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Étude de la fonction des cellules dendritiques dans la réponse immunitaire cutanée de type 2

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À mes parents

À mes grands-parents

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Résumé de la thèse

Introduction

La dermatite atopique (DA) est l'une des maladies inflammatoires de la peau les plus communes; définie par une inflammation chronique de la peau, un syndrome hyper IgE et une réponse immunitaire de type 2, caractérisée par l'infiltration de lymphocytes Th2, d'éosinophiles, de basophiles et de mastocytes. La prévalence de la DA a augmenté, le nombre d'enfants souffrant d'AD ayant triplé dans les pays industrialisés au cours des trente dernières années, atteignant maintenant 15-30% des enfants et 2-10% des adultes. De plus, la DA peut représenter la première étape d'une progression appelée marche atopique et qui conduit vers d'autres maladies atopiques, telles que l'asthme. Mon laboratoire de thèse a montré précédemment le rôle central de la lymphopoïétine stromale thymique (TSLP), une cytokine exprimée par les kératinocytes de l'épiderme, dans la promotion de la réponse de type 2 et le déclenchement de la pathogenèse de la DA, ainsi que dans la marche atopique, grâce à son rôle crucial dans la sensibilisation aux allergènes dans la peau.

Il a été suggéré, chez souris et chez l'humain, que TSLP exerce ses fonctions sur la différentiation des lymphocytes T auxiliaires par l'intermédiaire des cellules dendritiques de la peau. Toutefois, les cellules dendritiques de la peau forment un ensemble complexe et hétérogène comprenant les cellules de Langerhans (LCs) de l'épiderme, ainsi que les cellules dendritiques (DCs) conventionnelles de type 1 (cDC1), les cDC2 et des DCs dérivées de monocytes dans le derme. Le rôle joué par les différentes populations de cellules dendritiques cutanées dans le cadre de la DA n'a pas encore été élucidé.

Mon travail de thèse s'axe sur deux parties : dans la partie 1, nous étudions le rôle des cellules dendritiques dermiques langerine⁺ et des LCs dans la différentiation des lymphocytes auxiliaires folliculaires (Tfh) dans la pathogenèse de la DA. Dans la partie 2, nous explorons l'expression d'OX40L dans les sous-populations de DCs et la fonction de cette molécule dans la différentiation des lymphocytes T et la réponse immunitaire dans la DA.

PARTIE 1 : le rôle des cellules de Langerhans dans la différentiation des lymphocytes Tfh induite par TSLP dans la dermatite atopique chez la souris

Nous avons dans un premier temps utilisé un modèle établi par le laboratoire, et bien caractérisé, de surexpression de TSLP par les kératinocytes de la peau par application topique de MC903. Dans ce contexte, nous avons démontré une augmentation de la fréquence et du nombre de lymphocytes Tfh, définis comme des lymphocytes CD4$^+$ CXCR5$^+$ PD-1$^+$, ainsi que leur production d'IL-4 chez les souris sauvages traitées au MC903. L'IL-4 est un acteur clé des interactions entre les Tfh et les lymphocytes B des centres germinatifs (GC), et forme un signal de différentiation pour les lymphocytes B. En accord avec ces résultats, la réponse humorale semblait augmentée, les souris traitées présentant un nombre plus élevé de lymphocytes B des centres germinatifs (GC) ainsi que des lymphocytes B IgG1$^+$ ou IgE$^+$. L'ensemble de la réponse Tfh/GC était largement diminué au sein des souris déficientes pour TSLP (*Tslp$^{-/-}$*), démontrant que TSLP promeut la réponse Tfh/GC après traitement au MC903. De façon intéressante, nous avons trouvé une diminution drastique du nombre de lymphocytes Tfh ainsi que leur production d'IL-4 au sein des souris LangDTR, chez qui les LCs et les cellules langerine$^+$ du derme sont supprimées par l'injection de toxine diphtérique. Ceci s'accompagnait également d'un nombre réduit de lymphocytes B des centres germinatifs et IgG1$^+$ après traitement au MC903, par rapport aux souris sauvages. Afin de différentier les cellules de Langerhans des cellules langerine$^+$ du derme, nous avons utilisé deux méthodes pour ne supprimer que les cellules de Langerhans lors de notre traitement : d'une part, le différentiel de temps de reconstitution entre ces deux populations après injection de DT, et d'autre part, l'utilisation de souris human LangDTR, chez qui seules les LCs sont supprimées par injection de DT. Dans ces deux cas, les données suggèrent un rôle crucial des LCs dans la réponse Tfh/GC induite par la surexpression de TSLP.

Nous avons alors souhaité valider l'implication de l'axe TSLP-LCs dans la différentiation des lymphocytes Tfh dans le contexte de la sensibilisation allergique par la peau. Dans ce but, nous avons élaboré un nouveau modèle innovant pour délivrer un allergène, l'ovalbumine (OVA), à des souris dont la barrière cutanée a été détériorée de façon contrôlée et précise à l'aide de microporations par laser afin de mimer la peau défective des patients atteints de DA,. Nous avons montré que le

traitement à l'OVA induit une inflammation allergique cutanée, ainsi qu'une sensibilisation allergique qui peut conduire à la marche atopique, comme observé en clinique. Nous avons montré que la réponse Tfh/GC est abrogée chez les souris *Tslp*$^{-/-}$, indiquant un rôle crucial de TSLP dans la réponse Tfh/GC induite par les allergènes. Toutefois, au contraire de ce que nous avons observé dans le modèle MC903, les souris chez qui les cellules langerine$^+$ ont été supprimées ne présentent pas de diminution de la réponse Tfh/GC, mais une tendance à l'augmentation. Cette augmentation était d'autant plus significative lorsque l'OVA était appliquée sur une peau où la barrière épidermique était détériorée par des microporations plus superficielles, avec une augmentation de la fréquence des Tfh, des lymphocytes B des GC, IgG1$^+$ et IgE$^+$ et des immunoglobulines spécifiques de l'OVA dans le sérum des souris LangDTR. Des données similaires ont été obtenues chez des souris human LangDTR, suggérant que les LCs ne sont pas nécessaires pour l'induction de la réponse Tfh/GC provoquée par la sensibilisation à l'ovalbumine, mais semblent plutôt contrecarrer celle-ci. En outre, la réponse inflammatoire au niveau de la peau montre également une augmentation des infiltrations cellulaires dans le derme, incluant notamment des lymphocytes Th2, des éosinophiles et des basophiles, chez les souris au sein desquelles les LCs ont été supprimées. Ces données suggèrent que les LCs limitent à la fois la réponse Tfh/GC et la réponse allergique inflammatoire dans un contexte de sensibilisation allergique par la peau.

Nos études soulignent donc le double visage des LCs dans deux contextes de pathogenèse de DA dépendante de TSLP, d'une part en induisant la réponse Tfh dans un contexte de DA induite par la surexpression de TSLP et d'autre part en limitant cette réponse dans le cadre de la DA induite par une sensibilisation allergique cutanée. De façon intéressante, ces deux contextes nous fournissent une opportunité parfaite pour étudier au niveau moléculaire l'expression des gènes par les les DCs du ganglion drainant, incluant les LCs, afin de mieux comprendre leur rôle dans la promotion ou la limitation de la réponse Tfh. Pour cela, nous avons utilisé les souris rapportrices Langerine-GFP et avons trié les DC migratoires Langerine-GFP$^+$ du ganglion de souris traitées au MC903, sensibilisées à l'OVA ou non traitées, avant de réaliser une analyse transcriptomique. Nous avons découvert que les cellules Langerine-GFP$^+$ issues de souris traitées au MC903, mais pas LMP/OVA, présentent une augmentation de

l'expression de CD80 et CD86, montrant un état activé de la DC, mais également une induction de l'expression d'IL-6, une cytokine ayant été liée à l'induction de la différenciation des lymphocytes Tfh.

Ces données nous donnent de nouvelles perspectives concernant le rôle des LCs dans la régulation de la réponse Tfh/GC dans la pathogenèse de la DA. Ceci pourrait ouvrir la voie à de nouveaux concepts thérapeutiques, notamment dans les immunothérapies des allergies, ainsi qu'à la vaccination transcutanée, deux domaines au sein desquels la réponse Tfh/GC est d'une importance cruciale.

PARTIE 2 : Etude de l'axe de signalisation TSLP-TSLPR-OX40L dans la différentiation des lymphocytes T auxiliaires dans un modèle murin de dermatite atopique

Notre analyse de la différenciation des lymphocytes T après traitement au MC903 montre que TSLP induit une augmentation de la fréquence des lymphocytes $ST2^+$ $CXCR5^-$, décrits par la littérature comme des lymphocytes Th2. Afin d'étudier l'implication du récepteur à TSLP des DCs dans ce phénomène, et donc la signalisation directe de TSLP sur les cellules $CD11c^+$, incluant les DCs, nous avons généré un nouvel outil génétique murin présentant un gène *Tslpr* floxé, et nous l'avons croisé avec une souris CD11c-Cre. L'augmentation de la fréquence des lymphocytes T $ST2^+$ $CXCR5^-$ induite par le MC903 est abrogée chez les souris $Tslpr^{CD11c-/-}$, de même que chez les souris $Tslp^{-/-}$ et $Tslpr^{-/-}$, montrant l'implication des cellules $CD11c^+$ et de la signalisation de TSLP *via* les cellules $CD11c^+$ dans l'induction des lymphocytes T $ST2^+$ $CXCR5^-$. Toutefois, nous avons observé par marquage intracellulaire, et à l'aide de souris rapportrices, que les cellules $ST2^+$ ne produisent que peu de cytokines Th2 (IL-4, IL-13). De plus amples analyses ont montré que la majorité des cellules $ST2^+$ induites par le traitement au MC903 expriment FoxP3, CD25, GATA3 et OX40, récapitulant le phénotype de lymphocytes T régulateurs (Tregs) $ST2^+$. Il semble donc que le traitement au MC903 augmente de façon sélective la population de Tregs $ST2^+$ *via* un axe TSLP-TSLPR sur les cellules $CD11c^+$, vraisemblablement spécifiquement *via* les DCs.

Les lymphocytes $ST2^+$ $FoxP3^+$ exprimant fortement OX40, nous avons étudié l'implication de l'axe OX40L-OX40 dans le développement et la maintenance de ces

cellules grâce à un nouvel outil génétique développé par le laboratoire et permettant de traquer les cellules exprimant OX40L grâce à la protéine fluorescente BFP (*Tsnfsf4^{KI/+}*), mais aussi de supprimer l'expression d'OX40L (*Tnfsf4^{KI/KI}*). Dans un premier temps, nous avons voulu déterminer si les DCs exprimaient OX40L après traitement au MC903 et nous avons identifié une population de DC migratoires dont l'expression d'OX40L est augmentée après traitement au MC903. Afin d'étudier le rôle de la signalisation OX40L-OX40, nous avons traité des souris *Tnfsf4^{KI/KI}* avec le MC903. De façon intéressante, nous avons constaté que l'augmentation de la fréquence des lymphocytes ST2$^+$ FoxP3$^+$ était abrogée chez les souris n'exprimant pas OX40L. Ceci suggère donc que TSLP, par l'intermédiaire du TSLPR des DCs, induit la différenciation de lymphocytes T ST2$^+$ FoxP3$^+$ grâce à la signalisation OX40L. Nous décrivons donc un rôle non identifié de l'axe TSLP-TSLPR-OX40L dans la promotion des lymphocytes Tregs ST2$^+$, ce qui soulève des questions intéressantes, notamment concernant la fonction régulatrice de ces cellules, leur relation avec les cellules Th2, ainsi que leur contribution dans pathogenèse de la DA.

Main abbreviations

A
AD: atopic dermatitis
AK2: adenylate kinase 2
AP-1: activator protein 1
APC: antigen-presenting cell

B
BAC: bacterial artificial chromosome
BAL: bronchoalveolar lavage
BATF: basic leucine zipper transcription factor ATF-like
BM: bone marrow

C
cDC: conventional dendritic cell
CDP: common dendritic cell progenitor
Clec9A: C-type lectin domain containing 9A
CLR: C-type lectin receptor
CCR: C-C chemokine receptor
CHS: contact hypersensitivity
CLE: cutaneous lupus erythematous
CSF1R: colony-stimulating factor receptor 1
CT: control
CTL: cytotoxic T lymphocytes
CTLD: C-type lectin-like domain
CXCR: C-X-C chemokine receptor

D
DAMP: danger-associated molecular pattern
DC: dendritic cell
DNA: desoxyribonucleic acid
DT: diphteria toxin
DTR: diphteria toxin receptor

E
EDLN: ear-draining lymph node
eEF-2: eukaryotic elongation factor 2
ELISA: Enzyme-linked immunosorbent assay
ER: endoplasmic reticulum

F
FcR: fragment crystallizable region receptor
FDC: follicular dendritic cell

G
GC: germinal center
GM-CSF: granulocyte-macrophage colony-stimulating factor

H
HAMA: human anti-mouse antibody

I
IC: immune complex
IDO: 2,3-Dioxygenase
IFN: interferon
IgE: immunoglobulin E
IgG: immunoglobulin G
IgSF: immunoglobulin superfamily
IHC: immunohistochemistry
IL: interleukin
ILC: innate lymphoid cell
IRF: interferon regulatory factor
IS: immune synapse

K
K14: keratin 14
Klf4: Kruppel-like factor 4

L
LC: Langerhans cell
LCH: Langerhans cell histocytiosis
LMP: laser microporation
LN: lymph node
LTβ : lymphotoxin β
LTβR : lymphotoxin β receptor

M
mAb: monoclonal antibody
MAL: MyD88 adaptator-like
MAPK: mitogen-activated protein kinase
MBP: major basic protein
MCPT8: mast cell protease 8
MGL2: macrophage galactose-type C-type lectin 2
MHC: major histocompatibility complex
MHC-I: MHC class I
MHC-II: MHC class II
MLR: mixed lymphocyte reaction
MMP: metalloprotease
moDC: monocyte-derived dendritic cell
MyD88: myeloid differentiation factor

N
NF-κB: nuclear factor κB
NIK: NF-κB-inducing kinase
NLR: NOD-like receptor
NOD: nucleotide-binding oligomeriztion domain

O
OVA: ovalbumin

P
PAMP: pathogen-associated molecular pattern
pDC: plasmacytoid dendritic cell
PRR: pattern-recognition receptor
PTEN: phosphatase and tensin homolog

R
RA: retinoic acid
RER: rough endoplasmic reticulum
RIG-I: retinoic acid-inducible gene I
RLR: RIG-I-like receptor
RNA: ribonucleic acid
RT-qPCR: reverse transcription − quantitative polymerase chain reaction
Runx3: runt related transcription factor 3

S
SCID: severe combined immunodeficiency
sLO: secondary lymphoid organ

T
TCR: T-cell receptor
TGF: transforming growth factor
Tfh: T follicular helper
Tfr: T follicular regulatory
Th: T helper
TIR: Toll/Interleukin-1 receptor
TLR: toll-like receptor
TNF: tumor necrosis factor
TNFSF: tumor necrosis factor superfamily
Treg: regulatory T cell
TRIF: TRIF-related adaptor molecule
TSLP: thymic stromal lymphopoietin

W
WAS: Wiskott-Aldrich Syndrome
WT: wild type

X
XCR1: X-C motif chemokine receptor 1

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INTRODUCTION

A. History of dendritic cells studies

a. Discovery of *in vivo* classical dendritic cells

The first ever observation of a dendritic cell (DC) was performed more than 100 years before the understanding of their function, in 1868, by Paul Langerhans, who described what he called Langerhans cells (LCs) as non-pigmented cells of the epidermis [1]. Due to their dendritic morphology, he suggested that they may exert extracutaneous sensing in the skin.

It is only in 1973 that Ralph Steinman and Zanvil Cohn described large adherent nucleated cells in mouce spleen and lymph nodes (LNs), known to be attached to the structure of the organ because it shows a better recovery after collagenase digestion. These new cells are distinct from other adherent cells by their dendritic morphology in culture [2] and were thus named dendritic cells. Steinman continued to characterize mouse dendritic cells [3, 4, 5] and this work, besides an extensive description of the morphology of DCs, raised hypotheses and conceptual considerations regarding the function and the denomination of this new cell type. DCs were characterized as hematopoietic radiosensitive cells, different from macrophages and lymphocytes because of their low phagocytosis and the absence of lymphocytes surface markers. However, it was suggested that DCs could be the counterpart of another cell type, the "antigen-retaining reticular cells" nowadays known as follicular dendritic cells (FDC). We now know that FDCs, unlike DCs, are from stromal origin [6] and present immune complexes useful for efficient germinal center (GC) reaction and antibody response [7].

In parallel, the concept of antigen presentation was being raised by Rosenthal and Shevach [8], and was rapidly linked with the major histocompatibility complex (MHC) [9] and MHC restriction [10]. It was postulated that antigen-presentation, between histocompatible lymphocytes and antigen-presenting cells (APCs), was necessary for efficient adaptative response.

In the following years, functional assays, especially mixed lymphocyte reaction (MLR) demonstrated that DCs represent the most potent inducers of adaptive immunity. Steinman and Witmer [11] first showed that mouse low-density spleen cells greatly enhanced lymphocyte proliferation in MLR and then demonstrated that this effect was due to DCs and

not macrophages. Inaba and colleagues [12] took advantage of the development of monoclonal antibodies (mAbs) to perform elegant studies using cell depletion strategies. They demonstrated that DCs and not macrophages increase anti-sheep red blood cells response not only in mouse, but also in human by using cells purified from blood.

mAbs were also used, along with the development of flow cytometry, for immunophenotyping of DCs. DC populations were thus described using the clone 33D1 (against DCIR2), NLDC-145 (against DEC205) and, importantly, N418 which binds to CD11c [13, 14]. DCs were rapidly defined as CD11c$^+$ and MHC class II (MHC-II)$^+$ cells, surface markers that allowed better in depth study of these cells and are still used today, despite a well recognized lack of specificity of these markers that can be expressed by several other cell types, including macrophages or activated T cells. It was also noted that DCs found in the T cell zone, called "interdigitating cells", were expressing CD205 and required collagenase digestion to be recovered from the LNs, whereas those isolated from the tissue or without enzymatic digestion of the lymphoid organs, called "interstitial cells" were rather DCIR2$^+$ [13, 14].

Vremec and colleagues found that DCs could be subdivided in populations according to their expression of CD4, CD8 and CD11b [15]. This lead to the idea of a "lymphoid" DC population expressing CD8 but negative for CD4 and CD11b, and a "myeloid" DC population that expresses CD11b together with CD4 or not, but that does not express CD8. Together these cells form the conventional dendritic cells (cDCs). However, this classification induced several confusions in the field and was changed several years later.

b. Studies of plasmacytoid dendritic cells

A peculiar population called either "plasmacytoid T cells" or "plasmacytoid monocytes" was known to be found in secondary lymphoid organs, especially under pathological conditions. Those cells were first isolated from human blood and tonsil, and presented a small, round lymphoid shape as well as developed rough endoplasmic reticulum (RER). They were distinguished from antibody-secreting plasma cells by surface expression of several markers and their function was thus highly mysterious. Grouard and colleagues reported in 1997 [16] that those cells, after interleukin (IL-)3 and CD40-ligand treatment, developed dendrites, lost

RER structure and displayed poor phagocytosis but strongly stimulated naïve T cells. Because of those particularities, researchers first classified these plasmacytoid dendritic cells (pDCs) as precursors of IL-3 dependent DCs and their mouse homologues were identified a few years later [17, 18, 19]. Further studies have revealed that pDCs represent a highly specialized dendritic cell subset producing important amounts of type I interferon (IFN) and equipped with specific receptors to detect viral infections [20].

c. Development of *in vitro* differentiated dendritic cells

The critical role played by DCs in immunobiology urged researchers to find quick, reliable and relatively cheap ways to generate large number of cells *in vitro*, isolation of primary cells from animals being laborious in matter of time, work and animals. DCs being of hematopoietic origin, several teams cultured bone-marrow cells of various species and eventually obtained DC-like cells by culturing rat, mouse or human bone-marrow cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) supplemented medium [21, 22, 23, 24]. In parallel, other researchers generated DC-like cells from human monocytes in culture [25, 26] and the possibility of generating monocyte-derived DCs (moDCs) raised the hypothesis that monocytes could be able to differentiate in both macrophages and DCs.

Despite a keen interest from the scientific and medical communities in *in vitro* differentiated DCs, which were used in clinical trials and contributed to our knowledge of DC development and function, several limitations of this method were pointed. First, bone marrow-derived DCs and moDCs can functionally differ from lymphoid organ-resident DCs. Second, cytokine dependence, and their required concentration for DC development, varies between *in vivo* and *in vitro* context. Finally, the surface markers found on *in vitro* differentiated cells do not recapitulate faithfully those found on *ex vivo* isolated DCs [27, 28, 29].

B. Dendritic cells function as antigen-presenting cells

a. Antigen capture

Peripheral DCs in the various non lymphoid tissues of the organism present an immature state in which they have the capacity to recognize and capture microbes. This recognition leads to the internalization and destruction of the pathogen, and ultimately, to the presentation of the uptaken antigens.
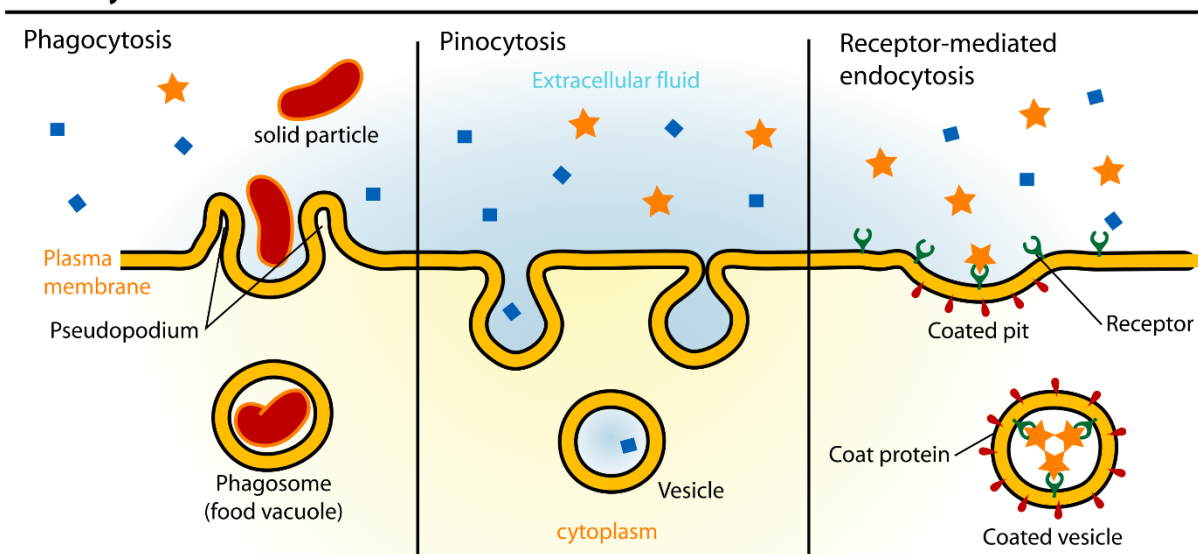
Figure 1: The three main mechanisms of endocytosis [30].
Endocytosis can be performed through three main mechanisms. Phagocytosis (left) is the process of engulfing large particles. Pinocytosis (middle) allows the cell to sample small amount of their surrounding fluidic milieu. Receptor-mediated endocytosis is the process of endocytosis triggered by binding of a specific ligand, like a neurotransmitter or a protein, to its receptor.

Antigen capture can be achieved by DCs by a mechanism called endocytosis. Endocytosis is a process of invagination of the cell membrane which results in the confinement of the target molecule, cell or microbe in a vesicle inside the DC. We can distinguish three main types of endocytosis:

- Pinocytosis, which etymologically means "cell drinking", is a process which allows the cell to sample its surrounding fluidic environment inside small vesicles. This process is non-specific for the substances that it transports. The vesicles will eventually be

addressed to hydrolase-containing lysosomes leading to the digestion of their content. This process is used for clearing and renewing the extracellular fluid and for immunological surveillance.

- Phagocytosis, this time meaning "cell eating", is the process of engulfing large particles such as cells. Phagocytosis can be performed by various cell types, but professional phagocytic cells include macrophages, neutrophils and DCs. The process leads to the formation of a particle-containing vesicle called phagosome which will fuse to a lysosome, forming the phagolysosome where the phagocyted body will be destroyed.

