mémoire dans les cellules T CD4+ *in vitro*. Nous avons constaté qu'à long terme, les cellules T déficientes pour l'autophagie présentaient un défaut de survie sous traitement à IL-7. Il semble donc que l'autophagie est essentielle à la fonction normale des cellules T mémoires.



Ma deuxième préoccupation était de mieux comprendre les voies de signalisation conduisant à l'induction de l'autophagie après stimulation du récepteur des cellules T (TCR). Nous avons activé ou inhibé les voies principales induites après l'activation du TCR, afin d'évaluer leur impact sur l'activité autophagique. Pour le moment nous avons ont pu montrer que la voie du calcium est principalement impliquée dans l'induction de ce processus. Cela nous a amené à

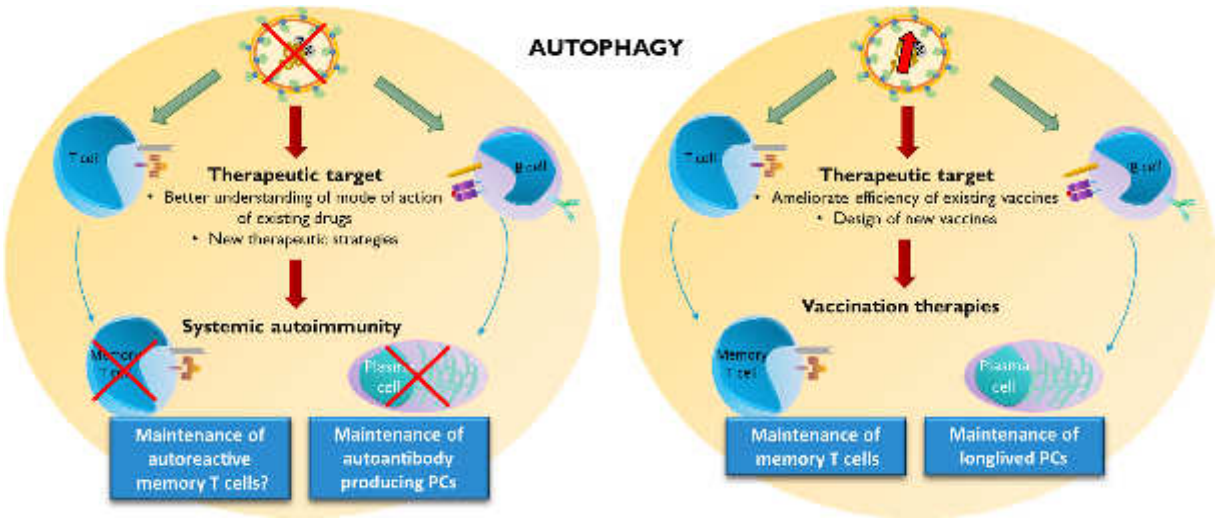
penser qu'en aval de la voie Ca2+, le facteur de transcription nucléaire des cellules T activées (NFAT) pourrait être impliqué dans la régulation de certains gènes liés à l'autophagie (les *Atg*). Cependant, les expériences de PCR quantitative n'ont pas confirmé cette hypothèse. Nous avons donc commencé à explorer une possible régulation post-transcriptionnelle ou post-traductionnelle de protéines ATG par l'activation de la voie calcique. Nous nous sommes concentrés plus spécifiquement sur la régulation de la protéine de la chaîne légère 3 associé aux microtubules (LC3). L'expression de cette protéine ATG varie en effet le plus dans nos conditions lorsque la voie Ca2+ est stimulée ou inhibée.

Les résultats préliminaires obtenus en utilisant des inhibiteurs pharmacologiques de la transcription et de la traduction indiquent que la régulation pourrait se produire au niveau de la traduction. Nous avons l'intention de poursuivre cette voie dans le but d'identifier les acteurs impliqués dans ce règlement.



L'étude du rôle de l'autophagie dans les lymphocytes mémoires dans le maintien d'une maladie auto-immune pourraient conduire à la conception de nouveaux médicaments ciblant spécifiquement ces cellules mémoires, contribuant ainsi à la mise au point de traitements plus précis et diminueraient les effets secondaires. En effet il semble que l'autophagie n'est pas essentielle dans les réponses lymphocytaires précoce, de ce fait inhiber ce processus

n’affecterait pas leur fonction primaires et secondaires contrairement aux médicaments immunosuppresseurs. Autophagie, à cet égard est un bon candidat. Néanmoins, cibler le compartiment mémoire n'est bien sûr pas totalement inoffensif. Il est possible en effet que les patients soient plus sensibles aux infections à la suite d’un tel traitement. Mais étant donné que les patients atteints de lupus sont souvent jeunes et ils auraient la possibilité de reconstituer un compartiment mémoire compétent après le traitement. Toutefois, afin d'évaluer l'impact de l'autophagie des lymphocytes T dans les paramètres auto-immuns, il nécessiterait, comme pour l'étude sur les lymphocytes B, la génération de souris transgéniques avec une délétion spécifique de l’autophagie dans les cellules T. Cela pourrait donner un premier aperçu sur l’éventuelle implication de l’autophagie au niveau auto-immun. Dans un autre contexte, induire l’autophagie pour conduire à l’amélioration de l’efficacité de certains vaccins étant donné que cela contribuerait à un maintien des cellules mémoires.



Diane MURERA

Study of lymphocyte autophagy in normal and autoimmune responses

Résumé

L'autophagie est un processus catabolique lié aux lysosomes, essential à la l'homéostasie cellulaire notamment dans les lymphocytes. Elle est impliquée dans la pathogenèse de nombreuses maladies et pourrais jouer un rôle dans le développement de maladies auto-immunes. Nous avons voulu étudier son rôle in vivo dans les lymphocytes B et T. Nous avons généré des souris déficientes en autophagie spécifiquement dans ces cellules et montré que l'autophagie n'est pas essentiale au développement des LB, mais que dans un contexte auto-immun la persistance de plasmocytes et la production d'autoanticorps été diminuée. Cela démontre un rôle de l'autophagie dans les réponses à long terme. Les réponses humorales à long-terme T dépendantes sont également impactées. De plus des souris transplantées avec des LT CD4+ déficients en autophagie montrent une réponse humorale mémoire diminuée. Nous nous sommes également intéressé aux voies de signalisation conduisant à l'induction de l'autophagie en réponse à une stimulation du TCR dans des LT normaux et pathologiques. Nos résultats préliminaires montrent une implication de la voie calcique.

Mots clés : Macroautophagie, lymphocytes T et B, mémoire, autoimmunité, lupus, modèles murins

Résumé en anglais

Autophay is a catobolic lysosomal process essentail for cellular maintenance and fucntion such as lymphocyte homeosatsis. The generation of mice models with an Atg5 conditional knock-out in B and T cells respectively, have allowed us to study autophagy requirements of those immune cells in vivo. We have demonstrated that autophagy was dispensable for B cell development but that in autoimmune settings B cell autophagy was required for the maintenance of long-lived plasma cells and for the production of autoantibodies. In mice deficient for autophagy in T cells, long-term tumoral response to a T-dependent antigen is decreased. We also showed that in mice adoptively transferred with autophagy deficient CD4 T cells, the antigen specific memory humoral immune response was impaired. We also investigated the signaling pathways leading to autophagy induction upon TCR stimulation in normal and lupus T cells and showed that the calcium signaling is highly involved.

Key Words : Macroautophagy, T and B lymphocytes, memory, autoimmunity, lupus, mouse models