- The third type is called "receptor-mediated endocytosis". In this process, the cell will absorb a specific molecule, such as a protein or a hormone, after its binding on a cell receptor. It is thus also a substance-specific process.

b. Classical antigen presentation pathways

DCs express both type I and type II MHC at their surface, allowing them to present antigens to CD8$^+$ and CD4$^+$ T cells respectively. Classical pathways of antigen presentation lead to the processing of exogenous antigens (from microbes but also apoptotic or infected cells) toward MHC-II whereas endogenous antigens will be presented through MHC class I (MHC-I) [Reviewed in 31]. If all nucleated cells from the organism can present through MHC-I, MHC-II presentation is the hallmark of professional APCs such as macrophages and DCs.
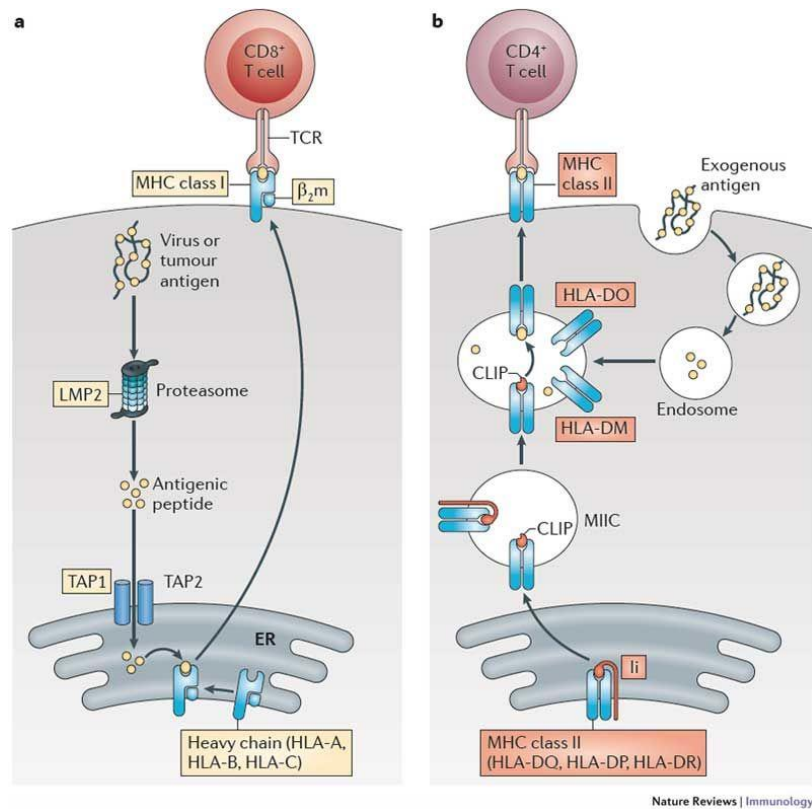
Figure 2: Classical antigen presentation pathways on MHC-I (a) and MHC-II (b) [32].
Antigen presentation is a process in which endogenous or exogenous antigens are bound to MHC-I and MHC-II respectively. Presentation on MHC-I is done by all nucleated cells and involves proteasome degradation of misfolded proteins before their presentation at cell surface (a). MHC-II-mediated presentation is done by professional APCs: exogenous antigens are endocyted, degraded and the remaining peptides are presented at the cell surface.

MHC-I is composed by two chains, a α chain, highly polymorphic, and the β2 microglobulin, lighter and more or less invariant. Both chains meet in the endoplasmic reticulum (ER) where their interaction is stabilized by the presence of a peptide in the MHC-I pocket of the α chain. These peptides are synthesized in the cytosol of the cells and are degraded by the proteasome either because they form incompletely translated products or misfolded proteins. Proteasome products are then translocated in the ER lumen by TAP1 and TAP2 transporters. Dedicated complexes will then load the 8 to 9 amino acid peptide in the MHC-I before quality control and addressing it to the plasma membrane. This mechanism is critical for cytotoxic T cells to check for viral infection or cell transformation but represents a major limitation for compatibility in tissue grafting.

MHC-II complex is composed by two subunits, α and β, both highly polymorphic. The peptide-binding pocket is composed by both chains. Since binding of endogenous peptide is not

wanted in the ER, an invariant chain (CD74) stabilizes the complex and prevents any premature loading of an undesired peptide in the complex. MHC-II is then translocated to the late endosomes through cell surface. Proteases from the late endosomal compartment will hydrolyze the invariant chain, leaving only CLIP peptide in the peptide-binding pocket. Exogenous peptides produced by degradation of endocyted products are also present in the late endosomes and can be loaded into the MHC-II pocket by HLA-DM, a protein which will promote loading of peptide onto the MHC-II by exchanging it with CLIP. MHC-II:peptide complexes are then translocated back to the cell surface where they will trigger antigen-specific CD4^{+} cell stimulation.

Once the peptide is presented on MHC-II at the surface, DCs will crawl between T cells in order to find one that express a TCR with a strong affinity for this antigen. Interaction between TCR and MHC-I is stabilized by CD8 coreceptor, whereas MHC-II interaction is stabilized by CD4, which results in a preferential presentation of MHC-I bound peptides to CD8^{+} T cells and MHC-II bound peptides to CD4^{+} T cells. Stable interactions will last longer and induce the formation of an immune synapse (IS) between the DC and the T cells where signals will be given to instruct T cell activation.

c. Antigen cross-presentation

DCs have the unique capability of loading exogenous antigens, from bacteria, infected or dying cells, onto MHC-I before cell surface presentation. This mechanism is important to trigger cytotoxic responses to viruses that contaminated and killed a cell that the DC engulfed or a tumorigenic cell that was endocyted by the DC.

Since its discovery more than 40 years ago [33] and up to now, this pathway has remained under heavy investigations and is not yet fully understood. Two main mechanisms that may coexist are proposed for cross-presentation: the vacuolar pathway and the cytosolic pathway.

The hypothesis of the vacuolar pathway was raised after the observation that cross-presentation of ovalbumin (OVA)-E. coli Crl fusion protein by mouse DCs was unaffected by proteasome inhibitors or brefeldin A (inhibitor of ER-Golgi transport) and was only partially impaired in TAP-deficient background [34]. Overall, these data suggested a mechanism which

is lysosome-mediated and researchers postulated that soluble antigens can be degraded in endosomes where they meet MHC-I that was brought back from the cell membrane. Antigen-derived peptides can then bind in the MHC-I pocket before their presentation at the cell surface.

On the other hand, concomitant mouse studies with particulate antigens like bead-bound OVA showed that proteasome inhibition, TAP-deficiency and brefeldin A treatment disrupted cross-presentation [35]. This study demonstrated the existence of another pathway, probably involving export of the antigens from the lysosome to the cytosol before loading it onto MHC-I in the more classical ER pathway.

d. Danger sensing receptors

Antigen presentation is the first key signal given by DCs to T cells in order to mount an efficient immune response. It is therefore referred to as "signal 1". Costimulatory signals, also called "signal 2", are critical to both induce and polarize helper and cytotoxic responses. In order to give the most appropriate signals to T cells, DCs display a panel of receptors allowing for danger evaluation.

One type of receptors used to evaluate danger is opsonin receptors. Opsonization is the process of coating a pathogen with molecules such as antibodies or complement, thus facilitating its recognition and phagocytosis by macrophages and dendritic cells for instance. The most studied opsonin receptors on DCs are antibodies receptors, also called Fragment crystallizable region receptors (FcR), which capture antigen-bound antibodies, also known as immune complexes (IC). Mouse and human DCs have been shown to express FcRs, especially FcγR that bind immunoglobulin G (IgG) [36, 37, 38]. FcγR expression varies depending on the subset of DCs; classical DCs express low amounts of activating FcγR in physiological conditions, both in human and mouse. On the other hand, moDCs express activating FcγRI and FcγRIII in mouse and FcγRIIB in human, which have been shown to be involved in IC-dependent DC activation. Binding of ICs to FcRs induces the internalization of the IC and triggers subsequent antigen presentation both on MHC-II and MHC-I. In addition, all DCs express inhibitory FcγRIIB that may counteract activating receptors signaling. The T helper (T_h) polarization induced by

IC-stimulated DCs is still not well understood with few evidences suggesting that stimulation of activating FcγR leads to T_h1 [39, 40] or T_h2 induction [41].

Another class of DC-activating receptors that have been well studied is formed by pattern-recognition receptors (PRRs). Pathogen-associated molecular patterns (PAMPs) at the surface of all microorganisms and danger-associated molecular patterns (DAMPs) released from damaged cells are recognized by the PRRs expressed by DCs, discriminating "non-self" and "damaged-self" respectively. Unlike the receptors of adaptive immunity, PRRs are germline encoded receptors of broad specificities which recognize conserved motifs on microorganisms or damaged cells. We can distinguish four types of PRR:

- Toll-like receptors (TLRs) are transmembrane proteins that recognize nucleic acids or microbial components, either at the surface of the cell or inside pathogen-containing endosomes. TLR1-9 are conserved between mouse and human, whereas TLR8 and 10 are nonfunctional in mouse and TLR11-13 are absent in human [reviewed in 42]. Most of the TLRs have identified ligands: receptors present at the cell surface recognize components of the surface of microbes whereas endosomal TLRs recognize nucleic acids (see table 1). Binding of their cognate ligand will induce the dimerization of the ectodomain of two TLRs, and allow their interaction with the Toll/interleukin-1 receptor (TIR) domain of intracellular adaptor molecules. To start the signaling cascade, TLR-1/2 and TLR-2/6 interact with the myeloid differentiation factor (MyD)D88/MyD88 adaptor-like (MAL) complex; TLR-3 interacts with TIR domain-containing adaptor-inducing IFN-β (TRIF); TLR-4 interacts with MyD88/MAL and TRIF/ TRIF-related adaptor molecule (TRAM) complexes; all other TLRs signal with MyD88 alone. TLR signaling can activate the transcription factors nuclear factor (NF)-κB, activator protein (AP)-1 and Interferon regulatory factor (IRF), thus inducing the immune response depending on the context.

Toll-like receptor	M	H	Exogenous ligand	Pathogen	Hematopoietic cell distribution
TLR-1:TLR-2 dimer	Y	Y	Lipomamans; lipoproteins; lipoteichoic acids; β-glucans; zymosan	Gram-negative bacteria	Monocytes, dendritic cells, mast cells, eosinophils, basophils
TLR-2:TLR-6 dimer	Y	Y			
TLR-3	Y	Y	Double-stranded (ds)RNA; Poly I:C	Viruses with dsRNA genome (e.g. rotavirus)	Macrophages, dendritic cells, gut epithelium
TLR-4 (+ MD-2/CD14)	Y	Y	Lipopolysaccharides; lipoteichoic acids	Gram-negative bacteria	Macrophages, dendritic cells, mast cells, eosinophils
TLR-5	Y	Y	Flagellin	Bacteria	Macrophages, dendritic cells, gut epithelium
TLR-7	Y	Y	Single-stranded (ss)RNA	Viruses with ssRNA genome (e.g. orthomyxovirus)	Macrophages, plasmacytoid dendritic cells, eosinophils, B cells
TLR-8	Y	Y	Single-stranded (ss)RNA	Viruses with ssRNA genome (e.g. orthomyxovirus)	Macrophages, neutrophils
TLR-9	Y	Y	Unmethylated CpG	Viruses/bacteria	Plasmacytoid dendritic cells, eosinophils, B cells, basophils
TLR-10		Y	Unknown	Unknown	Plasmacytoid dendritic cells, eosinophils, B cells, basophils
TLR-11	Y		Flagellin, Profilin	Becteria, Protozoa (e.g. *Toxoplasma gondii*)	Macrophages, dendritic cells
TLR-12	Y		Profilin	Protozoa (e.g. *Toxoplasma gondii*)	Macrophages, dendritic cells
TLR-13	Y		Single-stranded RNA (bacterial ribosomal RNA)	Bacteria	Macrophages, dendritic cells

Table 1: List of TLR, their exogenous ligands and expression pattern (from Murphy K., Weaver C. (2017) Janeway's Immunobiology. Garland Science).

- C-type lectin receptors (CLR) are also transmembrane proteins that contains C-type lectin-like domain (CTLD) which normally binds sugars through conserved amino acid motifs. However, several studies have shown that these receptors evolved to also bind lipids, proteins and even inorganic compounds [reviewed in 43]. One of the members of this family is CD207, also known as Langerin, expressed by LCs.

- In addition to those receptors present at the cell surface and in endosomes, there are cytosolic receptors such as retinoic acid-inducible gene I (RIG-I)-like receptors (RLR). RIG-I, a member of the RLR family, recognizes the 5' triphosphate group of RNA, which is capped by a 7-methylguanosine in the nucleus of eukaryotic cells, but often left free on viral RNA that replicate in the cell cytoplasm. Through its helicase domain, melanoma differentiation associated (MDA)-5 is able to recognize double stranded (ds)RNA, which should not be found freely accessible in the cytoplasm of eukaryotic cells. Activation of RIG-I or MDA-5 leads to the production of type I interferons and the antiviral response.

- Nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) are cytosolic receptors which recognize components of intracellular bacteria, such as peptidoglycans of gram-negative bacteria (NOD1 and NOD2), potassium influx (NLRP3) or DNA (AIM2) for instance. As reviewed in [44], NLR can detect both viral and bacterial products in the cell cytoplasm. NLR family can be further subdivided according to the protein domains expressed by its members, and trigger either MAPK/NF-κB pathways, or the activation of the inflammasome, which in turn cleaves and activates the caspase 1 and leads to production of active IL-1β and eventually apoptosis of the cell.

Sensing of danger signals will induce the expression of costimulatory signals by DCs. These signals are required for T cell priming, both for activation and polarization of T_h response.

e. Migration of DCs to the draining LN

Upon activation, DCs emigrate from the skin to the skin-draining LN. The process of DC migration is complex and well regulated.

The first step is the mobilization and detachment of DCs. While PAMPs and danger signals will induce DC activation and subsequent costimulatory signaling, it seems that the mobilization of DCs required signaling by Tumor necrosis factor (TNF)-α and IL-1β. To demonstrate this, IL-1β and TNF-α were neutralized by antibodies [45, 46] showing decreased sensitization and LC mobilization in the draining LN respectively. This hypothesis was confirmed by the genetic deletion or the antibody-mediated blocking of TNF-α type II receptors and IL-1 type 1 receptor [47, 48] which showed depressed LC migration to LN associated with lower contact hypersensitivity. Conversely, these two cytokines are not only necessary, but also sufficient for inducing DC mobilization as subcutaneous administration of either of these cytokines promote rapid DC translocation to the LN [49]. This feature of TNF-α and IL-β could suggest the possibility of a differential regulation of DC activation and migration which would explain the detection of DCs in the draining lymph node even in absence of tissue inflammation. Whether maturation of DCs and chemotaxis are coupled or not necessarily associated remains under debate.

Their quality of immune sentinels require DCs to stay in the non-lymphoid tissue for a relatively long period of time, especially in the skin [50]. To stay in the tissue, DCs are anchored in their surrounding environment by cell-cell adhesion molecules. The best studied example is the E-cadherin expression by LCs [51], which allow them to be attached to surrounding keratinocytes and maintain the tissue architecture. The critical importance of E-cadherin expression is illustrated by the spontaneous migration of LCs without expression of costimulatory signals upon disruption of E-cadherin interaction by injecting a neutralizing antibody [52]. Interestingly, disruption of cell-cell contact spontaneously induce the upregulation of C-C chemokine receptor type 7 (CCR7) by LCs, a chemokine receptor whose importance will be further discussed below. Transforming growth factor (TGF)-β is a key player in LCs homeostasis and it was reported that this cytokine upregulates the expression of E-cadherin by LCs while downregulating CCR7. Conversely, TNF-α and IL-1β induce downregulation of E-cadherin and stimulates CCR7 expression.
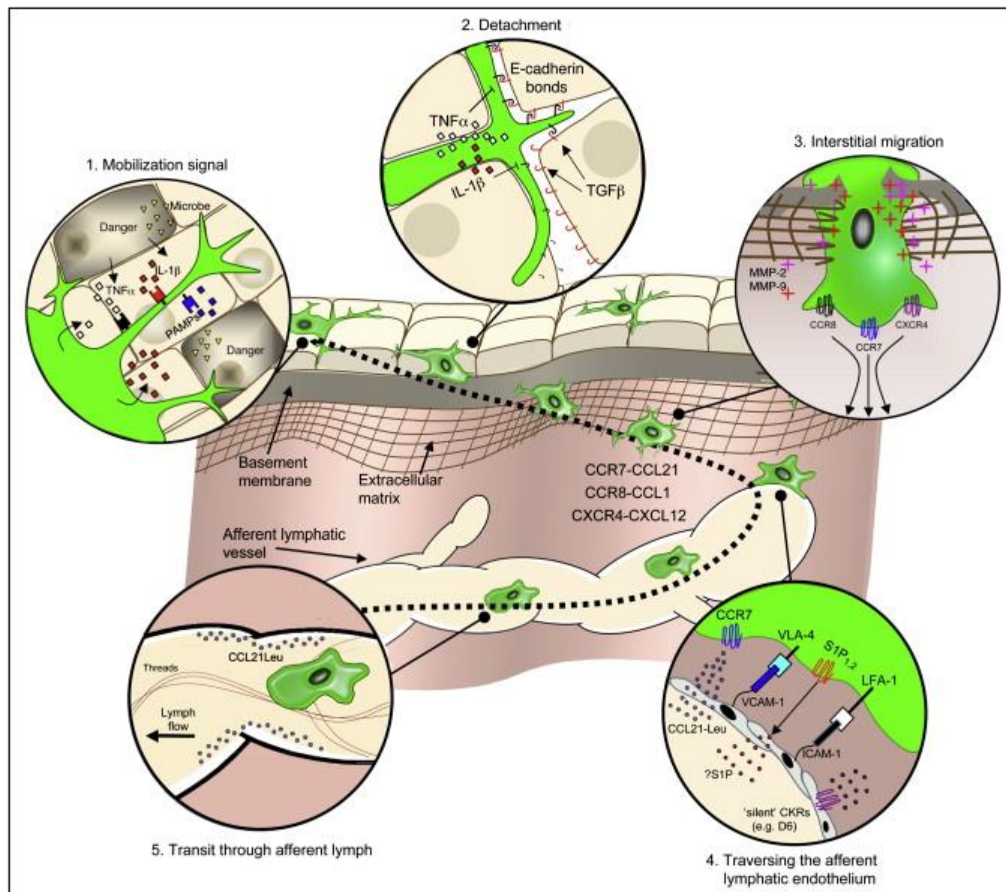
Figure 3: Migration of DCs to lymphoid organs [53].
Schematic model of DCs migration from peripheral non-lymphoid tissue to the draining lymph node. The process of migration could be divided in 5 sequential steps of 1) mobilization, 2) detachment, 3) interstitial migration, 4) entering the lymphatic vessel and 5) transit through the lymphatic system.

Both LC and dermal DCs need to crawl through relatively solid tissues composed of collagen and fibronectin and even the basal membrane for LCs. This process requires the expression of enzymes that will partially digest the extracellular matrix and the best known are matrix metalloproteases (MMPs). In inflammatory conditions, such as hapten stimulation, LCs will upregulate their expression of MMP-9, suggesting a role of this enzyme for LC migration [54]. Using broad spectrum inhibitors of MMPs, MMP-specific antibodies, and MMP-deficient mice, Ratzinger and colleagues confirmed the requirement of both MMP-9 and MMP-2 not only for LCs, but also for dermal DCs [55].

Digestion of the extracellular matrix thus seems to be the first important step allowing DCs to migrate out of the tissue. However, an efficient relocation of DCs to LNs requires DCs to be guided to the closest lymphatic vessel by chemotaxis. Even though the first mobilization of LCs from the epidermis to the dermis is independent of CCR7, cytokine signals given to DCs will

induce upregulation of CCR7, both at the transcriptional level and at the cell surface, and induce their migration into the lymphatic vessels, toward the draining lymph node in a CCL19- and CCL21-dependant manner [56].

CCL19 and CCL21 are two chemokines that are well conserved in vertebrates and signal through CCR7 to mediate various effects in the immune system [reviewed in 57]. Both chemokines are in the same cluster of genes located on human chromosome 9 and mouse chromosome 4. Of note, mouse CCL21 genes underwent duplication along evolution and two forms of CCL21 can now be found in *Mus musculus* with one amino acid of difference: CCL21Leu that is expressed by afferent lymphatic vessels and CCL21Ser that is expressed by both lymphatic vessels and LN stromal cells [reviewed in 53].

Kabashima and colleagues [58] suggested that CXCR4 blockade also reduced antigen-bearing migration to the skin-draining lymph node, raising the hypothesis of a redundant or complementary axis with CCR7-mediated chemitaxis.

f. TCR and costimulatory signals

Naïve T cell activation requires a three signal interaction between DCs and T cells. Signal 1 is given by the interaction of TCR with peptide:MHC complex. Signal 2 depends on costimulatory signals. As we have seen above, stable interaction of a T cell with a cognate peptide-presenting DC will induce the formation of an immune synapse where costimulatory molecules will be recruited to allow stronger signaling. As observed by the team of Schwartz, absence of secondary signals following TCR engagement will lead to T cell anergy or unresponsiveness [59, 60], which raised interesting perspectives for novel treatments in allergy and autoimmunity, but also in the field of cancer where it opened the way for immunotherapy [61]. The third signal is given by the cytokines in the microenvironment which will define the polarization of the differentiating CD4$^+$ and CD8$^+$ T cell.

Costimulatory receptors on T cells can be subdivided in two different families:

- Immunoglobulin superfamily (IgSF) containing Ig-like domains.
- TNF superfamily (TNFSF).

Costimulatory signals of the immunoglobulin superfamily

Costimulatory pathways from the IgSF can be either activator or repressor of T cell activation. I will go through some members of this family.

CD80/CD86 – CD28

One of the best studied costimulatory signals given by DCs to T cells transduce through CD28. It is the first member of the subfamily of costimulatory molecules containing Ig-like domain in their extracellular part. The ligands for CD28 are CD80 (B7-1) and CD86 (B7-2). They are not functionally equivalent [62] and they present different kinetics of expression by APCs since CD80 is upregulated later than CD86 [63], thus adding another layer of complexity in the activation of CD4$^+$ T cells. Signaling through CD28 will strengthen TCR signaling, provide T cell survival signals and induce the expression or the upregulation of other costimulatory receptors.

CD80/CD86 – CTLA4

CTLA4 is a receptor with high homology with CD28. They compete for the same ligands, CD80 and CD86 [64]. Interestingly, CTLA4 presents a higher affinity for both CD80 and CD86 than CD28, thus exerting an immunosuppressive effect by limiting the activation signaling through CD28 [65]. Of note, fusion proteins (including abatacept and belatacept) containing the recombinant extracellular domain of CTLA4 have proved efficacy in clinic to control some forms of arthritis [66, 67], type 1 diabetes [68] and organ rejection [69]. Of note, it was shown that CTLA4 signaling is bidirectional: upon ligation of CD86 and CD86 with CTLA4, DC will upregulate the expression of Indoleamine 2,3-Dioxygenase (IDO). It is thought that IDO will induce a deprivation of tryptophan in the surrounding environment, thus acting in trans on the T cell to induce anergy [70].

ICOSL – ICOS

ICOS (also known as CD278) is a member of the IgSF which shows relatively low sequence homology with CD28, both at the nucleic and amino acid sequence levels. However, it still displays the typical features of the IgSF members, such as the immunoglobulin IgV-like domain and the homology of its cytoplasmic domain. Its ligand, ICOSL, was first named B7 homology 2 (B7H2) because of its relative similarity with CD80 and CD86, however their sequence homology is very weak and this denomination was abandoned in favor of ICOSL (or CD275). ICOS knockout mice show protection in various autoimmune disease models and deficient humoral responses, whereas patients deficient for ICOS suffer from common variable immunodeficiency, with defective antibody response.

MHCII – LAG-3

LAG-3 presents even less homology than ICOS with CD28. The structure of LAG-3 consists of four extracellular Ig-domain very similar to those of the CD4. Upon TCR:peptide/MHCII biding, CD4 will also bind to MHCII, at another place than the TCR. An efficient TCR signaling requires the recruitment of the tyrosine kinase Lck, which is mediated by both the TCR and CD4. When expressed by Tregs, LAG-3, because of its higher affinity for MHCII compared with CD4, will prevent the binding of CD4 and thus largely diminish the strength of TCR signaling, thus acting as an immune regulator. Several clinical trials are going on to assess the potential of LAG-3 as a therapeutic target in cancer (e.g. NCT01968109, NCT02460224).

PD-L1/PD-L2 – PD-1

PD-1 (also known as CD279) is a transmembrane protein which relates to CD28 for its structure and function. It is expressed by T cells during thymic development, where it mediates the death of autoreactive T cells [71]. It has two identified ligands: PD-L1 (CD274; B7-H1) and PD-L2 (CD273; B7-DC). While the first can be expressed by various cells from both hematopoietic and non-hematopoietic cells, PD-L2 was more restricted to antigen-presenting cells, although its expression can be induced in both immune and non-immune cells by various microenvironmental stimuli. Upon binding of its ligand, PD-1 recruits SHP-1 and SHP-2, two phosphatase which will dephosphorylate the signaling molecules downstream of the TCR. In addition, PD-1 activates PTEN which will lead to an inhibition of the activating PI3K/Akt

pathway. Targeting PD-1 during cancer to relieve the immune suppression and allow an effective response has given great results already in responding patients [72].

CD80 – PDL1

Another layer of regulation of T cell induction is added by the interaction between CD80 and PD-L1 in *cis* at the surface of the DC. This interaction has been reported to limit the PD-1 signaling given by to the T cell, and thus to limit the inhibitory effect of PD-L1 [73]. This interaction could have therapeutic interest since it could be limiting T cell function in cancer, autoimmunity or chronic inflammation.

Costimulatory signals of the tumor necrosis factor superfamily

The TNFSF contains 19 members that signal through 29 receptors, exerting both pro- and anti-inflammatory effects [74, 75]. Despite an overall low sequence homology of the members of the TNFRSF, once engaged they trigger common or overlapping signaling pathways [76, 77, 78] as shown in figure 4.
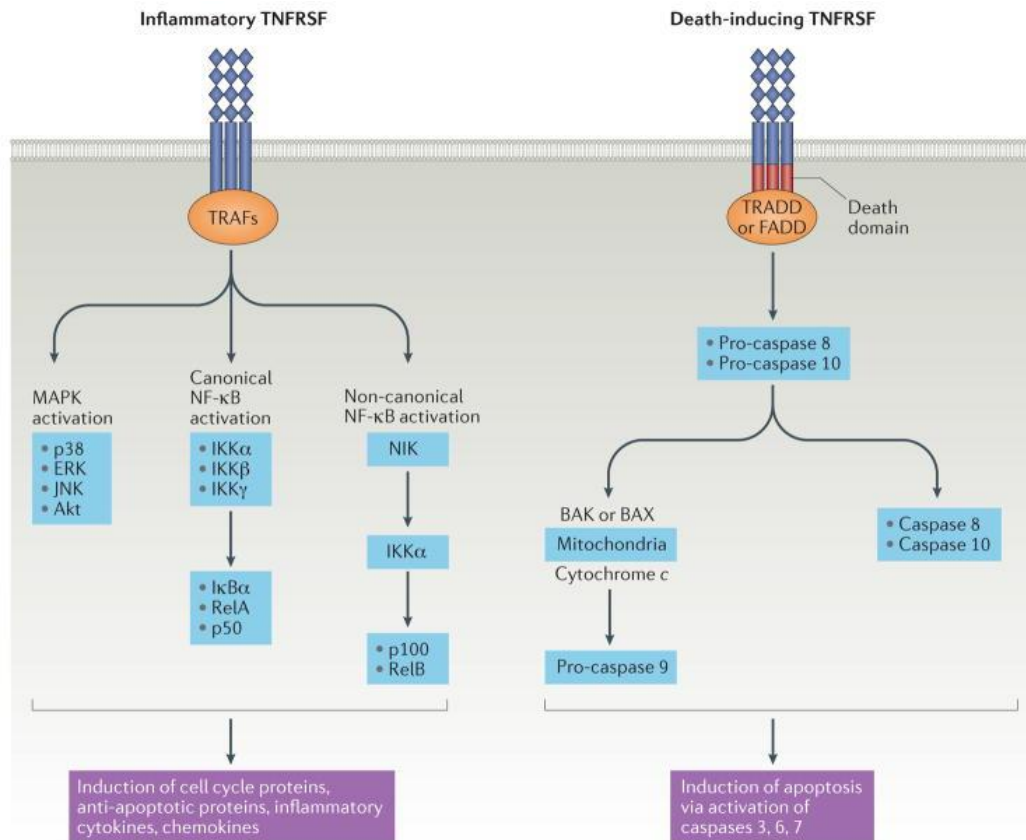
Figure 4: Signaling pathways downstream of TNFRSF members [75].
Schematic drawing of the signaling pathways triggered by TNFRSF. Inflammatory pathways signal through TRAFs which mediate MAPK, canonical and non-canonical NF-κB activation. Anti-inflammatory effect is mediated by inducing the death of the immune cells through TRADD or FADD death dromains which trigger the activation of caspase 8 and 10, leading to cell apoptosis.

FasL – Fas and TRAIL – TRAILR1/TRAILR2

Signaling through Fas (also known as TNFRSF6) and TRAILR1 or TRAILR2 (also known as TNFRSF10A or TNFRSF10B respectively) has been shown to induce cell apoptosis. These molecules are expressed, among other cells, by T cells and defective activity of FasL-Fas and TRAIL-TRAILR pathways are linked with an increased susceptibility to autoimmune diseases, especially auto-immune lymphoproliferative syndrome, both in mouse and human [79, 80, 81], suggesting that these pathways are immunosuppressive and control autoreactive cells. Thus researchers wonder about the relevance of targeting these two pathways in clinic and, despite encouraging data from preclinical models in rodents, human clinical trials generated mixed results [82].

CD70 – CD27, TL1A – DR3 and 4-1BBL – 4-1BB

CD27 (also known as TNFRSF7) is a receptor constitutively expressed by most T cells. Its ligand CD70 (also known as TNFSF7) can be expressed by APC upon activation and allow a bidirectional signaling which further enhances the activation of the APC. Death receptor (DR)3 (also known as TNFRSF25) and 4-1BB (also known as TNFRSF9) are inducible receptors on T cells that are expressed upon TCR and CD28 signaling. Their cognate ligands (TL1A (TNFSF15) and 4-1BBL (TNFSF9)) are expressed by activated professional APCs, such as DCs and macrophages. All these receptors signal through the pathways as indicated in Figure 4 and they are involved in various autoimmune syndromes [75, 83].

OX40L being a part of my thesis project, the bigger and more exhaustive part will be devoted to this member of the TNF superfamily.

g. OX40-OX40L signaling

i. Structure of OX40-OX40L

Members of the TNFSF can be subdivided into three groups: the conventional, the EF-disulfide-containing and the divergent groups. Even though the members of TNFSF form homotrimers, these groups diverge from each other in their structure and the way they bind to their respective receptors: the first group contains pyramidal trimers, the second one more globular structure and the third group contain ligands that are divergent from the members of the others groups, but also from each other. As a member of the divergent group, OX40L monomers form homotrimeric complexes at the cell membrane, even though the interface between the different units is smaller than other members of this superfamily.

The trimeric OX40L will bind to three units of OX40 to form the OX40L-OX40 complex. Each copy of OX40 will bind at the interface of two OX40L monomers. Interestingly, despite a relatively moderate similarity between mouse and human OX40L, mouse OX40L binds to human OX40 thanks to two well conserved residues critical for ligand-receptor interaction [84].

ii. OX40 expression

OX40-bearing cells are mostly lymphocytes and mainly CD4$^+$ T cells, but OX40 can also be expressed by mouse and human natural killer (NK) cells [85, 86], NKT cells [87, 88] and by human neutrophils [89]. Upon activation, CD4$^+$ and CD8$^+$ T cell will transiently upregulate OX40. Primary TCR interaction and secondary signal through CD28 are required for OX40 induction on T cells, as shown in CD28-deficient mice [90]. Of note, mouse regulatory T cells (T$_{regs}$) constitutively express OX40, whereas OX40 is only induced on activated human T$_{regs}$.

iii. OX40-Ligand expression

OX40-Ligand (OX40L) is not constitutively expressed but can be induced on APCs such as B cells [91], macrophages [92] and DCs [93]. OX40L expression was also reported on a broad variety of cell types, including endothelial cells, mast cells and even T cells, in the context of T cell-T cell interaction. It is interesting to point that type 2 and 3 innate lymphoid cells (ILCs) have also been reported to express OX40L [94, 95, 96, 97]. Besides the interaction of TCR with peptide:MHC and costimulatory signals, expression on DCs can be triggered by soluble molecules like Prostaglandin E2 [98] or cytokines including IFNγ [99] and TSLP [100].

Few studies have assessed the expression of OX40L on skin LCs. A first paper from Sato and colleagues in 2002 [101] presented data from a model of hapten (FITC)-induced allergic response. They used LCs isolated from WT, OX40L-deficient and OX40L-overexpressing mice to decipher the expression and the function of the expression of OX40L on LCs. From an MLR experiment, they concluded that "the requirement of OX40L during the antigen presentation function of LC in T cell priming [was] demonstrated". However, the provided data were not sufficient to reach such a statement providing that the authors used FITC$^+$ CD11c$^+$ cells from the LNs as LCs to reach their conclusion. Besides, their MLR reaction with LCs isolated from WT or OX40L-deficient mice reached similar results, OX40L-overexpressing LCs being the only ones able to induce efficient MLR. Another paper was published by Yoshiki and colleagues in 2010 [102] with more convincing data. They presented flow cytometry analysis of epidermis cells and draining LN stained with anti-OX40L antibody. If the LN data show an increased OX40L expression in EpCAM$^+$ Langerin$^+$ DCs that may not be only due to LCs [103], the authors also show that UVB treatment induces the expression of OX40L on epidermal LCs.

iv. Function of OX40-OX40L signaling

In vitro studies using $Ox40^{-/-}$ T cells showed that OX40L signal, given by APCs [104] but also T cells [105], is not important for early proliferation of activated T cells but controls late activation and proliferation. In addition, OX40L transfected human moDCs [106] and mouse fibroblasts [107] showed higher activation of T cells together with induction of interleukin (IL)-4 and IL-13 production. These observations were confirmed by *in vivo* studies which demonstrated, using agonistic anti-OX40 antibodies, that OX40-OX40L signaling is enhancing antigen-specific T cell response [108, 109]. Correspondingly, genetic mouse tools overexpressing OX40L in DCs [110] and T cells [111] showed an increased number of activated $CD4^+$ T cells.

Some reports suggested that OX40 signaling could instruct for specific T_h polarization. The idea was raised because several studies reported that mouse and human OX40L-stimulated T cells were producing high amounts of IL-4 and upregulating GATA3 expression [112, 113, 114] suggesting a T_h2 polarization of these cells.

However, these conclusions are still debated and some works report that OX40L could also enhance T_h1 polarization in presence of IL-12 [100] or the recently discovered T_{fh} polarization [115, 116]. Overall, all these reports could possibly suggest that OX40L-OX40 signaling simply enhance any ongoing polarization defined by the cytokine milieu.

The role played by OX40 signaling in T_{regs} is paradoxical in the way that OX40L signal given by effector T cells boosts T_{regs} proliferation [117] and $Ox40^{-/-}$ mice present decreased T_{regs} in the spleen and thymus at young age but not in older mice [118]. However, natural $CD25^+$ $FoxP3^+$ T_{regs} highly express OX40 and it was reported that OX40L stimulation impaired the protective role of T_{regs} in inflammatory bowel disease [118] or skin transplant [119]. This paradoxical role is even clearer when we focus on experimental autoimmune encephalomyelitis where OX40L ligation during antigen-sensitization increased T_{regs} proliferation and induced amelioration of the disease, whereas treatment at the onset of the disease worsen the phenotype [120]. This observation is confirmed in the development of diabetes in NOD mice [121].

ILC2-derived OX40L signaling has been reported to induce both T_h2 and T_{reg} differentiation *in vivo* [94, 95]. Halim and colleagues pointed that deficiency of OX40L specifically in ILC2s would

induce a reduction of GATA3$^+$ T$_{regs}$ upon IL-33 instillation in the lung. Interestingly, data from the literature suggest that IL-33 treatment would not require DCs, whereas antigens or other inflammatory molecules would rather exert their effect through DCs. Together, these observations suggest that the source of costimulation and the primary source of OX40L could be different depending on the tissue environment.

ILC3s are also able to express OX40L and intestinal ILC3-derived OX40L signaling has been shown to be required for intestinal T$_{regs}$ homeostasis [122].

Overall, all these reports highlight a critical role of OX40-OX40L signaling in T cell costimulation and in regulation of T$_{regs}$ proliferation and function, even though we do not yet fully understand what other signals drive preferentially effector or regulatory cell polarization.

v. Clinical relevance

Immunotherapies targeting OX40 or OX40L emerged few years ago in the treatment of several inflammatory diseases and malignancies.

Following the observation that high levels of OX40 were found on tumor infiltrating T cells, preclinical trials were started on mice. Promising results were obtained from the treatment of intracranial and lung sarcomas [123, 124], melanoma and melanoma-derived mammary metastasis [124, 125] as well as colon carcinoma [124, 126] with either agonistic OX40 antibody or soluble OX40L. Several OX40 stimulating compounds were subsequently generated and used in human clinical trials.

One of the first strategies was to develop mouse anti-human OX40. For instance, the clone 9B12, also called MEDI6469, is an agonistic mouse IgG1 anti-human OX40 used in several phase 1 clinical trials (ClinicalTrials.gov identifiers NCT02559024, NCT01303705, NCT02274155, NCT01644968, NCT02205333, NCT01862900, reference 127]. All results are not publicly available yet, but researchers raised concerned about the induction of human anti-mouse antibodies (HAMA) leading to decreased treatment efficacy.

To overcome such an issue, two different drugs were developed, including the constant fragment of human IgG1 fused with OX40L extracellular domain by an isoleucine zipper (hFcILZOX40L). The aim of this strategy was not only to overcome HAMA, but also to increase

the crosslinking of OX40 on T cells. Anti-OX40 antibodies are dimeric units that are expected to bind two OX40 units whereas natural ligand binds three of them. Analysis of hFcILZOX40L by chromatography, sedimentation analysis and electron microscopy revealed a hexameric complex upon self-assembly of hFcILZOX40L units which may lead to more efficient clustering of OX40 leading to a subsequent activation of the cell.

Another strategy used to avoid HAMA-mediated drug inhibition was to replace the mouse Fc of MEDI6469 by human IgG1 or IgG4 Fc, giving birth to MEDI6383 and MEDI0562 respectively. A few clinical trials have been performed for these two drugs, however their efficacy in cancer treatment remains under investigation. There are a few other drugs that were tested against malignancies through trials, including MOXR0916 and PF-04518600, humanized IgG1 and IgG2a agonistic anti-human OX40 respectively.

Besides its role in malignancies, the OX40L-OX40 axis has been studied in other mouse models of pathologies, including skin inflammatory diseases and airway allergies.

In the early 2000s, the generation of OX40 and OX40L knock-out mice revealed a crucial role of this axis in the generation of allergen-induced asthmatic response [128, 129, 130]. However, the use of germline knock-out mice does not allow for dissecting the role of this axis in the different phases of the allergic response, which are the sensitization and the challenge. To overcome this issue, anti-OX40L antibodies were injected either at the time of the sensitization or the challenge. This method confirmed the importance of OX40L signaling during the sensitization phase, but conflicting results were reported for the challenge in which the importance of OX40L for type 2 inflammation was debated [130, 131].

Only limited data from human clinical trials were gathered concerning the use of OX40L-OX40 as therapeutic target in asthma. To my knowledge, there was only one clinical trial [132, NTC: NCT00983658] which generated paradoxical results: anti-OX40L treatment induced reduced sputum eosinophils, mildly affected blood IgE, but did not decrease airway hyperresponsiveness.

Other clinical trials targeting OX40L by neutralizing antibodies in atopic dermatitis (AD) [NCT03754309, NCT03096223] did not yet give results (or not accessible online). A humanized anti-OX40 antibody (GBR 830) gave promising results in a phase 2a study [133,

NCT02683928], showing reduced cytokines expression in lesional skin as well as improved skin shape.

C. Dendritic cell populations in skin immunity

The skin forms the most exposed barrier between the inner body and the environment. It is composed of two anatomical distinct parts: 1) the dermis, the inner part, which contains fibroblasts, immune cells, blood vessels and nerves and 2) the epidermis, a highly differentiated stratified squamous keratinized epithelium containing a restricted number of immune cells under homeostatic conditions.

a. Mouse dendritic cells

In skin immunity, DCs can be first divided between skin DCs and lymph-node resident DCs. Secondary lymphoid organs contain resident plasmacytoid DCs and two populations of conventional DCs (cDCs): $XCR1^+$ cDC1s and $CD11b^+$ cDC2s. These two populations are homologs of the dermis cDC1 and cDC2 respectively, despite some differences in their surface expression of markers, such as the $CD8\alpha\alpha^+$ on the surface of lymph nodes cDC1s. Lymph node-resident DCs (resDCs) can be distinguished from skin-emigrating DCs (migDCs) according to their relative expression of CD11c and MHCII: resDCs are defined as $CD11c^{hi}$ $MHCII^{int}$ whereas migDCs are $CD11c^{int}$ $MHCII^{hi}$. We will here focus on the DC populations found in the skin, which will fall into the migDCs upon migration toward the lymph nodes.

There are some evidences that different DC populations are specialized in inducing different adaptive responses through different T_h cell polarization.

Various mouse models were developed to assess the specific role of DC populations, with elegant depletion techniques, mixing the selective ablation of transcription factors and diphtheria toxin-mediated depletion. I propose an updated and reorganized version of the review from Durai, V., and Murphy, K.M. (2016) in Immunity [134] in annex of this introduction.

i.   Langerhans cells

The epidermis represents the outer part of our skin and forms a barrier preventing both water loss and the invasion by exogenous pathogens. As the most exposed interface with our environment, highly colonized by the bacteria, viruses and fungi of the skin microbiota, it is critical to maintain immunosurveillance in this tissue. LCs represent about 5% of epidermal cells and the only populations of DCs in the epidermis in homeostatic conditions. After the reclassification from the nervous system to the immune system as DC, they were rapidly used as a model cell for antigen uptake and presentation. The embryonic epidermis is colonized by LC progenitors which are yolk sac-derived macrophages and fetal liver monocytes [135]. Early studies in adults have shown that LCs are derived from circulating bone-marrow progenitors [136, 137] but can also repopulate by proliferation [138, 139]. Further studies by Merad and colleagues [140], using bone-marrow transfer and parabiotic mice, showed that LCs are maintained by *in situ* cell proliferation under homeostatic conditions, whereas they are renewed from blood precursors under inflammatory conditions, especially from GR-1^{hi} circulating monocytes [141]. These precursors would enter the epidermis through a process controlled by the hair follicle-produced CCR2 and CCR6 chemokines [142] and generate a first wave of "short-term" LCs, which do not required the transcription factor Id-2 and present lower expression of EpCAM, CD24 and Langerin compared to classical LCs. In a second time, "long-term" LCs, which are dependent on Id-2 would reappear and repopulate the epidermis through a mechanism which is not yet fully understood [143].
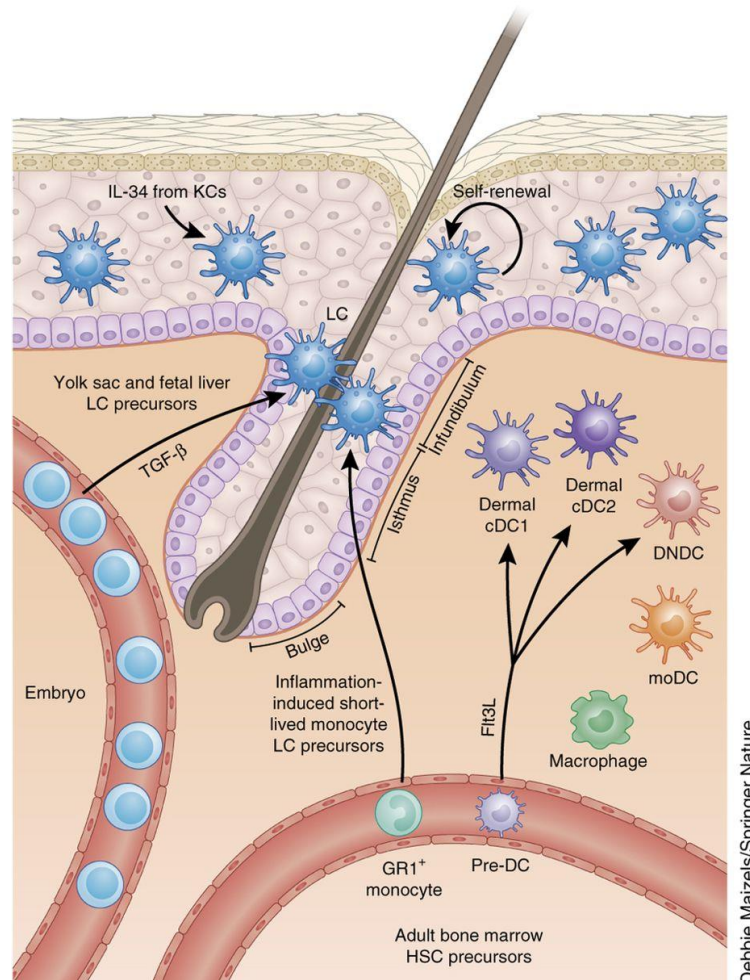
Figure 5: Skin dendritic cells in mouse dermis [144].
LCs are the only APCs of the epidermis in steady state. They originally arise from emnbryonic monocytic precursors and maintain by self-renewal. Under inflammatory conditions, LCs can replenish from GR1+ monocytic precursors that can enter in the epidermis through hair follicle. The dermis contain three populations of resident DCs that depend on Flt3/Flt3L signaling for their development and homeostasis: dermal cDC1, dermal cDC2 and CD11b- XCR1- double negative (DN) DCs.

Unlike dermal DCs, LCs do not require Flt3 signaling for their development. Development of LCs relies on several transcription factors, including Id-2, Runt Related Transcription Factor 3 (Runx3) and PU.1. Besides transcription factors, cytokines are also needed for LC development: keratinocytes-derived IL-34 signaling through colony-stimulating factor (CSF) receptor 1 (CSF1R) and TGF-β are both required for LCs homeostasis. In addition, LCs lacking TGF-β signaling because of an LC-specific deletion of *Tgfbr1*, *Tgfbr2* are not able to stay in epidermis and spontaneously migrate to the skin-draining LN [145, 146]. Interestingly, TGB-β signaling occurs at least partially in an autocrine manner, as shown by a reduced number of LC in LC-specific *Tgfb1* deletion in mice [145, 147]. Precisely, LC-derived TGF-β, secreted in its

inactive form, requires interaction with integrins expressed by keratinocytes to mature and retain LCs in the epidermis [148]. Thus, upon inflammatory conditions, keratinocytes could downregulate these integrins, indirectly triggering LC migration to skin-draining LN.

Several properties of LCs resemble those of macrophages and raise questions concerning their classification as DCs: they develop from embryonic Mafb-expressing precursors, their development is independent of Flt3-Flt3L but requires tissue-derived signals, and they are able of self-renewal but can be regenerated from monocyte precursors in inflammatory conditions. On the other hand, LCs express Zbtb46, a hallmark of DCs, present dendritic morphology, migrate to LN in a CCR7-dependent manner, express MHC-II, CD11c and efficiently stimulate T cells through antigen-presentation, which argues for a DC identity.
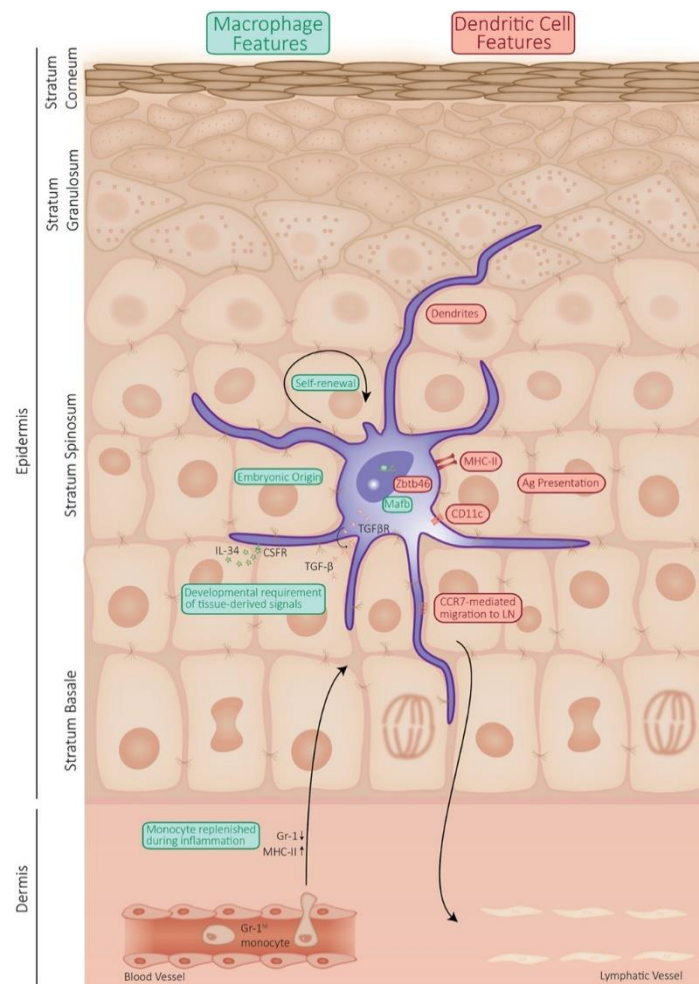
Figure 6: Langerhans cells at the border between dendritic cells and macrophages 149.
LCs form a unique population with overlapping features from both macrophages and dendritic cells. As macrophages, they derive from monocytic precursors that rely on tissue-derived signals to enter the tissue and

differentiate. Like dendritic cells, they have dendritic morphology, are able to present antigens and migrate to the draining lymph node upon CCR7 signaling.

The tools to assess the role of LC in skin immunology mostly rely on diphteria toxin (DT)-mediated depletion of LCs. Langerin-DTR mice (LangDTR) express the human DT receptor (DTR) under the control of Langerin promoter as mouse ortholog is significantly less sensitive to the toxin. In mice, this promoter is active in LCs and in Langerin expressing dermal DCs. Thus, these two populations are absent in DT-injected LangDTR mice [150]. To specifically study the role of LC, one solution is to leave the mice to rest for about two weeks before starting the treatment. This strategy allow for repopulation of dermal Langerin+ DCs but not LCs, which need up to one month to recover [103]. Another method is to generate bone marrow chimeras mixing LangDTR and WT mice; transferring WT bone marrow to lethally irradiated LangDTR mice will generate DT-sensitive radioresistant LCs but WT Langerin+ dermal DCs, whereas the transfer of LangDTR bone marrow into a WT mouse allows for the depletion of all Langerin-expressing cells but LCs. Alternatively, one can use another genetic mouse tool in which human DTR is expressed under the human Langerin promoter (HuLangDTR mice). For an unknown reason, using the human sequence triggers the expression of the receptor specifically in LCs [151]. However these models may raise controversial results: LangDTR mice, LangDTR with recovery and HuLangDTR respectively showed non changed, reduced and increased contact hypersensitivity (CHS), suggesting underlying mechanisms that are not yet well understood. TGF-β receptor 1 deletion specifically in Langerin-expressing cells, another model of LC suppression, also showed reduced CHS.

Antigen cross-presentation, as we have seen above, requires specific cellular mechanisms and thus cannot happen in all DCs. Several evidences have shown that LCs are able to cross-present skin-derived antigens. The first line of evidence comes from LCs isolated from keratin (K)14-OVA or wildtype (WT) mice that were *in vitro*-pulsed with ovalbumin or epidermal sheets that were cultured on an OVA-containing solution. LCs were subsequently co-cultured with OT-I CD8+ and OT-II CD4+ T cells and were able to induce proliferation of both populations [152]. Of note, epidermal delivery of nanoparticles induced cytotoxic T lymphocyte (CTL)-mediated response which was largely diminished in LC-depleted HuLangDTR mice [153] despite reports that LC-mediated cross-presentation after antigen targeting to Langerin without adjuvants leads to tolerance [154, 155].

LCs have also been implicated in the generation of skin T_h17 response against fungi and extracellular bacteria. Indeed, Kashem and colleagues [156] have shown that LCs are required for inducing a protective T_h17 response through IL-6 in a model of skin infection by *C. albicans*. These data have been extended to human by Mathers and colleagues [157] who used a model of skin explants to show that LC-derived IL-6 was required for an efficient T_h17 response. Moreover, similar results were obtained in a mouse model of *S. aureus* infection [158].

To my knowledge, state of the art literature does not suggest a role of LCs in the induction of T_h2 immunity: depletion of Langerin^{+} cells or LCs before HDM [159] or papain [160] sensitization respectively do not impair the T_h2 inflammation upon challenge.

Several reports suggested a role of LCs in triggering humoral immunity and antibody production. In a model of *S. aureus* skin infection, Ouchi and colleagues [161] showed that LCs capture extracutaneous antigens that did not penetrate into the epidermis and can trigger subsequent antigen-specific antibody response. In addition, Zimara [162] and Levin [163] showed, with intradermal injection of *L. major* or nanoparticles, that depletion of Langerin[+] DCs impairs T cell-B cell interaction and germinal center formation.

DCs have been shown in the beginning of the 2000s to be able to induce CD4 [164] and CD8-mediated tolerance [165]. Several studies have demonstrated a role of LCs in tolerance induction. Shklovskaya and colleagues [166] demonstrated, using WT mice and bone marrow chimeras in which the experimental antigen can only be specifically presented by LCs, that LCs were regulating T cell activation. Kautz-Neu and colleagues [167] have shown, using a mouse model of Leishmania infection, that LCs are involved in the induction of T cell tolerance and linked with a stronger pathologic phenotype. In the context of allergy, Agüero and colleagues [168] found that depletion of Langerin[+] DCs breaks tolerance to hapten and Dioszeghy showed that LC depletion reduces T_{reg} population upon transcutaneous ovalbumin sensitization [169]; Strandt and colleagues showed that neoantigens presented by LCs only induce CD8^{+} tolerance and CD4^{+} T_{regs} accumulation in the skin. Kitashima and colleagues [170] suggested that LCs could then prevent autoimmunity against epidermal antigens through presentation by LCs and subsequent induction of polyclonal tolerance. This property was confirmed in human skin by

Seneschal and colleagues [171] who demonstrated that human LCs can induce the proliferation of skin-resident T_{regs} *in vitro*.

The migratory capacity of LCs, which was debated in a first place due to their macrophagic origin, is now well established. However, whether LCs need to migrate to exert their function is usually not assessed with caution. Despite the remarkable and increasing amount of data that was generated on the regulatory role of skin LCs, it is rather unclear whether their tolerogenic role is exerted locally in the skin or in the draining lymph node. Pieces of evidence, using mice lacking a protein required for the remodeling of the cytoskeleton, suggest that LCs which fail to migrate from the epidermis have impaired regulatory function [172] but it remains undetermined whether migration to the dermis or to the draining LN is required for tolerance induction. Another study, using a model of contact hypersensitivity [173], suggest that cognate interaction between LCs and T cell is critical, because mouse lacking MHCII are not able to induce tolerance, but the authors did not find any difference in the FoxP3$^+$ population in LN upon depletion of LCs, which could mean that the interaction actually happened with skin T_{regs}. On the other hand, another study in which RANKL overexpression is induced in the epidermis showed an increased T_{reg} population in the spleen and the draining-lymph node [174] but these results do not allow to distinguish a direct induction in the lymphoid organs or a rehoming of skin T_{regs}. It was also shown that expression of neoantigens by LCs could lead to skin accumulation of T_{regs} [155] but the implication of LCs on the activation of those cells remains to be determined, as well as the exact mechanism of their accumulation in the tissue.

Senechal and colleagues showed with human cells that LCs could activate skin-resident T_{regs} *in vitro*, but such an evidence is lacking in mice and could probably be dependent on the nature of the antigen.

Overall, the review of the existing literature concerning the role of LCs in T_h cell polarization shows a strong regulatory phenotype under homeostatic conditions that can be either direct or mediated by T_{regs}. Once LCs receive inflammatory stimuli, they switch to a pro-effector phenotype that depends on their environment.

ii. Dendritic cell populations in the dermis

Mononuclear phagocytes from the dermis form a more complex network than that of epidermis. Steady state dermis contains three populations of conventional dendritic cells (cDCs), which are XCR1^{+} (cDC1s), CD11b^{+} (cDC2s) or double-negative cDCs (DN cDCs), and monocyte-derived DCs (mo-DCs). In addition to dermal DCs, "en route" LCs that cross the dermis on their way to the skin-draining lymph node can be detected in the dermis.

All these DCs express thee pan-classical DC transcription factor Zinc finger and BTB domain containing 46 (Zbtb46) and all, but the mo-DCs, are dependent on FMS-like tyrosine kinase 3 ligand (Flt3L) signaling, as opposite to tissue resident monocytes/macrophages which rely on CCR2 signaling.

Dermal cDC1

Dermal cDC1s form a population of about 5% of dermal DCs [103] and express specific surface markers such as the X-C motif chemokine receptor 1 (XCR1, receptor of XCL1 chemokine), the C-type lectin domain containing 9A (Clec9A, a receptor for F-actin), CD103 (also kown as integrin alpha E; ITGAE) and CD24. Of note, mouse but not human cDC1s express the marker Langerin, creating confusion in several studies that used Langerin as a critical marker specific for LCs.

The homeostasis of cDC1s requires signaling through Flt3 [175] and the development of these cells partially requires, among others, the transcription factors basic leucine zipper transcription factor, ATF-like 3 (Batf3) [176, 177, 178], Id-2 [178] and IRF8 [179]. The dependence on specific transcription factors for the development of cDC1s led to the engineering of knockout mice to study the functional importance of cDC1s in different contexts. Other strategies were developed, like the use of chimeras in which LangDTR bone marrow (BM) is injected into wild type mice. Upon DT-injection, Langerin^{+} cDC1 would then be depleted but not LC.

An overwhelming amount of data demonstrate the ability of cDC1 to cross-present antigens and induce CD8^{+} T cell response. Henri and colleagues demonstrated that dermal CD103^{+} cDC1s are efficient at cross-presenting keratinocyte-derived antigens in mouse [103], while

Bedoui et al. showed that CD103$^+$ cDC1s can also cross-present viral-derived antigens [180]. Review of the literature reveals that the cDC1 counterparts found in lung or lymph nodes efficiently stimulated antitumor response [176, 181, 182], possibly involving Clec9A, which could allow cDC1s to collect damaged or necrotic cells through binding of filamentous actin.

In addition to its cross-presenting capacities that allow for CD8$^+$ T cell activation, cDC1-derived IL-12 has also been reported to be critical for T$_h$1 polarization [183, 184]. This point is interesting in the way that cDC1s would then form a unique subset that is highly efficient in inducing both T$_h$1 response and CTL response through IL-12 production and cross-presentation respectively. The dependence of these DCs on IFN type I [181, 182] suggest the need of a synergistic activation of cDC1s and IFN type I producing cells, such as pDCs, to induce an immune response, which could represent a safety mechanism against auto-immunity.

Overall, these data raised a keen interest from the community which eventually led to the initiation of several preclinical trials, including one [185] in which mice were pretreated or not with influenza hemagglutinin linked to XCL1, acting as a cDC1-targeted vaccine. Results were very promising as vaccinated mice were fully protected against a lethal challenge with influenza virus.

The ability of cDC1 to induce T$_h$1 response and to activate CTL response generated great promises concerning the development of so called "cancer vaccines", a form of active specific immunotherapy. State of the art literature, as well as the advances and remaining obstacles in this field have been recently reviewed in ref 185.

Dermal CD11b$^+$ cDC2

Type 2 cDCs (cDC2s) form the biggest DC population in both human and mouse. They are characterized by the expression of CD11b, MHC-II and no expression of CD24 or Langerin [103].

Their expression of CX$_3$CR1, SIRPα and CCR2 generated difficulties in the identification of dermal CD11b$^+$ cDC2s because of the presence of dermal resident macrophages that shares these parameters. Several studies assessing the requirement of different cytokines or transcription factors for the development or the homeostasis of cDC2 were thus probably

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biased by the remaining MHC-II^{+} CD11b^{+} macrophages which are impossible to distinguish from cDC2 without specific monocytes/macrophages markers such as CD64 and Ly-6C [188].

The development of dermal cDC2s also relies on Flt3 signaling [175]. Their development is dependent on PU.1 [189], V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog B (RelB) [190] and Recombining binding protein suppressor of hairless (RBP-J) [191]. Both human and mouse cDC2s express IRF4 and mouse studies have shown that *Irf4*^{-/-} mice still develop dermal cDC2s but these cells harbor impaired migration under steady state and inflammatory conditions [192].

The function of CD11b^{+} cDC2s has been assessed in different anatomical locations, including gut and lungs. It is important to note that the phenotype of DCs can slightly vary between different tissues: one clear example of this variability is illustrated by the expression of CD103 by IRF4-dependant cDC2s in the gut of both human and mouse. Functional studies concerning the role of cDC2s across tissues should thus take these phenotypic specializations into consideration.

In contrast to the role of cDC1s in inducing T_h1 and CTL responses, cDC2 have been reported to be specialized in T_h2 and T_h17 induction by using DC-specific deletion of *Irf4*. Several teams have confirmed these findings in mouse models for gut [193], skin [194] and lung [195, 196] inflammation. Kumamoto and colleagues [197] have proposed the use of CD301, also known as macrophage galactose-type C-type lectin 2 (MGL2), as a surface marker to discriminate for cDC2 and they generated Mgl2DTR mice that allow DT-mediated depletion of those cells. Data obtained from Mgl2DTR mice confirmed the T_h2-inducing capacities of CD301b^{+} cDC2s but the specificity of this depletion strategy should lead to cautious interpretation of the results as LCs and skin macrophages are also affected. Pushing further the delineation of subpopulations inside cDC2s, Tussiwand and colleagues [198] identified a subset of CD11b^{+} DCs whose development depends on Kruppel-like factor 4 (Klf4). Using mutant mice harboring a DC-specific deletion of this gene, they demonstrated that CD11b^{+} IRF4-dependant cDC2s contain a population of T_h2-inducing Klf4-dependant DCs.

In addition, IRF4-deficient mice also present a defective T_h17 response in the gut [199, 200] due to the lack of IL-23 produced by CD11b^{+} DCs [201]. Spleen CD11b^{+} can be separated into

two subpopulations, one presenting a high expression of ESAM, whose development is impaired in Notch2-deficient mice [202]. Even though this effect may not be a direct effect on CD4$^+$ T cells but rather a DC-ILC-T cells axis, data indicate that in absence of these DCs, we observe a lower number of IL-17 producing T$_h$17 cells in the intestine [202]. Of note, CD11b$^+$ cDC2s may not be the only subset responsible for T$_h$17 induction, as Batf3-dependant cDC1s have been reported to be necessary for IL-17 production by CD8$^+$ cells in the skin [203].

In addition, cDC2s could be involved in the process of immune response regulation. Guilliams and colleagues [204] demonstrated that skin-derived CD11b$^+$ DCs express *Aldh1a2*, a gene encoding retinaldehyde dehydrogenase 2 which is involved in the production of retinoic acid (RA). RA is known to induce the formation of T$_{regs}$. This study was surprising and exciting because peripheral tolerance is usually generated in the gut where CD103$^+$ DCs express *Aldh1a2* and this regulatory activity could be important both under homeostatic conditions but could also be used in therapeutic approaches of desensitization.

Overall, the cDC2 population contains heterogeneous subtypes of DCs across different tissues. The heterogeneity reflects not only the lack of specific markers for cDC2s that would allow for a specific definition of these cell, but also the various states of activation of cDC2s, suggesting that those subtypes could be specialized in inducing different type of immune responses. However, this heterogeneity is also a challenge in the field and the development of tools to further delineate the ontogeny, the requirement of transcription factors and the function of these subsets will be of major interest in the next years.

Dermal double negative cDCs

Dermal DN DCs are characterized as CD11b^{lo} CD24$^-$ XCR1$^-$ cells. Their development relies on IRF4 and Flt3L, like CD11b$^+$ cDC2s and they express the cDC lineage-specific transcription factor Zbtb46. DN-cDCs are CCR2^{int} and CX$_3$CR1^{hi} whereas CD11b$^+$ cDC2s are CCR2^{hi} and CX$_3$CR1^{int} which can help to distinguish them.

Functional studies of this subset are difficult and results obtained from mouse models of *in vivo* depletion should be interpreted with caution since no specific marker was identified. Thus the use of the promoter of CCR2 and CX$_3$CR1 to direct the expression of human diphtheria

toxin receptor is an imperfect strategy that leads to the depletion of both DN-cDCs and CD11b$^+$ cDC2s in addition to subsets of monocytes/macrophages.

Monocyte-derived DCs

MoDCs are a challenging population to study. For years, we did not know the specific surface markers allowing to distinguish CD11b$^+$ DCs, moDCs from dermal monocytes and macrophages. In 2013, Tamoutounour et al. identified the different cell populations falling in the CD11b$^+$ gate in dermis and reported the antibody panel to be used to distinguish them [188]. Using gene profiling analysis, they reported that moDCs presented a strong "DC signature", like CD11b$^+$ cDC2s, whereas other CD11b$^+$ cells present rather a macrophage signature, demonstrating the heterogeneity of CD11b$^+$ cells. It was shown that *Ccr2$^{-/-}$* mice were deprived of moDCs and dermal monocytes, and that these dermal monocytes were the tissue precursors of moDCs. These cells have poorer migratory capacity compared to CD11b$^+$ cDC2s and, despite similar gene expression profile upon migration, present lower T cell activation capacity compared to CD11b$^+$ cDC2s. Interestingly, upon hapten-induced skin inflammation, an increased number of CD11b$^+$ monocytes was observed in the dermis and part of them initiated a maturation toward moDCs, but showed an incomplete maturation phenotype with an intermediate phenotype (e.g. they still express Ly-6C). In addition, both these moDCs and CD11b$^+$ cDC2s showed around 500 and 1000 genes differentially regulated respectively in hapten-treated inflamed skin with about 200 genes commonly changed. This suggest that, despite their completely different origins, moDCs and CD11b$^+$ cDC2s could exert overlapping functions in inflamed skin.

Plasmacytoid DCs

In contrast to other dendritic cells which can migrate to peripheral non-lymphoid organs upon release from the bone marrow, pDCs usually migrate to secondary lymphoid organs, such as lymph nodes. Indeed, pDCs are not found in steady state human or mouse skin, but can be recruited upon virus infection [206], skin injury, or chronic inflammation [207] including psoriasis [208]. These cells are the major source of type I IFNs under inflammatory conditions,

and they are well equipped to detect viral infections, as shown by their expression of endosomal TLR which recognize viral RNA (TLR-7) and DNA (TLR-9).

b. Human dendritic cells

The classification of human and mouse DCs has first suffered from the same limitation, which was the use of surface markers to define DC populations. At first, with the development of flow cytometry and monoclonal antibodies, this strategy allowed for the initial and critical steps for the characterization of dendritic cells and macrophages in the tissues. However, this may not only lead to an overestimation of the number of subsets, but also group the cells according to a parameter which does not reflect their functions, nor their ontogeny. And finally, the expression of surface markers may vary between species, for instance between human and mouse.

A unified classification for macrophages and dendritic cells was proposed a few years ago, based on the ontogeny and the dependence of several transcription factors, thus based on mouse data. This created the current classification of DCs that I used in this introduction since the beginning, including monocyte-derived cells, conventional DCs, pDCs and LCs [209]. However, the authors of this opinion paper suggested that this classification could be similar between mouse and human, because of previous gene expression analysis that revealed the close identity of DCs between these two species [210, 211] (and see figure 7 below), despite some interindividual variation observed in human [212].
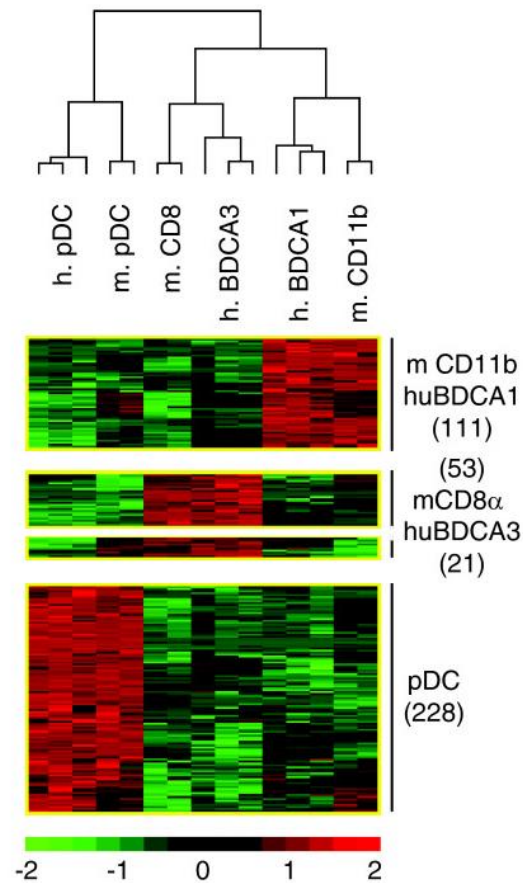
Figure 7: Comparison of the expression of selected genes between mouse and human DCs [223].

Human (h.) and mouse (m.) DC populations cluster close to each other in gene expression analyses, showing similar expression patterns.

The transcription factors and classical markers expressed by human DCs are summarized in the table below. Interestingly, a few surface markers are conserved between human and mouse (e.g. XCR1, EpCAM, DCIR) and the transcription factors are well conserved across the species (e.g. IRF4 and KLF4 for cDC2s, E2-2 for pDCs, IRF8 for cDC1s).

DC population	Differential TFs	Classical markers	Other markers
Langerhans cells	ID2 Runx3	CD207 CD1a E-Cadherin	EpCAM TROP2

50

cDC1s	ID2 IRF8 BATF3	CD141 / BDCA-3	CLEC9A XCR1 BTLA CADM1
cDC2s	ID2 ZEB2 IRF4 KLF4	CD1c / BDCA-1 CD11c CD11b	CD2 FCER1 SIRPA DCIR
pDCs	E2-2 ZEB2 IRF4 IRF8 …	CD123 BDCA-2 BDCA-4	FCER1
moDCs	MAFB KLF4	CD11c CD14	SIRPA CD206 DC-SIGN

Table 2: human skin DC populations, their transcription factors (TFs) and markers [197].

i. Langerhans cells

Like in mouse, human LCs are resident in skin epidermis and they express the C-type lectin langerin. Of note, a low expression of langerin was reported in several DC subsets, but to a much lower extend than that of LCs [213]. Moreover, LCs express the invariant MHC-I molecules CD1a and CD1c. They are retained in the epidermis by E-Cadherin and EpCAM, and express tight junction proteins claudin and occludin. In addition to their expression of CD1a and EpCAM, LCs can be distinguished from cDC2s by their low expression of CD11c and CD11b. However, they should not be confused with cDC1s which, in contrary to mouse DCs, do not express langerin. The close proximity of human and mouse LCs has recently been proven by comparative genomics analysis [214].

The function of human LCs is often assessed using *in vitro* derived LC-like DCs, often using TGF-β-induced differentiation, however whether this technique reflects the true potential of

LCs remains under debate. It was observed that higher frequency of LCs in human ulcerated skin in diabetic patients is associated with a better outcome [215], however, this association was not observed in PU.1 null mice which lack LCs [216], thus leaving the role of LCs in wound healing unresolved. Concerning their regulatory capacity, *in vitro* coculture studies have demonstrated that human immature LCs can activate and expand skin resident T_{regs} [171]. On the other hand, human LCs show an activated state in lesioned AD skin, with a higher expression of CD80 and CD86 [217], and in patients with defective epidermal barrier function (i.e. filaggrin mutations) [218] probably because of the high production of TSLP in AD skin [219, 220].

ii. Dermal dendritic cells

Despite the use of different surface markers to differentiate human and mouse dermal DCs, several populations can be described in the tissue of both species:

CD141+ XCR1+ cDC1s

As we have seen for mouse cDC1s, the main characteristic of these cells is their ability to cross-present antigens and induce the CD8+ T cell and Th1 response. The functional and phenotypic homology between mouse CD103+ non-lymphoid DCs and human CD141hi (BDCA3hi) DCs has been shown by Haniffa et al. [221] and later confirmed by genomics analysis from Carpentier et al. [214]. This last study also emphasizes the difference between cDC1s and LCs, both in human and mouse, where the surface markers have caused confusing results in previous studies.

CD11b+ cDC2s

Mice cDC2s have appeared to contain smaller subsets of Th2- and Th17-inducing DCs, as we have seen above. This capacity to induce Th17 cytokines, especially IL-17, has been shown in human IRF4-dependent CD11b+ cDC2s [222]. In addition, genomic expression analyses have shown the close proximity of CD11b+ CD1c+ (BDCA1+) cDC2 cells with mice CD11b+ cDC2 cells [223]. Of note it remains to determine whether cells classified as CD14+ cDC2s represent

monocyte-derived DCs or bona fide cDC2s, although gene expression analyses show a rather DC signature from these cells [224, 225].

Plasmacytoid dendritic cells

Human pDCs do not express CD11b, as seen in mice, nor CD11c although the latter is expressed at low level in mice. They rather keep the expression of CD123 (IL-3R) and B220 which are markers of hematopoietic precursors. In human, they express CD4 at a higher level than other DCs, and they express BDCA-2 and BDCA-4 (also known as CD303 and CD304 respectively). Functionally, they are closely related to mice pDCs with the expression of TLR7 and TLR9 to detect viral nucleic acids. Upon binding of their cognate ligands, TLR signaling will induce a high expression of type I and type III interferons by pDCs, and the initiation of antiviral response.

Conflicting results have been obtained for the role of pDCs in allergy [226, 227] and as tolerogenic cells in cancer [228].

Monocyte derived DCs

Like in mice, moDCs are already present in steady state human skin [229]. They are defined as $CD14^+$ $CCR2^+$ dermal $CD11c^+$ DCs but they express also CD11b and CD172a, which may cause confusion between moDCs and human dermal cDC2s. Their number if largely increased, upon inflammatory conditions like in eczema [230], psoriasis [231, 232] and even skin blister [233].

Overall, despite difference in the surface markers that have been used, DCs seem to be well conserved between mice and humans. In the next part, we will briefly see why the study of DCs is crucial, both for the understanding of human disease, but also for the development of new generation therapeutics.

D. Dendritic cells in disease

In this part, I would like to review two pathogenic contexts in which DCs are involved: the role of DCs in some disease pathogenesis and a few examples of genetic deficiencies in DCs. For the first part, I will focus exclusively on skin diseases.

a. Primary deficiency of dendritic cells

There are several syndromes, identified in human, in which DCs are more or less deeply impaired, either for their differentiation, accumulation or function. This presentation is mainly based on a recent review from Bigley et al [241].

Pancytopenia

A reduced number, or complete absence of DCs is observed in some human syndromes. Most of the time, it is due to an altered hematopoiesis with number of defects in the immune system. Here we will focus on the defects in DC differentiation of accumulation.

Mutations in adenylate kinase 2 (AK2) can lead to the reticular dysgenesis syndrome, which is characterized by a global loss of monocytes, DCs and LCs. It is the most severe form of severe combined immunodeficiency (SCID) with absence of secondary lymphoid organs and it systematically leads to death at birth or within days after birth [242, 243].

The biallelic mutation of IRF8 and the heterozygous mutation of GATA2 cause hematopoietic troubles which lead to complete absence of monocytes and DCs, but preserve tissue macrophages and LCs. As a consequence, patients are at higher risk of mycobacterial and viral infections, but haploinsufficiency of GATA2 was also linked with autoimmunity, myelodysplasia and acute myeloid leukemia.

Mutations in the chemokine receptor CXCR5 can cause the WHIM (warts, hypogammaglobulinaemia, infections and myelokathexis) syndrome. This is caused by an accumulation of hematopoietic cells in the bone marrow because of defective chemotaxis. Therefore, reduced number of monocytes and DCs are observed in periphery.

A specific absence of pDCs is observed in patients with heterozygous mutation of IKZF1, also called IKAROS; this is accompanied by an increase in cDC1s. Regarding the clinic, people suffering from IKZF1 haploinsufficiency develop a common variable immunodeficiency with impaired humoral response.

Functional deficiencies

Several mutations can alter the presentation of antigens through MHC-II and generate a SCID-like syndrome, with defective CD4^{+} T cell and antibody responses [244]. These patients present recurrent bacterial, fungal and viral infections and their lifespan is lowered to less than 10 years in absence of hematopoietic stem cell transplantation [245].

The Wiskott-Aldrich Syndrome is caused by a mutation in the WAS gene. This gene encodes a protein involved in the rearrangement of the cytoskeleton. This syndrome leads to defective DC migration, impaired DC:T cell interaction because of issues in the immune synapse which results in ineffective T cell and B cell responses [246].

Haploinsufficiency of TCF4 causes the Pitt-Hopkin's Syndrome in which the number of pDCs is reduced, and with defective IFNα production [247]. Patients do not present critical defects in immunity, and no other obvious impairment of the hematopoietic system.

Patients with IRF7 deficiency present impaired type I and type III IFN responses and, despite a normal number of circulating DCs, they are more susceptible to severe virus infections, such as influenza.

This list is not exhaustive. There are reported deficiencies downstream of TLR signaling for example, which cause severe immunodeficiencies due to impaired sensing of danger by dendritic cells. Other mutations in signaling pathways downstream of cytokines receptors, such as STAT3 mutations, lead to defects that alter Th17 response for example.

b. Role of dendritic cells in skin disease

Psoriasis

Psoriasis is a common chronic T-cell mediated disease. The current understanding of the pathogenesis of psoriasis points to multiple contributions of pDCs to the disease pathogenesis. The production of type I IFN by activated pDCs has been linked with the initiation of psoriasis [208], in which IFNα turns up the production of IL-23 and IL-17 in the skin, inducing Th17 cell differentiation, recruiting neutrophils and activating mast cells and γδ T cells. Several data suggest the strong role of pDCs in early psoriasis: the pathogenesis of the disease in mice can

be blocked by neutralizing type I IFN pathways [208]; in addition, psoriasis susceptibility has been linked with IRF5 [234] and the signaling pathway of TLR9 [235, 236], which are involved in type I IFN production by pDCs. The absence of pDCs from psoriatic lesion at later time points show that the importance of these cells decreases with time, suggesting that targeting these cells may only be useful in early pathogenesis.

Lupus erythematosus

Lupus erythematous (LE) is a complex autoimmune disease, which can range from localized skin lesions to systemic multiorgan invasion. Most of the patients develop characteristic antinuclear antibodies and patients suffering from cutaneous (C)LE often present anti-SSA/Ro and anti-SSA/La anti-ribonucleoprotein antibodies. Patients suffering from active CLE present elevated blood levels of type I IFNs and upregulated interferons-regulated genes. The role of pDCs is thus well established in CLE, and they represent now an interesting therapeutic target. Indeed, anti-interferons antibodies are now being developed and tested in humans to ameliorate the disease, and some studies even sensitized humans to develop anti-IFNα antibodies, which led to an ameliorated condition [237].

Langerhans cells histocytiosis

LC histocytiosis (LCH) is a myeloid neoplastic disorder characterized by an abnormal accumulation and behavior of these cells. It is rather a syndrome than a disease, since some patients will develop specific bone, skin or disseminated lesions, which vary in cellular composition and anatomical location. LCH is a pediatric granulomatous disease which affects 4 to 8 children per million, and 1 to 2 adults per million. LCH that affect specifically the skin are rare (2% of the cases) but the early symptoms of the disease usually start in the skin. The etiology and the pathogenesis of this threatening syndrome are not yet fully understood.

Lichen planus

Lichen planus is an autoimmune inflammatory disease in which CD8^{+} cytotoxic T cells cause the destruction of epidermal keratinocytes which has been associated with type I interferons

and pDC infiltration [238, 239, 240]. However, despite a strong association, the role played by pDC in the pathogenesis has not yet been elucidated.

c. Atopic dermatitis (AD)

AD, also known as atopic eczema, is common chronic inflammatory skin disease which affects about 10 to 20% of children worldwide and 1 to 3% of adults [249]. Of note, the prevalence of this disease increased two to three times over the last 30 years in the industrialized countries, creating major public-health concerns. Interestingly, the distribution of AD varies wildly across the word, ranging from about 1% in Iran to almost 17% in Japan [250] and showing broad disparity between northern and western countries of Europe, or Asia and Africa for instance [250]. Moreover, people with the same ethnic origin can present a higher risk of developing AD if they live in urban region, come from a higher social class, a smaller family or use more antibiotics [251, 252], suggesting that both genetics and environmental factors play a crucial role in AD pathogenesis.

From a clinical point of view, AD patients present a broad range of symptoms with varying severity from dry depigmented skin (pityriasis alba) to erythrodermic rashes. The diagnosis is mainly based on the pruritus and chronic or relapsing eczemateous lesions with a typical distribution on the face, the scalp, the extensor aspect of the knees and the shoulders in children which progress to the flexors and hands later in life. Chronic inflammation often leads to the lichenification of the erythematous and oedematous papules, a process involving the rubbing and itchiness. The onset of the disease usually takes place before 2 years of age, and typically between 2 and 6 months, although a recent meta-analysis suggested that up to 1 in 4 patients had an adult-onset of AD [253].

A large European study assessed the burden caused by AD to individual adults [254] using tools specifically designed to this aim and are questionaries which evaluate the quality of life, the anxiety and the severity as evaluated by the patient. This work showed that a dramatic 88% of patients with severe AD estimate that their disease at least partly compromised their ability to face life. In all AD patients, 57% were emotionally affected and reported issues with intimacy, trying to hide eczmateous lesions or feeling guilty about eczema and 10% showed

indications of depression which could be linked to loss of sleep. In addition to this terrible outcome on patients' life, about 57% of the responders reported to have missed at least 1 day of work in the previous year due to AD, causing an economic burden and underlining the public-health concern caused by AD. In addition, children with eczema present a higher prevalence and more severe asthma and increased risk to develop allergic rhinitis and food allergies later in life. Precisely, about 80% of children which develop AD will experience either asthma of allergic rhinitis [255], suggesting that skin sensitization could lead to respiratory diseases.

The pathogenesis of AD remains uncompletely understood, although it is well recognized that it relies on both the defects in epidermal skin barrier and an abnormal type 2 skin immune response. The importance of the epidermal barrier is suggested by genome wide association studies (GWAS) and single nucleotide polymorphism (SNP) which reveal a strong association between mutations in genes involved in the epidermal barrier such as fillagrin, SPINK5 or claudin-1, and AD. On the other hand, genes coding for immune players such as MHC-II, IL-4 or IL-4Rα were also strongly associated with AD [256].

Patients can be split into two subtypes: about 80% of AD patients present elevated blood IgE, referred to as an extrinsic endotype, whereas the remaining 20% show total and allergen-specific IgE within the normal range and is named intrinsic endotype. In parallel, peripheral blood monocytes from AD patients have a decreased capacity to produce interferon-γ, a higher frequency of IL-4-, IL-5- and IL-13-producing allergen-specific T cells and an increased expression of vascular adhesion molecules which facilitate the extravasation of immune cells, such as eosinophils, a hallmark cell of allergic inflammation. Altogether, these parameter show an increased type 2 systemic response in AD patients, accompanied with a lower type 1 environment.

Analysis of the skin of AD patients reveal the presence of IgE molecules both in the dermis and epidermis, which can readily trigger an acute response upon encounter with the cognate antigen [257]. These IgE bind to the recepteor FcϵR which is present on the surface of DCs, but also basophils and mast cells. Of note, chronic lichenified lesions have an increased number of IgE-bearing LCs as well as inflammatory dendritic cells, which express the receptor

for IgE and have been shown to be required for the inflammatory response in AD skin [258]. Non-lesioned skin of AD patients present an increased number of CD4$^+$ T cells in the dermis compared with skin from healthy donors, and these cells are found further increased in lesioned skin, associated with eosinophils which can contribute to the tissue inflammation through the production of reactive oxygen species, pro-inflammatory cytokines and the release of their granules.

First of all, it is recommended to atopic patients to avoid the triggering factor when it is identified and stressfull situations. These factors vary from patient to patient and often can include skin irritants (soap, detergents, clothing), sweat, dry air, food allergens (eggs, milk) or house dust mite and pets. The first line of treatment of AD consists of bathing/showering with emollients to reinforce the skin barrier impairment, in addition to topical corticosteroid to control the tissue inflammation. These can be accompanied by antihistamitics and even antibiotics on short periods to take care of any bacterial infection such as *Staphylococcus aureus* which colonize the skin of amost 90% of AD patients. Failure of these treatment may lead to more intensive therapies, ranging from intensive topical treatment with steroids to systemic immunosuppressive chemicals such as ciclospirin or methotrexate, but new therapies can also be investigated, such as wet wrap techniques or phototherapy. Recently, immunotherapies have been developed to treat atopic dermatitis. Dupilumab is a monoclonal antibody that recognizes the subunit IL-4Rα which is shared by the receptors to IL-4 and IL-13. Dupilumab showed a high efficacy in treatment of moderate-to-severe AD which did not respond to topical glucocorticoid treatment and calcineurin inhibitors [259].

The problematics of antigen densitization is of high interest. There is a complex relationship between levels of exposure and tolerance, and recent data suggest that the induction of tolerance requires the exposure to high doses of antigen. The first clinical trial on antigen densensitization, or allergen immunotherapy, dates back from 1955, and it showed that the administration of high doses of pollen was able to moderate hay fever [260]. This concept thus raises questions concerning the implementation of allergen avoidance in the every-day life of AD patients, as is could potentially prevent allergen desensitization.

As we have seen previously, DCs play a crucial role in allergen sensitization, both in the skin and airways. In the skin, the production of cytokines by the epithelium, such as TSLP or IL-33, play a crucial role in activating DCs toward a proinflammatory Th2 inducing phenotype. These DCs will then induce T cell maturation and thus allergen sensitization. As we have seen above, Tussiwand et al. [198] described two populations of IRF4-dependent cDC2s according to KLF4-dependence: KLF4-dependent cDC2s that trigger the Th2 response and the others that mediate the Th17 response. Deckers et al. observed indeed that both KLF4-dependent and KLF4-independent cDC2s can induce the allergic Th2 response depending on the context in which the allergen is provided, e.g. epicutaneously versus intradermally [159]. Concerning LCs, it has been shown that TSLP, a skin keratinocyte-derived alarmin, can trigger the activation of LCs and license them to induce a pro-allergic Th2 immune response [219, 220]. Moreover, Nakajima and colleagues showed that the TSLP-LCs axis is crucial for epicutaneous allergen sensitization [261].

E.   Dendritic cells as therapeutic targets

The pivotal role of DCs in the initiation of the immune response prompted researchers to use them to induce antigen-specific immune responses. Moreover, the increasing understanding of the secondary and third signals given by DCs to T cells, which not only activate but also polarize the adaptive response presents a tremendous interest not only to elicit type specific responses (e.g. cytotoxic, humoral, Th2, Th17…) but also for immune regulation by triggering regulatory T cell differentiation and expansion. Some current strategies for cancer treatment, including immune checkpoint blockade of CTLA4 already aim at increasing the activating capacity of DCs.

a. Immunotherapies for cancer, allergy or autoimmune diseases

In a recently published review, Sabado et al. [265] exposed their opinion on the current and future techniques of DC-based immunotherapies (as active antigen-specific immunotherapies, they call it "DC-based vaccines"). Nowadays, DC based immunotherapies consist of isolating hematopoietic precursors of DCs, either CD34$^+$ hermatopoietic precursors (from bone

marrow), or monocytes (from the blood). These cells can then be differentiated *in vitro* in DCs or moDCs, loaded with the desired antigen, activated using the desired stimuli (including cytokines and TLR ligands for example) and reinjected back into the patient. This approach, however, is time consuming, expensive and requires to use either the patient's DCs or HLA-matched allogenic DCs to induce a strong immune response.

Provenge is a FDA-approved treatment which include blood DCs, B cells, monocytes and NK cells activated *in vitro* and that showed some effect in treatment of hormone-refractory prostatic cancer. Several clinical trials are going on to test the effect of Provenge in combination with other immunotherapies (NCT01420965, NCT01832870, NCT01804465, NCT01881867).

Remarkably, there are also clinical trials using so-called "naturally occuring DCs" which were *ex vivo* isolated. As of April 2019 [248], three clinical trials were ended with published results [262, 263, 264], one was terminated before the end (ACTRN12607000450415) and 5 were still ongoing (NCT02574377, NCT02692976, NCT02993315, NCT03707808, NCT03747744) either alone or in combination with other cancer immunotherapies.
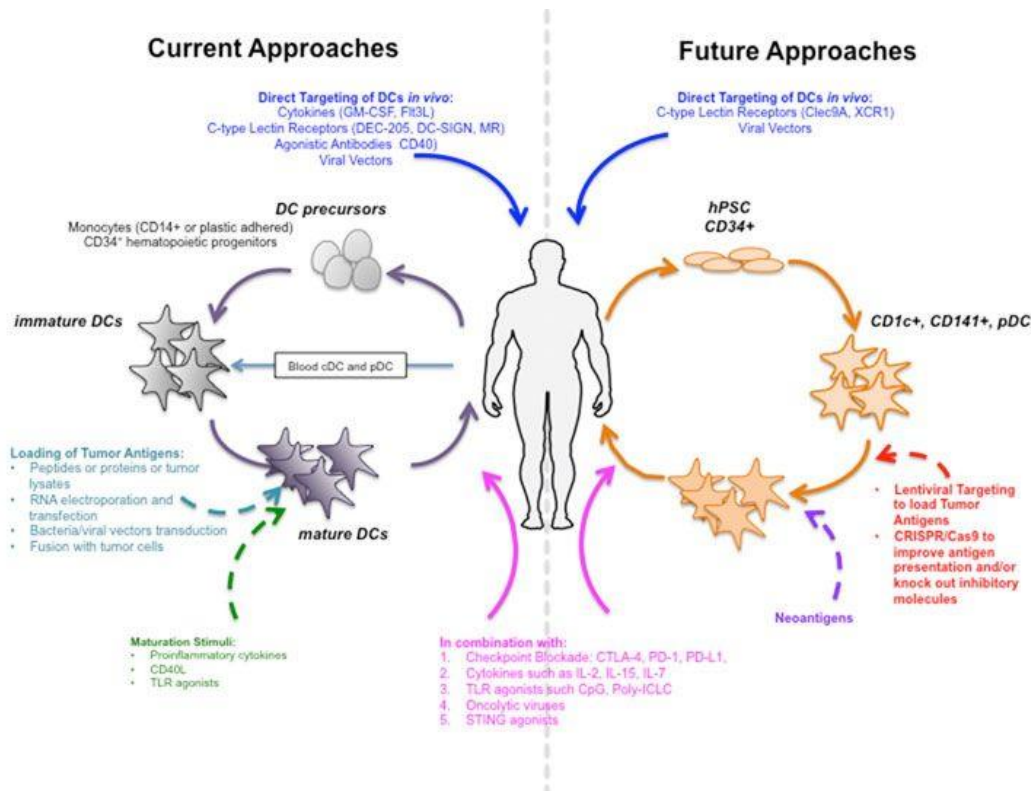

Figure 8: Current and future strategies for DC-based immunotherapies [265].

Current DC-vaccine based strategies include the isolation of DC precursors with subsequent antigen-loading and activation before reinjecting the cells in the patient. Future strategies aim at targeting specific DC subsets and controlling the costimulatory signals of the DC to induce the most precise adaptive response.

Importantly, the *in vitro* differentiation of either CD34$^+$ precursors or blood DCs can be tuned to preferentially induce the desired DC population (e.g. pDCs or CD141$^+$ cross-presenting DCs [28, 29]). Based on mouse data, this allows to test the specific effectiveness of each DC subset depending on the targeted cancer for example. However, blood-derived DCs provide a limited stock of precursors to induce the differentiation of these precursors in a particular population. The maturation stimuli are another important parameter to be adjusted; indeed, in addition to induce the maturation of DCs, with increased class II presentation and activating stimuli such as CD80 and CD86, it should aim a skew the DC toward Th1-inducing DCs for treating cancer and toward T$_{reg}$ inducing DCs for allergy or autoimmune diseases. PGE$_2$ for example, could induce the production of IDO by DCs and this helps for inducing T$_{regs}$ [266] but not IL-12 production [267].

Future strategies aim at avoiding the costly and time-consuming step of *in vitro* differentiation to directly target *in vivo* subsets of DCs. This will also allow to activate "natural" DCs at various anatomical location, which may have superior capacity to induce specific responses. This targeting can be performed using antibodies against specific receptors, or recombinant ligands, which can be chemically bound to activating signals. For example, NY-ESO-1 is a neoantigen observed in various forms of cancer and clinical trials are going on to target this neoantigen using an anti-DEC205 antibody in ovarian cancer (NCT02166905), AML (NCT01834248) and melanoma (NCT02129075).

b. Skin vaccination

Mass immunization programs have largely reduced the burden of infectious diseases worldwide and can overall be considered as one of the best successes for public health. However, global vaccination program suffers from logistic challenges because of the relative fragility of currently available vaccines that require to be kept in the cold chain, but without freezing temperature.

A wide range of techniques have been designed to overcome such issues, as well as the requirement for single-use needles, which may cause another logistical issue in third world countries. Besides classical intramuscular injections, skin as proven to be an accessible, highly immunogenic target for vaccination [268]. Microneedles patches represent a promising way to deliver antigens through the epidermal barrier into the skin where it can be taken up by skin DCs [269, 270] and the use of dried powder on the microneedle, which usually presents high thermostability could represent an alternative solution to the actual liquid containing vials. Now, the dissolving microneedles on patches seem to have promising immune-inducing capabilities and numerous preclinical trials have already shown the superiority of skin to induce efficient antibody responses compared to muscle [271].

Another strategy that is used to breach the epidermis is laser microporations (LMP). Several preclinical studies have assessed the efficacy of antigen delivery through microporated skin [272, 273, 275] and showed high level of neutralizing antibodies, as well as protection against lethal challenge with the pathogen. Now this strategy is reaching the human clinical trials, for example with the influenza vaccine (NCT02988739). Skin vaccination, with or without DC targeting may thus represent the future of technique of immunization.

Annex 1: Mouse models for dendritic cells studies

This part is based on the review published by Durai, V., and Murphy, K.M. (2016) in Immunity [134]. I chose to reorganize it and update it with minor corrections and modifications while focusing the scope on skin dendritic cells.

Functional studies of DC populations used to rely on *in vitro* coculture systems with T cells and subsequent analysis of the differentiated T cell population. However, the deeper understanding of the ontogeny of DCs and the development of genetic engineering in mice raised the possibility of *in vivo* cell depletion and cell specific gene depletion. Several mutant mouse models were created, using different strategies, allowing the studies of various DCs under homeostatic or inflammatory conditions. These models were reviewed in 2006 [134] and I propose here an updated and reorganized version of this review.

Germline versus conditional knock-out of a gene

The first knock-out strategies that were developed were based on the inactivation of the gene by inserting a sequence inside the gene, causing a frameshift during translation or leading the transcripts to be degraded by mechanisms such as the nonsense-mediated mRNA decay. However these methods generate a germline deletion which does not allow the study of the role of a specific cell population or of the function of a molecule in a specific cell type.

One of the most widely used strategies involves the deletion of genes in specific cell population, usually using the Cre-LoxP system. This often involves the expression of the bacteriophage P1 Cre recombinase under the control of the regulatory elements (usually the promoter) of a gene of interest. A small 34bp long sequence called LoxP is then inserted on both side of a gene or an exon which is thus qualified as "floxed". Crossing the Cre mouse with the floxed mouse will specifically induce the deletion of the floxed gene in the cells in which the promoter of the Cre is active. This is critically useful to assess the role of specific genes in the population of interest, but could also be used to remove transcription factors, subsequently impairing or even depleting cell populations.

Although the pioneer Cre-LoxP system remains the more broadly used worldwide, several alternatives have now been developed, including the Dre-rox system, which uses another

bacteriophage P1-like derived enzyme called Dre0 to mediate the recombination of rox sequences. Anoter site-specific recombinase, the FLP was identified in *Saccharomyces cerevisiae* and mediates the recombination of FRT sequences, however despite similarities with the Cre-LoxP system, the FLP-FRT system is less efficient in mammal cells due to poor thermostability of the enzyme at body temperature. To overcome this issue, a new codon-optimised and thermostable version of the FLP was developed and is thermed FLPo. Another remarkable player in the field is the φC31 integrase, also from a bacteriophage, which has the specificity to use dissimilar recombination sites *attB* and *attP*. After recombination, the flanking sites would be transformed into *attL* and *attR*, thus preventing any further rearrangement. This technology could be used both for integration of a DNA sequence into the genome or an excision.

Constitutive versus inducible gene knock-out or cell depletion

Another major difference between the different mouse genetic tools which allow for cell-specific depletion is that this depletion can either be constitutive or inducible. In the first case, those cells never appear in the organism from the developmental stage of the mouse, whereas the depletion of the cells is acutely induced at a defined time point in the latter. Two strategies have been widely used to specifically deplete cell types in mice. One can either rely on the deletion of a gene that is specifically required for the development of some cells or take advantage of the expression of an exogenous gene that mediates the death of the cell.

The constitutive depletion can thus be performed using a simple recombinase that excise transcription factors required for the development of the target cell type, as we will see later in this part or by expressing the subunit A of the diphtheria toxin (DTA) under the control of a promoter of interest, this latter strategy simply inducing the apoptosis of a cell as soon as the promoter is activated.

The inducible depletion of cell types is usually also performed by using the diphtheria toxin system, by using either knock-in or transgenic mice which express the human diphtheria toxin (DT) receptor (DTR) under the target promoter. Thus, upon DT injection, the cell in which the promoter is activated and that expression the human DTR will bind the DT and undergo apoptosis. Another technique would be to use an inducible integrase, such as the fusion

protein between Cre and the ligand binding domain or the oestrogen receptor (ER). Upon tamoxifen administration, a synthetic ligand of the ER, the Cre-ER complex would translocate into the nucleus, thus allowing the Cre activity and the deletion of the gene of interest, such as a transcription factor required for the development of the target cell. Of note, a fusion protein between the FLP and the ER has also been developed and is readily available.

Several other ligand-based induction systems have been developed. For the example remplacing the ER by the progesterone receptor, which can be translocated into the nucleus upon administration of the progesterone analog RU-486. More recently, a Cre allele which is fused to the *E. coli* dihydrofolate reductase (DHFR) protein has been created. The Cre/DHFR fusion protein is highly unstable and is rapidly degraded by the proteasome in absence of its ligand, the antibiotic trimethoprim (TMP). Upon administration of TMP, the Cre/DHFR complex is stabilized thus allowing the integrase to excise the floxed sequence.

Unlike the constitutive model, inducible depletion allow tu finely analyze the role of a cell type in particular complex, such as inflammation while making sure that the side effects during the hematopoiesis are minimal. However, the limitation of such depletion remains the specificity of the promoter being used, as well as the efficiency of the depletion, either because of a low activity of the integrase, or a low level of expression of the DTR.

Mouse models of DC selective gene mutation

a) Widely targeting DCs

The most classical marker for DC populations being CD11c, also known as Itgax, the first Cre-strain that was generated for deleting genes in DCs was based on CD11c expression. Two transgenic mouse strains of Itgax-Cre were generated. The first line was crossed with a *Rosa26*-STOP^fl-*yfp* strain to follow the Cre-mediated deletion and showed a high efficiency in both cDCs and pDCs. However, background deletion was noted in about 10% of T, B and NK cells as well as in some CD11c+ macrophage populations such as alveolar macrophages. The efficiency of the second mutant line was not assessed by the authors who used the GFP present in the construct to follow the Cre expression. Almost all cDCs and pDCs showed high expression of GFP, however this strategy does not allow for evaluating the efficiency of the recombination. Probst and colleagues generated a transgenic mouse line in which the Cre

fused to the ligand binding domain of the human oestrogen receptor was expressed under the control of the promoter of *Itgax* [274]. However, once crossed with an eGFP reporter line harboring a floxed STOP cassette before the eGFP sequence, only 5 to 8% of the CD11c-expressing cells became positive upon tamoxifen injection, showing a poor efficiency of Cre-mediated recombination. Of note, no eGFP signal was seen in the CD11c⁻ cells and this mouse tool could still be usefull in models which require a low level of recombination among CD11c⁺ cells, to mimick antigen presentation upon infection for example.

To address even more specifically cDCs, a *Zbtb46-cre* was generated. As expected, when crossed with a *Rosa26*-STOP^{fl}-*yfp* strain most of the excision occurred in DCs, although some excision was detected in erythroid lineage and endothelial cells. To circumvent this issue, it was suggested to create bone marrow chimeras with wildtype recipient mice. Of note, the efficiency of deletion of *Rosa26*-STOP^{fl}-*yfp* in cDCs was only about 65%, despite a high efficiency of 95% when this line was crossed with another strain harboring floxed MHC-II, suggestion a gene-dependent efficiency.

b) Targeting LCs and cDC1s

Several mouse lines were created and can be used to induce depletion of genes in LCs and/or langerin-expressing cDC1s. The first is the *Cd207-cre* in which the Cre sequence is inserted in the second exon of the langerin gene (CD207). The mouse showed high efficiency in both LCs and cDC1s in the dermis, secondary lymphoid organs (sLOs) and in the lung.

c) Targeting cDC1s

To specifically address cDC1s and delineate the role of this population, several mouse lines were generated: two *Xcr1-cre* lines in which CRE sequence replace the gene or is placed after the STOP codon with IRES and the *Karma-cre* in which an IRES-*cre* is placed after the STOP codon of the gene a530099j19rik which has been shown to be specifically expressed in cDC1s.

Before the discovery of the specific expression of XCR1 by cDC1s, several surface markers were used to identify this population. Clec9A was one of them and a *Clec9a-cre* mouse was generated. Because of its expression in hematopoietic precursors, it presents not only an efficient deletion in cDC1s, but also in 50% of cDC2s and in 20% of pDCs.

Cre deleter lines for conditional deletion of genes in dendritic cells			
Strain	**Affected cells**	**Comments**	**Reference**
Itgax-cre	DCs, pDCs, CD11c$^+$ Mφ, up to 10% of T, B and NK cells.	Itgax (CD11c gene) inserted in a BAC.	191
Itgax-cre	Not checked, most probably as above.	CD11c promoter and enhancer conducted Cre expression in transgenic mice.	276
Zbtb46-cre	>65% cDCs, <10% of other immune cells.	Also called zDC^{Cre} mouse. Knock-in mouse of Cre sequence in 3' UTR of Zbtb46 gene	277
Cd207-cre	Langerhans cells, Langerin$^+$ cDC1s (in tissue and sLOs).	Knock-in mouse of Cre sequence in exon 2 of langerin gene.	278
hCd207-creRT^{T2}	>75% of Langerhans cells, no other cell type in skin and skin-draining LN	Human langerin cre-ER^{T2} was generated by putting human langerin gene in a BAC and inserting cre-ER^{T2} sequence in the 3'UTR of langerin gene.	145
Crec9a-cre	100% of cDC1s, 50% of cDC2s and 20% of pDCs in sLOs.	Knock-in mouse in which cre sequence replace exon 1 and 2.	279
Xcr1-cre	Not checked, cDC1s, but others immune cells were not analyzed.	Knock-in mouse in which cre sequence replace Xcr1 gene.	280
Xcr1-cre	100% of cDC1s in skin, lung and liver and sLOs; <20% of CD4+ T cells and NKT cells in skin.	Knock-in targeting IRES-Cre sequence in exon 2 of Xcr1 gene after STOP codon.	281
Karma-cre	60 to 80% of cDC1s; 20% of migratory cDC2s, 10% Mφ from sLOs, and up to 60% of skin mast cells.	Knock-in targeting IRES-Cre sequence in exon 2 of a530099j19rik (Gpr141b/Karma) gene after STOP codon.	281
Siglech-cre	25% of pDCs, <2% of T, NK and NK-T cells.	Cre sequence was introduced in place of exon 1 in a bac containing SiglecH locus.	282

Mouse models of DC depletion by deleting transcription factors

    a)   Depleting cDC1s

It appeared quite early that the deletion of transcription factors may either deplete or impair specifically DC populations. One of the earliest example discovered is the deletion of IRF8 that

impairs the generation of cDC1s [283, 284]. However further studies demonstrated that germline deletion of IRF8 impairs other cell types, such as pDCs, but also B cells, eosinophils, basophils and monocytes, which showed that functional studies using deletion of transcription factors should be interpreted with caution and, if feasible, cell specific.

Besides *Irf8*^{-/-} mice, other knock-out mice are also deficient for cDC1s: *Id2*^{-/-} and *Nfil3*^{-/-} present a very similar phenotype as both strains lack cDC1s, but also present defective NK cells and ILCs. Batf3 is involved in the maintenance of IRF8 during the development of cDC1s and *Batf3*^{-/-} mice almost completely lack cDC1s in both lymphoid and non-lymphoid organs of Balb/c and 129SvEv mice, but with only partial ablation in C57Bl/6 mice. Of note, Batf3 is also expressed by other cells, including cDC2s and T cells, and its deletion may affect the immune system independently of the lack of cDC1s.

b) Targeting cDC2s

Interestingly, several members of the non-canonical NF-κB pathway have also been reported to be important for DC development and function. This pathway can be activated by the binding of lymphotoxin β (LTβ) on its receptor (LTβR) and will, through the stabilization and the phosphorylation of NF-κB-inducing kinase (NIK), activate the transcription factor RelB [285]. The importance of this pathway is demonstrated by the impaired function of cDC2s in *Ltbr*^{-/-} [286], *Nik*[DCΔ] [287] and *Relb*^{-/-} [190] mice. Due to multiple defect in various cell types and in the organogenesis of these mice, they do not represent a convenient model to study DC function. Further studies were performed with another technique allowing to assess the intrinsic or extrinsic role of hematopoietic versus stromal cells: bone marrow (BM) transplantation. First, *Relb*^{-/-} BM was transferred into an irradiated WT recipient (*Relb*^{-/-} → WT chimera) generating a mouse in which RelB is lacking only in the hematopoietic lineage. Strikingly, pDCs and cDCs were not defective in peripheral tissues or tissue-draining lymph nodes, but a population of splenic cDC2s was reduced [190, 288]. Moreover, antigen presentation to naïve T cells was not impaired in *Relb*^{-/-} → WT chimeric mice. Conversely, WT → *Relb*^{-/-} mice recapitulated the phenotype of germline knock-out mice, indicating that most of the observed defects were caused by deficient stromal cells and not intrinsic to the hematopoietic system. It is worth to point that the splenic cDC2 population affected by RelB

deficiency was also shown to required Notch2 signaling, as shown by DC-specific deletion of the notch signaling component RBP-J [191] and Notch2 [289].

Two more transcription factors have been shown to be important for the development and/or function of at least a part of cDC2s.

Kruppel-like factor 4 (Klf4) is a protein involved in the cell cycle and mice lacking this factor have very limited lifespan due to defective epidermal barrier causing excessive water loss. Mice harboring a deletion of *Klf4* in CD11c^{+} cells show partial depletion of the cDC2 subset, with reduced CD11b^{+} cDC2s in the spleen and sLO-resident DCs, and also completely lacking the CD11b^{-} cDC2 population in migratory DCs of skin-draining LNs as well as the Mgl2^{+} population in lung. It is unclear whether the absence of CD11b^{-} cDC2s in draining lymph node is due to the lack of these cells in the skin or to a defective migration toward the lymph node. Overall, the deletion of *Klf4* in DCs impairs the ability to mount Th2 responses [134].

Finally, *Irf4*^{-/-} mice show defective cDC2s. Ablation of this gene, either in the whole organism or preferentially in DCs, shows only a minor reduction of these cells in tissues like lung [200, 290], skin [192] or intestine [199, 200], but efficiently abrogates their migration toward the draining lymph node, particularly that of PDL2^{+} MGL2^{+} cDC2s, subsequently impairing the induction of Th2 responses [291].

c)   Targeting pDCs

Two mouse lines were generated in order to deplete pDCs. The first strain aimed at specifically deleting E2-2 (protein encoded by the gene *Tcf4*) [292] which is known to be important for pDC development. Bone marrow transplantation of E2-2^{+/+} and E2-2^{-/-} fetal liver cells into lethaly irradiated recipient mice showed a dramatic decrase of pDC development when the hematopoietic lineage lacks E2-2. This was confirmed using a tamoxifen-induced deletion of E2-2 in mice harboring floxed E2-2 and a knock-in of the CreER sequence in the widely expressed Rosa26 locus. Another transcription factor was Zeb2 and mice harboring a specific deletion of Zeb2 in CD11c^{+} cells present impaired pDC development [293]. However, it should be noted that Zeb2 is also important in the development of cDCs, especially the balance between cDC1s and cDC2s, thus data generated using this line should be interpreted with caution.

Finally, a recent study reported the critical role of Gata2 in DC development and showed that deletion of *Gata2* with *Rosa26-cre^{ER}* in adult mice reduce DC population from the hematopoietic stage. *In vitro* experiments suggest that this phenotype is cell intrinsic, but further studies using population-specific promoters need to be performed [294].

Transcription factor knock-out mice affecting DC development and/or function			
Strain	**Affected cells**	**Comments**	**Reference**
Irf8^{-/-}	cDC1s, pDC, B cells, eosinophils, basophils, monocytes	Reported affected cells are using germline knock-out, not DC-specific one.	283, 284
Id2^{-/-}	cDC1s, NK cells, ILCs	Reported affected cells are using germline knock-out, not DC-specific one.	295
Nfil3^{-/-}	cDC1s, NK cells, ILCs	Reported affected cells are using germline knock-out, not DC-specific one.	296
Batf3^{-/-}	cDC1s	Phenotype depends on mouse genetic background. Reported affected cells are using germline knock-out, not DC-specific one.	176
Relb^{-/-}	Splenic cDC2 subpopulation	Knock-out in the hematopoietic system after transfer into WT recipient.	288
Notch2^{-/-}	Splenic cDC2 subpopulation	Notch2 was specifically deleted in DCs using *itgax-cre*	202, 289
Klf4^{-/-}	CD11b^- cDCs from skin-draining LN.	Klf4 was depleted in DCs using *itgax-cre*	198
Irf4^{-/-}	Diminished tissue cDC2s, abrogates cDC2 migration to draining LN.	Phenotype observed in germline and DC-specific mutant mice. May affect CD11c^+ IRF4^+ MΦ.	192, 199, 200, 290, 291
Tcf4^{-/-}	60 to 80% of pDCs	E2-2 germiline KO mice die at birth. Results obtained with *Ro26-creER* and *itgax-cre*	292
Zeb2^{-/-}	pDCs, cDCs	DC-specific deletion using *itgax-cre* show reduced pDCs but also imbalanced cDC1/cDC2 development	293
Gata2^{-/-}	Spleen pDCs and cDCs	Adult deletion using *Rosa26-creER*	294

Combining the transcription factor deletion technique together with cell-specific cre strains allow for interesting and specific cell depletion, however this strategy raises difficulties in the interpretation of the results considering that the lack of transcription factors may not only

affect the development of the DC but also its function. Impaired migration is one example, but remains easily detectable as the cell population remains in the tissue but is absent from the draining LN, whereas more subtle changes in the gene expression could be harder to detect.

Mouse models of DC depletion using DT-based depletion system

This strategy can be used for constitutive or inducible depletion: constitutive depletion is achieved by expressing the diphtheria toxin (DT) fragment A (DTA) under le control of a cell-specific promoter [296]. Inducible depletion relies on the expression of the human or simian diphtheria toxin receptor (DTR) under the control of a cell-specific promoter [298] thus rendering DT-insensitive mice sensitive in specific cell populations.

Human DTR has been identified as a transmembrane heparin-binding EGF-like growth factor precursor. Upon injection of DT, the toxin will bind to its receptor on the surface of DTR-expressing cells. The binding process involves the cleavage of the toxin in two subunits: fragment A, containing the catalytic C domain, and fragment B, containing the R and T domains which allow for receptor binding and cell entry respectively. Upon fixation of the R domain on the receptor, the ligand-receptor complex will be endocytosed and the acidification of the vesicle will induce a conformational change of the T domain, forming a pore which will facilitate the translocation of the fragment A through the membrane, from the endosome to the cytoplasm. Once in the cytoplasm, the C domain will use NAD as a substrate to ADP-ribosylate eukaryotic elongation factor 2 (eEF-2), a critical player of the protein synthesis process. This will prevent protein synthesis by the host cell and thus its death by apoptosis.

a) Widely targeting DCs

The first and broader mouse strain using DTR-mediated cell depletion was a transgenic *Itgax*-DTR line [299] in which all CD11c$^+$ cells are efficiently depleted upon DT injection, including cDCs, but pDCs only to a lower extent, suggesting that mainly CD11c^{hi} cells would be killed. However, this strain presents several caveats, including the depletion of non-DC CD11c$^+$ cells, including macrophages, subsets of B cells and CD8$^+$ cells, but also the fact that repeated DT injection would induce the death of the mouse. Expression of DTR outside of the hematopoietic system was demonstrated by transferring WT bone marrow into irradiated

CD11c-DTR mice (WT → *Itgax*-DTR) and subsequently injecting several rounds of DT, which again led to the death of the animal. Conversely, *Itgax*-DTR → WT bone marrow transfer was a reliable model to induce DC depletion without killing the mouse.

This lethal phenotype was attributed to an off target expression of *Itgax* promoter lacking its regulatory elements. Thus, another model of *Itgax*-DTR was generated using a BAC strategy with more complete regulatory elements [300] conducting the expression of the DTR together with a fragment of ovalbumin and eGFP. This mouse, named *Itgax*-DOG, allows for depletion of classical dendritic cells and also target splenic macrophages without leading to the death of the mouse. Whether other cell types are affected remains to be determined.

To more specifically target cDCs, the idea was raised to use the transcription factor ZBTB46 to direct the expression of the DTR and an IRES leading the expression of a DTR-mCherry fusion protein was places in the 3' UTR sequence of the endogenous *Zbtb46* gene [301]. However, DT-injected *Zbtb46*-DTR mice died after a single dose, phenotype which could, once again, be compensate by *Zbtb46*-DTR → WT bone marrow transfer. In order to restrict the expression of the DTR to ZBTB46+ DCs, a floxed transcription Stop cassettes was placed before the IRES in the 3' UTR of *Zbtb46* gene [302]. Upon crossing with a Cre delete strain, one can target the DTR expression in specific cells. The authors used *Csf1r-cre* which is active in monocytes, macrophages and DC-precursors to induce the recombination of the Stop cassette, leading to the expression of DTR on the surface of cDCs. They showed an efficient and specific deletion of cDCs in the mouse.

Another marker used widely for staining dendritic cells is the C-type lectin receptor DEC-205, also known as CD205, encoded by the *Cd205* gene. A knock-in mouse was generated, in which the human DTR-*egfp* sequence was placed in the 3' UTR of *Cd205*, following an IRES [303]. DT injection was shown to efficiently eliminate CD205+ DCs in sLOs, which mainly represents cDC1s. Further data suggest CD205 expression by LCs, skin-migratory DCs and germinal center B cells, their depletion in this strain remains to be confirmed.

b) Targeting LCs and cDC1s

The next step was to address the specific depletion of DC subsets. Two knock-in mouse strains were generated in 2005 to induce depletion of langerin^{+} cells, including Langerhans cells and cDC1s. In the first construct, an IRES-DTR-*egfp* sequence was inserted in the 3' UTR of the gene *Cd207* [150] whereas the second construct aimed at inserting a DTR-*egfp* sequence in the second exon of *Cd207* [304]. In both strains, DT injection leads to efficient depletion of both Langerhans cells and cDC1s.

LC-specific depletion was obtained first in a constitutive depletion model in which DTA sequence was driven by an IRES and inserted in a BAC containing the human *Cd207* gene with its regulatory elements. For a still unknown reason, the human langerin gene is only expressed by LCs in mouse and this construct led to an LC-specific constitutive depletion [305]. It was later shown that, in addition to LCs, a subset of CD103^{+} cDC2 was also ablated in the intestinal lamina propria [306]. The same BAC strategy containing the human *Cd207* gene was used to generate an inducible DC-specific depletion by inserting a DTR sequence in the 3' UTR of the gene instead of DTA [307].

Of note, the peculiar nature of LCs as a radioresistant population derived from embryonic progenitors and self-renewing in adults allows for complex combination of knock-out or DT-mediated depletion with bone marrow transfer. For instance, transferring WT bone marrow into a lethally irradiated *Cd207*-DTR recipient (WT → *Cd207*-DTR) will allow for LC-specific depletion upon DT injection whereas *Cd207*-DTR → WT bone marrow transfer allow for the depletion of all langerin-expressing cells but LCs.

c) Targeting cDC1s

To specifically deplete cDC1s, several models were generated, according to the more and more precise identification markers that were discovered. The first line generated to this aim was the *Clec9a*-DTR, a BAC transgenic muse in which the first coding sequence of *Clec9a* was replaced with the coding sequence of DTR [308]. It efficiently targeted cDC1s, but showed reduced pDCs and cDC2s after several days, supposedly because of the expression of *Clec9a* by the common dendritic cell progenitor (CDP) and cDC1s. The replacement of the

endogenous *Xcr1* coding sequence by that of the DTR generated *Xcr1*-DTR line in which cDC1s are efficiently eliminated [309]. Another mouse targeting cDC1s is referred to as Karma mouse. In this mouse, an IRES followed by the sequence of tdTomato-2A-DTR is knock-in the 3' UTR of the gene *a530099j19rik* which was shown to be specifically expressed by cDC1s. Analysis showed a DC-restricted cell depletion [310].

        d)   Targeting cDC2s

Two different mouse strains were created to study the function of cDC2s by depleting them through DT-injection. The first one uses the gene *Clec4a4* which encodes DCIR2 to direct DTR expression thanks to a BAC [311]. DT injection proved to decrease cDC2 population in the lamina propria and mesenteric lymph nodes, as well as some macrophages, but characterization was not further extended outside of the intestine. Another mouse line targeting cDC2s is the *Mgl2*-DTR strain, in which a DTR-*gfp* cassette was inserted in the second exon of *Mgl2* gene. DT-injected *Mgl2*-DTR mice showed decreased population of dermal cDC2s [160]. Further studies confirmed the depletion of about 50% of lung PDL2$^+$ cDC2s [312] but the situation in other organs remains to be elucidated.

        e)   Targeting pDCs

Regarding pDC depletion, two mouse strains were generated using the gene *Siglech*. The first one targeted the endogenous locus and inserted an IRES-DTR-*egfp* sequence in the 3' UTR of the gene *Siglech* [313]. This strain allows for an efficient depletion of pDCs but algo targets other cell population [314]. In addition, the insertion of the cassette disrupted the gene expression and these mice are virtually *Siglech*$^{-/-}$ with implications besides the depletion of pDCs. The second construct was created by targeting the first coding exon of *Siglech* in a BAC with human DTR sequence [308]. It demonstrated an efficient pDC elimination upon DT injection, however the characterization of these mice remains limited and further studies should be performed. One surprising model used for pDC depletion relies on the human gene *CLEC4C* which encodes the human C-type lectin BDCA-2. This gene has no ortholog in mouse, but the promoter is still active and, like for the human langerin gene, it seems to be specific for pDCs. Indeed, transgenic mice which express human DTR under the control of *CLEC4C* promoter show complete depletion of pDCs upon DT injection with no reduction in B cells,

cDCs, T cells, NK cells, monocytes/macrophages or neutrophils [315]. The use of human promoter seems to be a promising way to dictate transgene expression when there is no known specific marker yet in mice.

Models of DTR-mediated depletion of dendritic cell			
Strain	**Affected cells**	**Comments**	**Reference**
***Itgax*-DTR**	cDCs, macrophages, plasma cells, activated CD8$^+$ T cells	Transgenic mouse. Depletion efficient in CD11c^{hi} cells, repeated treatments lead to death, controversies concerning depletion of pDCs	299
***Itgax*-DOG**	cDCs, splenic MΦ	BAC transgenic line. Efficient depletion of DCs from spleen, LN, BM and thymus. Induce expression of ovalbumin.	300
***Zbtb46*-DTR**	cDCs	Knock-in mouse. Efficient and complete depletion of cDCs but required bone marrow transfer	301
Zbtb46-LSL-DTR	cDCs	Knock-in mouse. Efficient depletion of *Zbtb* expressing cells. Requires CRE-mediated recombination.	302
***Cd205*-DTR**	cDC1s and part of cDC2s in sLOs	Knock-in mouse. Depletion of LCs, migratory DCs and GC B cells should be confirmed.	303
***Cd207*-DTR**	LCs and cDC1s	Knock-in mouse. Does not disturb endogenous expression.	150
***Cd207*-DTR**	LCs and cDC1s	Knock-in mouse. DTR sequence is inserted in the gene coding sequence.	304
***hCd207*-DTA**	LCs, a subset of CD103$^+$ intestinal cDC2s.	BAC transgenic line. Constitutive depletion. Efficient depletion of LCs but not cDC1s.	305
***hCd207*-DTR**	LCs, a subset of CD103$^+$ intestinal cDC2s.	BAC transgenic line. Inducible depletion. Efficient depletion of LCs but not cDC1s.	307
***Clec9a*-DTR**	cDC1s, part of cDC2s, CDP, part of pDCs	BAC transgenic line. Possibly target hematopoietic precursors, possibly create confusing results.	308
***Xcr1*-DTR**	cDC1s in resident and migratory part of mesenteric and skin-draining LNs.	Knock-in mouse. Disrupt the endogenous expression.	309
Karma-DTR	cDC1s, part of the mast cells	Knock-in mouse. Does not disturb endogenous gene.	310

Clec4a4-DTR	Populations of mesenteric and colonic cDC2s, colonic macrophages	BAC transgenic line. Characterization of the affected cells should be performed in other tissues.	311
Mgl2-DTR	Dermal cDC2s, 50% of lung PDL2$^+$ cDC2s	Knock-in mouse. Further analysis should confirm depletion of cDC2s in other organs.	160
Siglech-DTR	pDCs, pDCs progenitors, marginal zone macrophages and possibly microglia and cDC precursors.	Knock-in mouse. Endogenous Siglec-H expression is impaired despite the insertion in 3' UTR.	313, 314
Siglech-DTR	pDCs, pDCs progenitors, marginal zone macrophages and possibly microglia and cDC precursors.	BAC transgenic line. Further studies need to be performed.	308, 314
CLEC4C-DTR	pDCs	Transgenic strain expressing the human DTR under the control of human BDCA-2 promoter	315

Overall, the complexity of DC networks required the generation of state-of-the-art genetic tools that can be intercrossed to address the specific role not only of a DC population, but also of a specific molecule expressed by one or several populations of DCs.

1. Langerhans, P. Uber die Nerven der menschlichen Haut. Virchows. Arch. Pathol.44, 325–337 (1868).
2. Steinman RM, Cohn ZA.1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution .J Exp Med 137:1142–1162.
3. Steinman, R.M., and Cohn, Z.A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. J. Exp. Med. *139*, 380–397.
4. Steinman, R.M., Lustig, D.S., and Cohn, Z.A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. J. Exp. Med. *139*, 1431–1445.
5. Steinman, R.M., Adams, J.C., and Cohn, Z.A. (1975). Identification of a novel cell type in peripheral lymphoid organs of mice. IV. Identification and distribution in mouse spleen. J. Exp. Med. *141*, 804–820.
6. Krautler, N.J., Kana, V., Kranich, J., Tian, Y., Perera, D., Lemm, D., Schwarz, P., Armulik, A., Browning, J.L., Tallquist, M., et al. (2012). Follicular dendritic cells emerge from ubiquitous perivascular precursors. Cell *150*, 194–206.
7. Nossal, G.J., Abbot, A., Mitchell, J., and Lummus, Z. (1968). Antigens in immunity. XV. Ultrastructural features of antigen capture in primary and secondary lymphoid follicles. J. Exp. Med. *127*, 277–290.
8. Rosenthal, A.S., and Shevach, E.M. (1973). Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. J. Exp. Med. *138*, 1194–1212.
9. Shevach, E.M., and Rosenthal, A.S. (1973). Function of macrophages in antigen recognition by guinea pig T lymphocytes. II. Role of the macrophage in regulation of genetic control of the immune response. J Exp Med *138*, 1213–1229.
10. Zinkernagel RM, Doherty PC.1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature 248:701–702.
11. Steinman, R.M., and Witmer, M.D. (1978). Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. Proc. Natl. Acad. Sci. U.S.A. *75*, 5132–5136.
12. Inaba, K., Steinman, R.M., Van Voorhis, W.C., and Muramatsu, S. (1983). Dendritic cells are critical accessory cells for thymus-dependent antibody responses in mouse and in man. Proc. Natl. Acad. Sci. U.S.A. *80*, 6041–6045.
13. Metlay, J.P. (1990). The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. Journal of Experimental Medicine *171*, 1753–1771.
14. Crowley, M., Inaba, K., Witmer-Pack, M., and Steinman, R.M. (1989). The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. Cell. Immunol. *118*, 108–125.
15. Vremec, D., Pooley, J., Hochrein, H., Wu, L., and Shortman, K. (2000). CD4 and CD8 Expression by Dendritic Cell Subtypes in Mouse Thymus and Spleen. The Journal of Immunology *164*, 2978–2986.
16. Grouard, G., Rissoan, M.C., Filgueira, L., Durand, I., Banchereau, J., and Liu, Y.J. (1997). The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. J. Exp. Med. *185*, 1101–1111.

17. Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Brière, F., et al. (2001). Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. Nat. Immunol. *2*, 1144–1150.

18. Björck, P. (2001). Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. Blood *98*, 3520–3526.

19. Nakano, H., Yanagita, M., and Gunn, M.D. (2001). CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. J. Exp. Med. *194*, 1171–1178.

20. Reizis, B. (2019). Plasmacytoid Dendritic Cells: Development, Regulation, and Function. Immunity *50*, 37–50.

21. Bowers, W.E., and Berkowitz, M.R. (1986). Differentiation of dendritic cells in cultures of rat bone marrow cells. J. Exp. Med. *163*, 872–883.

22. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. *176*, 1693–1702.

23. Siena, S., Di Nicola, M., Bregni, M., Mortarini, R., Anichini, A., Lombardi, L., Ravagnani, F., Parmiani, G., and Gianni, A.M. (1995). Massive ex vivo generation of functional dendritic cells from mobilized CD34+ blood progenitors for anticancer therapy. Exp. Hematol. *23*, 1463–1471.

24. Caux, C., Dezutter-Dambuyant, C., Schmitt, D., and Banchereau, J. (1992). GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. Nature *360*, 258–261.

25. Romani, N., Reider, D., Heuer, M., Ebner, S., Kämpgen, E., Eibl, B., Niederwieser, D., and Schuler, G. (1996). Generation of mature dendritic cells from human blood An improved method with special regard to clinical applicability. Journal of Immunological Methods *196*, 137–151.

26. Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. Journal of Experimental Medicine *179*, 1109–1118.

27. O'Neill, H.C., and Wilson, H.L. (2004). Limitations with in vitro production of dendritic cells using cytokines. Journal of Leukocyte Biology *75*, 600–603.

28. Balan, S., Arnold-Schrauf, C., Abbas, A., Couespel, N., Savoret, J., Imperatore, F., Villani, A.-C., Vu Manh, T.-P., Bhardwaj, N., and Dalod, M. (2018). Large-Scale Human Dendritic Cell Differentiation Revealing Notch-Dependent Lineage Bifurcation and Heterogeneity. Cell Rep *24*, 1902-1915.e6.

29. Kirkling, M.E., Cytlak, U., Lau, C.M., Lewis, K.L., Resteu, A., Khodadadi-Jamayran, A., Siebel, C.W., Salmon, H., Merad, M., Tsirigos, A., et al. (2018). Notch Signaling Facilitates In Vitro Generation of Cross-Presenting Classical Dendritic Cells. Cell Rep *23*, 3658-3672.e6.

30. LadyofHats, M.R.V. (2007). Endocytosis (IPA: [ɛndəʊsaɪˈtəʊsɪs]) is a process whereby cells absorb material (molecules such as proteins) from the outside by engulfing it with their cell membrane. It is used by all cells of the body because most substances important to them are polar and consist of big molecules, and thus cannot pass through the hydrophobic plasma membrane.

31. Mantegazza, A.R., Magalhaes, J.G., Amigorena, S., and Marks, M.S. (2013). Presentation of Phagocytosed Antigens by MHC Class I and II. Traffic *14*, 135–152.

32. Kobayashi, K.S., and van den Elsen, P.J. (2012). NLRC5: a key regulator of MHC class I-dependent immune responses. Nature Reviews Immunology *12*, 813–820.

33. Bevan, M.J. (1976). Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. J. Immunol. *117*, 2233–2238.

34. Song, R., and Harding, C.V. (1996). Roles of proteasomes, transporter for antigen presentation (TAP), and beta 2-microglobulin in the processing of bacterial or particulate antigens via an alternate class I MHC processing pathway. J. Immunol. *156*, 4182–4190.

35. Kovacsovics-Bankowski, M., and Rock, K.L. (1995). A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science *267*, 243–246.

36. Fanger, N.A., Wardwell, K., Shen, L., Tedder, T.F., and Guyre, P.M. (1996). Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells. The Journal of Immunology *157*, 541–548.

37. Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Théry, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P., et al. (1999). Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. J. Exp. Med. *189*, 371–380.

38. Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. Journal of Experimental Medicine *179*, 1109–1118.

39. Rafiq, K., Bergtold, A., and Clynes, R. (2002). Immune complex–mediated antigen presentation induces tumor immunity. J Clin Invest *110*, 71–79.

40. Yada, A., Ebihara, S., Matsumura, K., Endo, S., Maeda, T., Nakamura, A., Akiyama, K., Aiba, S., and Takai, T. (2003). Accelerated antigen presentation and elicitation of humoral response in vivo by FcγRIIB- and FcγRI/III-mediated immune complex uptake. Cellular Immunology *225*, 21–32.

41. Bandukwala, H.S., Clay, B.S., Tong, J., Mody, P.D., Cannon, J.L., Shilling, R.A., Verbeek, J.S., Weinstock, J.V., Solway, J., and Sperling, A.I. (2007). Signaling through FcγRIII is required for optimal T helper type (Th)2 responses and Th2-mediated airway inflammation. J Exp Med *204*, 1875–1889.

42. Satoh, T., and Akira, S. (2016). Toll-Like Receptor Signaling and Its Inducible Proteins. Microbiology Spectrum *4*.

43. Zelensky, A.N., and Gready, J.E. (2005). The C-type lectin-like domain superfamily. The FEBS Journal *272*, 6179–6217.

44. Chen, G., Shaw, M.H., Kim, Y.-G., and Nuñez, G. (2009). NOD-like receptors: role in innate immunity and inflammatory disease. Annu Rev Pathol *4*, 365–398.

45. Cumberbatch, M., and Kimber, I. (1995). Tumour necrosis factor-alpha is required for accumulation of dendritic cells in draining lymph nodes and for optimal contact sensitization. Immunology *84*, 31–35.

46. Enk, A.H., Angeloni, V.L., Udey, M.C., and Katz, S.I. (1993). An essential role for Langerhans cell-derived IL-1 beta in the initiation of primary immune responses in skin. J. Immunol. *150*, 3698–3704.

47. Cumberbatch, M., Dearman, R.J., and Kimber, I. (1999). Langerhans cell migration in mice requires intact type I interleukin 1 receptor (IL-1RI) function. Arch Dermatol Res *291*, 357–361.

48. Wang, B., Fujisawa, H., Zhuang, L., Kondo, S., Shivji, G.M., Kim, C.S., Mak, T.W., and Sauder, D.N. (1997). Depressed Langerhans cell migration and reduced contact hypersensitivity response in mice lacking TNF receptor p75. The Journal of Immunology *159*, 6148–6155.

49. Cumberbatch, M., Dearman, R.J., and Kimber, I. (1997). Interleukin 1β and the stimulation of Langerhans cell migration: comparisons with tumour necrosis factor α. Arch Dermatol Res *289*, 277–284.

50. Ruedl, C., Koebel, P., Bachmann, M., Hess, M., and Karjalainen, K. (2000). Anatomical origin of dendritic cells determines their life span in peripheral lymph nodes. J. Immunol. *165*, 4910–4916.

51. Tang, A., Amagai, M., Granger, L.G., Stanley, J.R., and Udey, M.C. (1993). Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. Nature *361*, 82–85.

52. Jiang, A., Bloom, O., Ono, S., Cui, W., Unternaehrer, J., Jiang, S., Whitney, J.A., Connolly, J., Banchereau, J., and Mellman, I. (2007). Disruption of E-Cadherin-Mediated Adhesion Induces a Functionally Distinct Pathway of Dendritic Cell Maturation. Immunity *27*, 610–624.

53. Alvarez, D., Vollmann, E.H., and Andrian, U.H. von (2008). Mechanisms and Consequences of Dendritic Cell Migration. Immunity *29*, 325–342.

54. Kobayashi, Y. (1997). Langerhans' cells produce type IV collagenase (MMP-9) following epicutaneous stimulation with haptens. Immunology *90*, 496–501.

55. Ratzinger, G., Stoitzner, P., Ebner, S., Lutz, M.B., Layton, G.T., Rainer, C., Senior, R.M., Shipley, J.M., Fritsch, P., Schuler, G., et al. (2002). Matrix metalloproteinases 9 and 2 are necessary for the migration of Langerhans cells and dermal dendritic cells from human and murine skin. J. Immunol. *168*, 4361–4371.

56. Ohl, L., Mohaupt, M., Czeloth, N., Hintzen, G., Kiafard, Z., Zwirner, J., Blankenstein, T., Henning, G., and Förster, R. (2004). CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. Immunity *21*, 279–288.

57. Comerford, I., Harata-Lee, Y., Bunting, M.D., Gregor, C., Kara, E.E., and McColl, S.R. (2013). A myriad of functions and complex regulation of the CCR7/CCL19/CCL21 chemokine axis in the adaptive immune system. Cytokine & Growth Factor Reviews *24*, 269–283.

58. Kabashima, K., Shiraishi, N., Sugita, K., Mori, T., Onoue, A., Kobayashi, M., Sakabe, J., Yoshiki, R., Tamamura, H., Fujii, N., et al. (2007). CXCL12-CXCR4 Engagement Is Required for Migration of Cutaneous Dendritic Cells. The American Journal of Pathology *171*, 1249–1257.

59. Jenkins, M.K., Ashwell, J.D., and Schwartz, R.H. (1988). Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. J. Immunol. *140*, 3324–3330.

60. Mueller, D.L., Jenkins, M.K., and Schwartz, R.H. (1989). An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. The Journal of Immunology *142*, 2617–2628.

61. Schwartz, R.H. (1992). Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. Cell *71*, 1065–1068.

62. Borriello, F., Sethna, M.P., Boyd, S.D., Schweitzer, A.N., Tivol, E.A., Jacoby, D., Strom, T.B., Simpson, E.M., Freeman, G.J., and Sharpe, A.H. (1997). B7-1 and B7-2 Have Overlapping, Critical Roles in Immunoglobulin Class Switching and Germinal Center Formation. Immunity *6*, 303–313.

63. Sharpe, A.H., and Freeman, G.J. (2002). The B7–CD28 superfamily. Nature Reviews Immunology *2*, 116.

64. Linsley, P.S., Clark, E.A., and Ledbetter, J.A. (1990). T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. Proc. Natl. Acad. Sci. U.S.A. *87*, 5031–5035.

65. Engelhardt, J.J., Sullivan, T.J., and Allison, J.P. (2006). CTLA-4 Overexpression Inhibits T Cell Responses through a CD28-B7-Dependent Mechanism. The Journal of Immunology *177*, 1052–1061.

66. Genovese, M.C., Becker, J.-C., Schiff, M., Luggen, M., Sherrer, Y., Kremer, J., Birbara, C., Box, J., Natarajan, K., Nuamah, I., et al. (2005). Abatacept for Rheumatoid Arthritis Refractory to Tumor Necrosis Factor α Inhibition. New England Journal of Medicine *353*, 1114–1123.

67. Ruperto, N., Lovell, D.J., Quartier, P., Paz, E., Rubio-Pérez, N., Silva, C.A., Abud-Mendoza, C., Burgos-Vargas, R., Gerloni, V., Melo-Gomes, J.A., et al. (2010). Long-term safety and efficacy of abatacept in children with juvenile idiopathic arthritis. Arthritis & Rheumatism *62*, 1792–1802.

68. Orban, T., Bundy, B., Becker, D.J., DiMeglio, L.A., Gitelman, S.E., Goland, R., Gottlieb, P.A., Greenbaum, C.J., Marks, J.B., Monzavi, R., et al. (2011). Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial. The Lancet *378*, 412–419.

69. Vincenti, F., Blancho, G., Durrbach, A., Friend, P., Grinyo, J., Halloran, P.F., Klempnauer, J., Lang, P., Larsen, C.P., Mühlbacher, F., et al. (2010). Five-Year Safety and Efficacy of Belatacept in Renal Transplantation. JASN *21*, 1587–1596.

70. Munn, D.H., Sharma, M.D., and Mellor, A.L. (2004). Ligation of B7-1/B7-2 by Human CD4+ T Cells Triggers Indoleamine 2,3-Dioxygenase Activity in Dendritic Cells. The Journal of Immunology *172*, 4100–4110.

71. Blank, C., Brown, I., Marks, R., Nishimura, H., Honjo, T., and Gajewski, T.F. (2003). Absence of programmed death receptor 1 alters thymic development and enhances generation of CD4/CD8 double-negative TCR-transgenic T cells. J. Immunol. *171*, 4574–4581.

72. Gong, J., Chehrazi-Raffle, A., Reddi, S., and Salgia, R. (2018). Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: a comprehensive review of registration trials and future considerations. Journal for ImmunoTherapy of Cancer *6*, 8.

73. Sugiura, D., Maruhashi, T., Okazaki, I., Shimizu, K., Maeda, T.K., Takemoto, T., and Okazaki, T. (2019). Restriction of PD-1 function by cis-PD-L1/CD80 interactions is required for optimal T cell responses. Science *364*, 558–566.

74. Aggarwal, B.B. (2003). Signalling pathways of the TNF superfamily: a double-edged sword. Nat. Rev. Immunol. *3*, 745–756.

75. Croft, M., and Siegel, R.M. (2017). Beyond TNF: TNF superfamily cytokines as targets for the treatment of rheumatic diseases. Nat Rev Rheumatol *13*, 217–233.

76. Karin, M., and Gallagher, E. (2009). TNFR signaling: ubiquitin-conjugated TRAFfic signals control stop-and-go for MAPK signaling complexes. Immunol. Rev. *228*, 225–240.

77. Micheau, O., and Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell *114*, 181–190.

78. Silke, J., and Brink, R. (2010). Regulation of TNFRSF and innate immune signalling complexes by TRAFs and cIAPs. Cell Death Differ. *17*, 35–45.

79. Fisher, G.H., Rosenberg, F.J., Straus, S.E., Dale, J.K., Middleton, L.A., Lin, A.Y., Strober, W., Lenardo, M.J., and Puck, J.M. (1995). Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell *81*, 935–946.

80. Rieux-Laucat, F., Le Deist, F., Hivroz, C., Roberts, I.A., Debatin, K.M., Fischer, A., and de Villartay, J.P. (1995). Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science *268*, 1347–1349.

81. Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A., and Nagata, S. (1992). Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature *356*, 314–317.

82. Stuckey, D.W., and Shah, K. (2013). TRAIL on Trial: Preclinical advances for cancer therapy. Trends Mol Med *19*.

83. Croft, M. (2009). The role of TNF superfamily members in T-cell function and diseases. Nat. Rev. Immunol. *9*, 271–285.

84. Compaan, D.M., and Hymowitz, S.G. (2006). The crystal structure of the costimulatory OX40-OX40L complex. Structure *14*, 1321–1330.

85. Liu, C., Lou, Y., Lizée, G., Qin, H., Liu, S., Rabinovich, B., Kim, G.J., Wang, Y.-H., Ye, Y., Sikora, A.G., et al. (2008). Plasmacytoid dendritic cells induce NK cell-dependent, tumor antigen-specific T cell cross-priming and tumor regression in mice. J. Clin. Invest. *118*, 1165–1175.

86. Zingoni, A., Sornasse, T., Cocks, B.G., Tanaka, Y., Santoni, A., and Lanier, L.L. (2004). Cross-talk between activated human NK cells and CD4+ T cells via OX40-OX40 ligand interactions. J. Immunol. *173*, 3716–3724.

87. Marschner, A., Rothenfusser, S., Hornung, V., Prell, D., Krug, A., Kerkmann, M., Wellisch, D., Poeck, H., Greinacher, A., Giese, T., et al. (2005). CpG ODN enhance antigen-specific NKT cell activation via plasmacytoid dendritic cells. Eur. J. Immunol. *35*, 2347–2357.

88. Zaini, J., Andarini, S., Tahara, M., Saijo, Y., Ishii, N., Kawakami, K., Taniguchi, M., Sugamura, K., Nukiwa, T., and Kikuchi, T. (2007). OX40 ligand expressed by DCs costimulates NKT and CD4+ Th cell antitumor immunity in mice. J. Clin. Invest. *117*, 3330–3338.

89. Baumann, R., Yousefi, S., Simon, D., Russmann, S., Mueller, C., and Simon, H.-U. (2004). Functional expression of CD134 by neutrophils. Eur. J. Immunol. *34*, 2268–2275.

90. Walker, L.S., Gulbranson-Judge, A., Flynn, S., Brocker, T., Raykundalia, C., Goodall, M., Förster, R., Lipp, M., and Lane, P. (1999). Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. J. Exp. Med. *190*, 1115–1122.

91. Stüber, E., Neurath, M., Calderhead, D., Fell, H.P., and Strober, W. (1995). Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. Immunity *2*, 507–521.

92. Weinberg, A.D., Wegmann, K.W., Funatake, C., and Whitham, R.H. (1999). Blocking OX-40/OX-40 ligand interaction in vitro and in vivo leads to decreased T cell function and amelioration of experimental allergic encephalomyelitis. J. Immunol. *162*, 1818–1826.

93. Ohshima, Y., Tanaka, Y., Tozawa, H., Takahashi, Y., Maliszewski, C., and Delespesse, G. (1997). Expression and function of OX40 ligand on human dendritic cells. J. Immunol. *159*, 3838–3848.

94. Drake, L.Y., Iijima, K., and Kita, H. (2014). Group 2 innate lymphoid cells and CD4+ T cells cooperate to mediate type 2 immune response in mice. Allergy *69*, 1300–1307.

95. Halim, T.Y.F., Rana, B.M.J., Walker, J.A., Kerscher, B., Knolle, M.D., Jolin, H.E., Serrao, E.M., Haim-Vilmovsky, L., Teichmann, S.A., Rodewald, H.-R., et al. (2018). Tissue-Restricted Adaptive Type 2 Immunity Is Orchestrated by Expression of the Costimulatory Molecule OX40L on Group 2 Innate Lymphoid Cells. Immunity *48*, 1195-1207.e6.

96. Kim, M.-Y., Gaspal, F.M.C., Wiggett, H.E., McConnell, F.M., Gulbranson-Judge, A., Raykundalia, C., Walker, L.S.K., Goodall, M.D., and Lane, P.J.L. (2003). CD4(+)CD3(-) accessory cells costimulate primed CD4 T cells through OX40 and CD30 at sites where T cells collaborate with B cells. Immunity *18*, 643–654.

97. Kim, S., Han, S., Withers, D.R., Gaspal, F., Bae, J., Baik, S., Shin, H.-C., Kim, K.-S., Bekiaris, V., Anderson, G., et al. (2011). CD117$^+$ CD3$^-$ CD56$^-$ OX40Lhigh cells express IL-22 and display an LTi phenotype in human secondary lymphoid tissues. Eur. J. Immunol. *41*, 1563–1572.

98. Krause, P., Bruckner, M., Uermösi, C., Singer, E., Groettrup, M., and Legler, D.F. (2009). Prostaglandin E2 enhances T-cell proliferation by inducing the costimulatory molecules OX40L, CD70, and 4-1BBL on dendritic cells. Blood *113*, 2451–2460.

99. Kurche, J.S., Haluszczak, C., McWilliams, J.A., Sanchez, P.J., and Kedl, R.M. (2012). Type I IFN-dependent T cell activation is mediated by IFN-dependent dendritic cell OX40 ligand expression and is independent of T cell IFNR expression. J. Immunol. *188*, 585–593.

100. Ito, T., Wang, Y.-H., Duramad, O., Hori, T., Delespesse, G.J., Watanabe, N., Qin, F.X.-F., Yao, Z., Cao, W., and Liu, Y.-J. (2005). TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. J. Exp. Med. *202*, 1213–1223.

101. Sato, T., Ishii, N., Murata, K., Kikuchi, K., Nakagawa, S., Ndhlovu, L.C., and Sugamura, K. (2002). Consequences of OX40-OX40 ligand interactions in Langerhans cell function: enhanced contact hypersensitivity responses in OX40L-transgenic mice. European Journal of Immunology *32*, 3326–3335.

102. Yoshiki, R., Kabashima, K., Sakabe, J., Sugita, K., Bito, T., Nakamura, M., Malissen, B., and Tokura, Y. (2010). The mandatory role of IL-10-producing and OX40 ligand-expressing mature Langerhans cells in local UVB-induced immunosuppression. J. Immunol. *184*, 5670–5677.

103. Henri, S., Poulin, L.F., Tamoutounour, S., Ardouin, L., Guilliams, M., de Bovis, B., Devilard, E., Viret, C., Azukizawa, H., Kissenpfennig, A., et al. (2010). CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. J. Exp. Med. *207*, 189–206.

104. Rogers, P.R., Song, J., Gramaglia, I., Killeen, N., and Croft, M. (2001). OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. Immunity *15*, 445–455.

105. Soroosh, P., Ine, S., Sugamura, K., and Ishii, N. (2006). OX40-OX40 ligand interaction through T cell-T cell contact contributes to CD4 T cell longevity. J. Immunol. *176*, 5975–5987.

106. Dannull, J., Nair, S., Su, Z., Boczkowski, D., DeBeck, C., Yang, B., Gilboa, E., and Vieweg, J. (2005). Enhancing the immunostimulatory function of dendritic cells by transfection with mRNA encoding OX40 ligand. Blood *105*, 3206–3213.

107. Gramaglia, I., Weinberg, A.D., Lemon, M., and Croft, M. (1998). Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. J. Immunol. *161*, 6510–6517.

108. Gramaglia, I., Jember, A., Pippig, S.D., Weinberg, A.D., Killeen, N., and Croft, M. (2000). The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. J. Immunol. *165*, 3043–3050.

109. Maxwell, J.R., Weinberg, A., Prell, R.A., and Vella, A.T. (2000). Danger and OX40 receptor signaling synergize to enhance memory T cell survival by inhibiting peripheral deletion. J. Immunol. *164*, 107–112.

110. Brocker, T., Gulbranson-Judge, A., Flynn, S., Riedinger, M., Raykundalia, C., and Lane, P. (1999). CD4 T cell traffic control: in vivo evidence that ligation of OX40 on CD4 T cells by OX40-ligand expressed on dendritic cells leads to the accumulation of CD4 T cells in B follicles. Eur. J. Immunol. *29*, 1610–1616.

111. Murata, K., Nose, M., Ndhlovu, L.C., Sato, T., Sugamura, K., and Ishii, N. (2002). Constitutive OX40/OX40 ligand interaction induces autoimmune-like diseases. J. Immunol. *169*, 4628–4636.

112. Flynn, S., Toellner, K.M., Raykundalia, C., Goodall, M., and Lane, P. (1998). CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blr-1. J. Exp. Med. *188*, 297–304.

113. Ohshima, Y., Yang, L.P., Uchiyama, T., Tanaka, Y., Baum, P., Sergerie, M., Hermann, P., and Delespesse, G. (1998). OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. Blood *92*, 3338–3345.

114. So, T., Song, J., Sugie, K., Altman, A., and Croft, M. (2006). Signals from OX40 regulate nuclear factor of activated T cells c1 and T cell helper 2 lineage commitment. Proc. Natl. Acad. Sci. U.S.A. *103*, 3740–3745.

115. Pattarini, L., Trichot, C., Bogiatzi, S., Grandclaudon, M., Meller, S., Keuylian, Z., Durand, M., Volpe, E., Madonna, S., Cavani, A., et al. (2017). TSLP-activated dendritic cells induce human T follicular helper cell differentiation through OX40-ligand. Journal of Experimental Medicine *214*, 1529–1546.

116. Tahiliani, V., Hutchinson, T.E., Abboud, G., Croft, M., and Salek-Ardakani, S. (2017). OX40 Cooperates with ICOS To Amplify Follicular Th Cell Development and Germinal Center Reactions during Infection. J. Immunol. *198*, 218–228.

117. Baeyens, A., Saadoun, D., Billiard, F., Rouers, A., Grégoire, S., Zaragoza, B., Grinberg-Bleyer, Y., Marodon, G., Piaggio, E., and Salomon, B.L. (2015). Effector T Cells Boost Regulatory T Cell Expansion by IL-2, TNF, OX40, and Plasmacytoid Dendritic Cells Depending on the Immune Context. The Journal of Immunology *194*, 999–1010.

118. Takeda, I., Ine, S., Killeen, N., Ndhlovu, L.C., Murata, K., Satomi, S., Sugamura, K., and Ishii, N. (2004). Distinct Roles for the OX40-OX40 Ligand Interaction in Regulatory and Nonregulatory T Cells. The Journal of Immunology *172*, 3580–3589.

119. Vu, M.D., Xiao, X., Gao, W., Degauque, N., Chen, M., Kroemer, A., Killeen, N., Ishii, N., and Li, X.C. (2007). OX40 costimulation turns off Foxp3+ Tregs. Blood *110*, 2501–2510.

120. Ruby, C.E., Yates, M.A., Hirschhorn-Cymerman, D., Chlebeck, P., Wolchok, J.D., Houghton, A.N., Offner, H., and Weinberg, A.D. (2009). Cutting Edge: OX40 Agonists Can

Drive Regulatory T Cell Expansion if the Cytokine Milieu Is Right. The Journal of Immunology *183*, 4853–4857.

121. Haddad, C.S., Bhattacharya, P., Alharshawi, K., Marinelarena, A., Kumar, P., El-Sayed, O., Elshabrawy, H.A., Epstein, A.L., and Prabhakar, B.S. (2016). Age-dependent divergent effects of OX40L treatment on the development of diabetes in NOD mice. Autoimmunity *49*, 298–311.

122. Deng, T., Suo, C., Chang, J., Yang, R., Li, J., Cai, T., and Qiu, J. (2019). ILC3-derived OX40L is essential for homeostasis of intestinal Tregs in immunodeficient mice. Cell. Mol. Immunol.

123. Kjaergaard, J., Peng, L., Cohen, P.A., Drazba, J.A., Weinberg, A.D., and Shu, S. (2001). Augmentation Versus Inhibition: Effects of Conjunctional OX-40 Receptor Monoclonal Antibody and IL-2 Treatment on Adoptive Immunotherapy of Advanced Tumor. The Journal of Immunology *167*, 6669–6677.

124. Weinberg, A.D., Rivera, M.-M., Prell, R., Morris, A., Ramstad, T., Vetto, J.T., Urba, W.J., Alvord, G., Bunce, C., and Shields, J. (2000). Engagement of the OX-40 Receptor In Vivo Enhances Antitumor Immunity. The Journal of Immunology *164*, 2160–2169.

125. Morris, A., Vetto, J.T., Ramstad, T., Funatake, C.J., Choolun, E., Entwisle, C., and Weinberg, A.D. (2001). Induction of anti-mammary cancer immunity by engaging the OX-40 receptor in vivo. Breast Cancer Res. Treat. *67*, 71–80.

126. Pan, P.-Y., Zang, Y., Weber, K., Meseck, M.L., and Chen, S.-H. (2002). OX40 ligation enhances primary and memory cytotoxic T lymphocyte responses in an immunotherapy for hepatic colon metastases. Mol. Ther. *6*, 528–536.

127. Search of: MEDI6469 - List Results - ClinicalTrials.gov. Retrieved from https://clinicaltrials.gov/ct2/results?term=MEDI6469. Accessed 05/16/2019.

128. Arestides, R.S.S., He, H., Westlake, R.M., Chen, A.I., Sharpe, A.H., Perkins, D.L., and Finn, P.W. (2002). Costimulatory molecule OX40L is critical for both Th1 and Th2 responses in allergic inflammation. Eur. J. Immunol. *32*, 2874–2880.

129. Jember, A.G., Zuberi, R., Liu, F.T., and Croft, M. (2001). Development of allergic inflammation in a murine model of asthma is dependent on the costimulatory receptor OX40. J. Exp. Med. *193*, 387–392.

130. Hoshino, A., Tanaka, Y., Akiba, H., Asakura, Y., Mita, Y., Sakurai, T., Takaoka, A., Nakaike, S., Ishii, N., Sugamura, K., et al. (2003). Critical role for OX40 ligand in the development of pathogenic Th2 cells in a murine model of asthma. Eur. J. Immunol. *33*, 861–869.

131. Salek-Ardakani, S., Song, J., Halteman, B.S., Jember, A.G.-H., Akiba, H., Yagita, H., and Croft, M. (2003). OX40 (CD134) controls memory T helper 2 cells that drive lung inflammation. J. Exp. Med. *198*, 315–324.

132. Gauvreau, G.M., Boulet, L.-P., Cockcroft, D.W., FitzGerald, J.M., Mayers, I., Carlsten, C., Laviolette, M., Killian, K.J., Davis, B.E., Larché, M., et al. (2014). OX40L blockade and allergen-induced airway responses in subjects with mild asthma. Clin. Exp. Allergy *44*, 29–37.

133. Guttman-Yassky, E., Pavel, A.B., Zhou, L., Estrada, Y.D., Zhang, N., Xu, H., Peng, X., Wen, H.-C., Govas, P., Gudi, G., et al. (2019). GBR 830, an anti-OX40, improves skin gene signatures and clinical scores in patients with atopic dermatitis. Journal of Allergy and Clinical Immunology.

134.   Durai, V., and Murphy, K.M. (2016). Functions of Murine Dendritic Cells. Immunity *45*, 719–736.

135.   Hoeffel, G., Wang, Y., Greter, M., See, P., Teo, P., Malleret, B., Leboeuf, M., Low, D., Oller, G., Almeida, F., et al. (2012). Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. J. Exp. Med. *209*, 1167–1181.

136.   Katz, S.I., Tamaki, K., and Sachs, D.H. (1979). Epidermal Langerhans cells are derived from cells originating in bone marrow. Nature *282*, 324–326.

137.   Tamaki, K., and Katz, S.I. (1980). Ontogeny of Langerhans cells. J. Invest. Dermatol. *75*, 12–13.

138.   Giacometti, L., and Montagna, W. (1967). Langerhans cells: uptake of tritiated thymidine. Science *157*, 439–440.

139.   Ghigo, C., Mondor, I., Jorquera, A., Nowak, J., Wienert, S., Zahner, S.P., Clausen, B.E., Luche, H., Malissen, B., Klauschen, F., et al. (2013). Multicolor fate mapping of Langerhans cell homeostasis. J. Exp. Med. *210*, 1657–1664.

140.   Merad, M., Manz, M.G., Karsunky, H., Wagers, A., Peters, W., Charo, I., Weissman, I.L., Cyster, J.G., and Engleman, E.G. (2002). Langerhans cells renew in the skin throughout life under steady-state conditions. Nat. Immunol. *3*, 1135–1141.

141.   Ginhoux, F., Tacke, F., Angeli, V., Bogunovic, M., Loubeau, M., Dai, X.-M., Stanley, E.R., Randolph, G.J., and Merad, M. (2006). Langerhans cells arise from monocytes in vivo. Nat. Immunol. *7*, 265–273.

142.   Nagao, K., Kobayashi, T., Moro, K., Ohyama, M., Adachi, T., Kitashima, D.Y., Ueha, S., Horiuchi, K., Tanizaki, H., Kabashima, K., et al. (2012). Stress-induced production of chemokines by hair follicles regulates the trafficking of dendritic cells in skin. Nat. Immunol. *13*, 744–752.

143.   Seré, K., Baek, J.-H., Ober-Blöbaum, J., Müller-Newen, G., Tacke, F., Yokota, Y., Zenke, M., and Hieronymus, T. (2012). Two distinct types of Langerhans cells populate the skin during steady state and inflammation. Immunity *37*, 905–916.

144.   Kaplan, D.H. (2017). Ontogeny and function of murine epidermal Langerhans cells. Nature Immunology *18*, 1068–1075.

145.   Bobr, A., Igyarto, B.Z., Haley, K.M., Li, M.O., Flavell, R.A., and Kaplan, D.H. (2012). Autocrine/paracrine TGF-β1 inhibits Langerhans cell migration. Proc. Natl. Acad. Sci. U.S.A. *109*, 10492–10497.

146.   Kel, J.M., Girard-Madoux, M.J.H., Reizis, B., and Clausen, B.E. (2010). TGF-beta is required to maintain the pool of immature Langerhans cells in the epidermis. J. Immunol. *185*, 3248–3255.

147.   Kaplan, D.H., Li, M.O., Jenison, M.C., Shlomchik, W.D., Flavell, R.A., and Shlomchik, M.J. (2007). Autocrine/paracrine TGFbeta1 is required for the development of epidermal Langerhans cells. J. Exp. Med. *204*, 2545–2552.

148.   Yang, Z., Mu, Z., Dabovic, B., Jurukovski, V., Yu, D., Sung, J., Xiong, X., and Munger, J.S. (2007). Absence of integrin-mediated TGFbeta1 activation in vivo recapitulates the phenotype of TGFbeta1-null mice. J. Cell Biol. *176*, 787–793.

149.   Deckers, J., Hammad, H., and Hoste, E. (2018). Langerhans Cells: Sensing the Environment in Health and Disease. Front. Immunol. *9*.

150.   Kissenpfennig, A., Henri, S., Dubois, B., Laplace-Builhé, C., Perrin, P., Romani, N., Tripp, C.H., Douillard, P., Leserman, L., Kaiserlian, D., et al. (2005). Dynamics and function of

Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity *22*, 643–654.

151. Kaplan, D.H., Jenison, M.C., Saeland, S., Shlomchik, W.D., and Shlomchik, M.J. (2005). Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. Immunity *23*, 611–620.

152. Stoitzner, P., Tripp, C.H., Eberhart, A., Price, K.M., Jung, J.Y., Bursch, L., Ronchese, F., and Romani, N. (2006). Langerhans cells cross-present antigen derived from skin. PNAS *103*, 7783–7788.

153. Zaric, M., Lyubomska, O., Poux, C., Hanna, M.L., McCrudden, M.T., Malissen, B., Ingram, R.J., Power, U.F., Scott, C.J., Donnelly, R.F., et al. (2015). Dissolving microneedle delivery of nanoparticle-encapsulated antigen elicits efficient cross-priming and Th1 immune responses by murine Langerhans cells. J. Invest. Dermatol. *135*, 425–434.

154. Flacher, V., Tripp, C.H., Mairhofer, D.G., Steinman, R.M., Stoitzner, P., Idoyaga, J., and Romani, N. (2014). Murine Langerin+ dermal dendritic cells prime CD8+ T cells while Langerhans cells induce cross-tolerance. EMBO Mol Med *6*, 1191–1204.

155. Strandt, H., Pinheiro, D.F., Kaplan, D.H., Wirth, D., Gratz, I.K., Hammerl, P., Thalhamer, J., and Stoecklinger, A. (2017). Neoantigen Expression in Steady-State Langerhans Cells Induces CTL Tolerance. The Journal of Immunology *199*, 1626–1634.

156. Kashem, S.W., Igyarto, B.Z., Gerami-Nejad, M., Kumamoto, Y., Mohammed, J.A., Jarrett, E., Drummond, R.A., Zurawski, S.M., Zurawski, G., Berman, J., et al. (2015). Candida albicans morphology and dendritic cell subsets determine T helper cell differentiation. Immunity *42*, 356–366.

157. Mathers, A.R., Janelsins, B.M., Rubin, J.P., Tkacheva, O.A., Shufesky, W.J., Watkins, S.C., Morelli, A.E., and Larregina, A.T. (2009). Differential Capability of Human Cutaneous Dendritic Cell Subsets to Initiate Th17 Responses. The Journal of Immunology *182*, 921–933.

158. Kobayashi, T., Glatz, M., Horiuchi, K., Kawasaki, H., Akiyama, H., Kaplan, D.H., Kong, H.H., Amagai, M., and Nagao, K. (2015). Dysbiosis and Staphylococcus aureus Colonization Drives Inflammation in Atopic Dermatitis. Immunity *42*, 756–766.

159. Deckers, J., Sichien, D., Plantinga, M., Moorleghem, J.V., Vanheerswynghels, M., Hoste, E., Malissen, B., Dombrowicz, D., Guilliams, M., Bosscher, K.D., et al. (2017). Epicutaneous sensitization to house dust mite allergen requires interferon regulatory factor 4–dependent dermal dendritic cells. Journal of Allergy and Clinical Immunology *140*, 1364-1377.e2.

160. Kumamoto, Y., Linehan, M., Weinstein, J.S., Laidlaw, B.J., Craft, J.E., and Iwasaki, A. (2013). CD301b+ dermal dendritic cells drive T helper 2 cell-mediated immunity. Immunity *39*, 733–743.

161. Ouchi, T., Kubo, A., Yokouchi, M., Adachi, T., Kobayashi, T., Kitashima, D.Y., Fujii, H., Clausen, B.E., Koyasu, S., Amagai, M., et al. (2011). Langerhans cell antigen capture through tight junctions confers preemptive immunity in experimental staphylococcal scalded skin syndrome. J. Exp. Med. *208*, 2607–2613.

162. Zimara, N., Florian, C., Schmid, M., Malissen, B., Kissenpfennig, A., Männel, D.N., Edinger, M., Hutchinson, J.A., Hoffmann, P., and Ritter, U. (2014). Langerhans cells promote early germinal center formation in response to Leishmania-derived cutaneous antigens. European Journal of Immunology *44*, 2955–2967.

163.   Levin, C., Bonduelle, O., Nuttens, C., Primard, C., Verrier, B., Boissonnas, A., and Combadière, B. (2017). Critical Role for Skin-Derived Migratory DCs and Langerhans Cells in TFH and GC Responses after Intradermal Immunization. J Invest Dermatol *137*, 1905–1913.

164.   Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J.V., Steinman, R.M., and Nussenzweig, M.C. (2001). Dendritic Cells Induce Peripheral T Cell Unresponsiveness under Steady State Conditions in Vivo. Journal of Experimental Medicine *194*, 769–780.

165.   Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M.C., and Steinman, R.M. (2002). Efficient Targeting of Protein Antigen to the Dendritic Cell Receptor DEC-205 in the Steady State Leads to Antigen Presentation on Major Histocompatibility Complex Class I Products and Peripheral CD8+ T Cell Tolerance. Journal of Experimental Medicine *196*, 1627–1638.

166.   Shklovskaya, E., O'Sullivan, B.J., Ng, L.G., Roediger, B., Thomas, R., Weninger, W., and Fazekas de St Groth, B. (2011). Langerhans cells are precommitted to immune tolerance induction. Proc. Natl. Acad. Sci. U.S.A. *108*, 18049–18054.

167.   Kautz-Neu, K., Noordegraaf, M., Dinges, S., Bennett, C.L., John, D., Clausen, B.E., and Stebut, E. von (2011). Langerhans cells are negative regulators of the anti-Leishmania response. Journal of Experimental Medicine *208*, 885–891.

168.   Agüero, M.G. de, Vocanson, M., Hacini-Rachinel, F., Taillardet, M., Sparwasser, T., Kissenpfennig, A., Malissen, B., Kaiserlian, D., and Dubois, B. (2012). Langerhans cells protect from allergic contact dermatitis in mice by tolerizing CD8$^+$ T cells and activating Foxp3$^+$ regulatory T cells. J Clin Invest *122*, 1700–1711.

169.   Dioszeghy, V., Mondoulet, L., Laoubi, L., Dhelft, V., Plaquet, C., Bouzereau, A., Dupont, C., and Sampson, H. (2018). Antigen Uptake by Langerhans Cells Is Required for the Induction of Regulatory T Cells and the Acquisition of Tolerance During Epicutaneous Immunotherapy in OVA-Sensitized Mice. Front Immunol *9*.

170.   Kitashima, D.Y., Kobayashi, T., Woodring, T., Idouchi, K., Doebel, T., Voisin, B., Adachi, T., Ouchi, T., Takahashi, H., Nishifuji, K., et al. (2018). Langerhans Cells Prevent Autoimmunity via Expansion of Keratinocyte Antigen-Specific Regulatory T Cells. EBioMedicine *27*, 293–303.

171.   Seneschal, J., Clark, R.A., Gehad, A., Baecher-Allan, C.M., and Kupper, T.S. (2012). Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells. Immunity *36*, 873–884.

172.   Loh, J.T., Lim, T.J.F., Ikumi, K., Matoba, T., Janela, B., Gunawan, M., Toyama, T., Bunjamin, M., Ng, L.G., Poidinger, M., et al. (2018). Ezh2 Controls Skin Tolerance through Distinct Mechanisms in Different Subsets of Skin Dendritic Cells. IScience *10*, 23–39.

173.   Igyarto, B.Z., Jenison, M.C., Dudda, J.C., Roers, A., Müller, W., Koni, P.A., Campbell, D.J., Shlomchik, M.J., and Kaplan, D.H. (2009). Langerhans Cells Suppress Contact Hypersensitivity Responses Via Cognate CD4 Interaction and Langerhans Cell-Derived IL-10. The Journal of Immunology *183*, 5085–5093.

174.   Loser, K., Mehling, A., Loeser, S., Apelt, J., Kuhn, A., Grabbe, S., Schwarz, T., Penninger, J.M., and Beissert, S. (2006). Epidermal RANKL controls regulatory T-cell numbers via activation of dendritic cells. Nat. Med. *12*, 1372–1379.

175. Kingston, D., Schmid, M.A., Onai, N., Obata-Onai, A., Baumjohann, D., and Manz, M.G. (2009). The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis. Blood *114*, 835–843.

176. Hildner, K., Edelson, B.T., Purtha, W.E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B.U., Unanue, E.R., Diamond, M.S., et al. (2008). Batf3 Deficiency Reveals a Critical Role for CD8α+ Dendritic Cells in Cytotoxic T Cell Immunity. Science *322*, 1097–1100.

177. Edelson, B.T., Bradstreet, T.R., Kc, W., Hildner, K., Herzog, J.W., Sim, J., Russell, J.H., Murphy, T.L., Unanue, E.R., and Murphy, K.M. (2011). Batf3-Dependent CD11blow/– Peripheral Dendritic Cells Are GM-CSF-Independent and Are Not Required for Th Cell Priming after Subcutaneous Immunization. PLOS ONE *6*, e25660.

178. Seillet, C., Jackson, J.T., Markey, K.A., Brady, H.J.M., Hill, G.R., Macdonald, K.P.A., Nutt, S.L., and Belz, G.T. (2013). CD8α+ DCs can be induced in the absence of transcription factors Id2, Nfil3, and Batf3. Blood *121*, 1574–1583.

179. Tailor, P., Tamura, T., Morse, H.C., and Ozato, K. (2008). The BXH2 mutation in IRF8 differentially impairs dendritic cell subset development in the mouse. Blood *111*, 1942–1945.

180. Bedoui, S., Whitney, P.G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R.S., Wojtasiak, M., Shortman, K., Carbone, F.R., et al. (2009). Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. Nat. Immunol. *10*, 488–495.

181. Diamond, M.S., Kinder, M., Matsushita, H., Mashayekhi, M., Dunn, G.P., Archambault, J.M., Lee, H., Arthur, C.D., White, J.M., Kalinke, U., et al. (2011). Type I interferon is selectively required by dendritic cells for immune rejection of tumors. J. Exp. Med. *208*, 1989–2003.

182. Fuertes, M.B., Kacha, A.K., Kline, J., Woo, S.-R., Kranz, D.M., Murphy, K.M., and Gajewski, T.F. (2011). Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells. J. Exp. Med. *208*, 2005–2016.

183. Mashayekhi, M., Sandau, M.M., Dunay, I.R., Frickel, E.M., Khan, A., Goldszmid, R.S., Sher, A., Ploegh, H.L., Murphy, T.L., Sibley, L.D., et al. (2011). CD8α(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites. Immunity *35*, 249–259.

184. Reis e Sousa, C., Hieny, S., Scharton-Kersten, T., Jankovic, D., Charest, H., Germain, R.N., and Sher, A. (1997). In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. J. Exp. Med. *186*, 1819–1829.

185. Fossum, E., Grødeland, G., Terhorst, D., Tveita, A.A., Vikse, E., Mjaaland, S., Henri, S., Malissen, B., and Bogen, B. (2015). Vaccine molecules targeting Xcr1 on cross-presenting DCs induce protective CD8+ T-cell responses against influenza virus. Eur. J. Immunol. *45*, 624–635.

186. Wculek, S.K., Amores-Iniesta, J., Conde-Garrosa, R., Khouili, S.C., Melero, I., and Sancho, D. (2019). Effective cancer immunotherapy by natural mouse conventional type-1 dendritic cells bearing dead tumor antigen. Journal for ImmunoTherapy of Cancer *7*, 100.

187. Cancel, J.-C., Crozat, K., Dalod, M., and Mattiuz, R. (2019). Are Conventional Type 1 Dendritic Cells Critical for Protective Antitumor Immunity and How? Front. Immunol. *10*.

188. Tamoutounour, S., Guilliams, M., Montanana Sanchis, F., Liu, H., Terhorst, D., Malosse, C., Pollet, E., Ardouin, L., Luche, H., Sanchez, C., et al. (2013). Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. Immunity *39*, 925–938.

189. Guerriero, A., Langmuir, P.B., Spain, L.M., and Scott, E.W. (2000). PU.1 is required for myeloid-derived but not lymphoid-derived dendritic cells. Blood *95*, 879–885.

190. Wu, L., D'Amico, A., Winkel, K.D., Suter, M., Lo, D., and Shortman, K. (1998). RelB Is Essential for the Development of Myeloid-Related CD8α– Dendritic Cells but Not of Lymphoid-Related CD8α+ Dendritic Cells. Immunity *9*, 839–847.

191. Caton, M.L., Smith-Raska, M.R., and Reizis, B. (2007). Notch–RBP-J signaling controls the homeostasis of CD8– dendritic cells in the spleen. Journal of Experimental Medicine *204*, 1653–1664.

192. Bajaña, S., Roach, K., Turner, S., Paul, J., and Kovats, S. (2012). IRF4 Promotes Cutaneous Dendritic Cell Migration to Lymph Nodes during Homeostasis and Inflammation. The Journal of Immunology *189*, 3368–3377.

193. Flores-Langarica, A., Cook, C., Müller Luda, K., Persson, E.K., Marshall, J.L., Beristain-Covarrubias, N., Yam-Puc, J.C., Dahlgren, M., Persson, J.J., Uematsu, S., et al. (2018). Intestinal CD103+CD11b+ cDC2 Conventional Dendritic Cells Are Required for Primary CD4+ T and B Cell Responses to Soluble Flagellin. Front Immunol *9*.

194. Gao, Y., Nish, S.A., Jiang, R., Hou, L., Licona-Limón, P., Weinstein, J.S., Zhao, H., and Medzhitov, R. (2013). Control of T Helper 2 Responses by Transcription Factor IRF4-Dependent Dendritic Cells. Immunity *39*, 722–732.

195. Williams, J.W., Tjota, M.Y., Clay, B.S., Vander Lugt, B., Bandukwala, H.S., Hrusch, C.L., Decker, D.C., Blaine, K.M., Fixsen, B.R., Singh, H., et al. (2013). Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. Nature Communications *4*, 2990.

196. Zhou, Q., Ho, A.W.S., Schlitzer, A., Tang, Y., Wong, K.H.S., Wong, F.H.S., Chua, Y.L., Angeli, V., Mortellaro, A., Ginhoux, F., et al. (2014). GM-CSF–Licensed CD11b+ Lung Dendritic Cells Orchestrate Th2 Immunity to Blomia tropicalis. The Journal of Immunology *193*, 496–509.

197. Kumamoto, Y., Linehan, M., Weinstein, J.S., Laidlaw, B.J., Craft, J.E., and Iwasaki, A. (2013). CD301b+ Dermal Dendritic Cells Drive T Helper 2 Cell-Mediated Immunity. Immunity *39*, 733–743.

198. Tussiwand, R., Everts, B., Grajales-Reyes, G.E., Kretzer, N.M., Iwata, A., Bagaitkar, J., Wu, X., Wong, R., Anderson, D.A., Murphy, T.L., et al. (2015). Klf4 expression in conventional dendritic cells is required for T helper 2 cell responses. Immunity *42*, 916–928.

199. Persson, E.K., Uronen-Hansson, H., Semmrich, M., Rivollier, A., Hägerbrand, K., Marsal, J., Gudjonsson, S., Håkansson, U., Reizis, B., Kotarsky, K., et al. (2013). IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. Immunity *38*, 958–969.

200. Schlitzer, A., McGovern, N., Teo, P., Zelante, T., Atarashi, K., Low, D., Ho, A.W.S., See, P., Shin, A., Wasan, P.S., et al. (2013). IRF4 Transcription Factor-Dependent CD11b+ Dendritic Cells in Human and Mouse Control Mucosal IL-17 Cytokine Responses. Immunity *38*, 970–983.

201. Kinnebrew, M.A., Buffie, C.G., Diehl, G.E., Zenewicz, L.A., Leiner, I., Hohl, T.M., Flavell, R.A., Littman, D.R., and Pamer, E.G. (2012). Interleukin 23 production by intestinal

CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. Immunity *36*, 276–287.

202. Lewis, K.L., Caton, M.L., Bogunovic, M., Greter, M., Grajkowska, L.T., Ng, D., Klinakis, A., Charo, I.F., Jung, S., Gommerman, J.L., et al. (2011). Notch2 Receptor Signaling Controls Functional Differentiation of Dendritic Cells in the Spleen and Intestine. Immunity *35*, 780–791.

203. Naik, S., Bouladoux, N., Linehan, J.L., Han, S.-J., Harrison, O.J., Wilhelm, C., Conlan, S., Himmelfarb, S., Byrd, A.L., Deming, C., et al. (2015). Commensal–dendritic-cell interaction specifies a unique protective skin immune signature. Nature *520*, 104–108.

204. Guilliams, M., Crozat, K., Henri, S., Tamoutounour, S., Grenot, P., Devilard, E., de Bovis, B., Alexopoulou, L., Dalod, M., and Malissen, B. (2010). Skin-draining lymph nodes contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. Blood *115*, 1958–1968.

205. Collin, M., and Bigley, V. (2018). Human dendritic cell subsets: an update. Immunology *154*, 3.

206. Gerlini, G., Mariotti, G., Bianchi, B., and Pimpinelli, N. (2006). Massive recruitment of type I interferon producing plasmacytoid dendritic cells in varicella skin lesions. J. Invest. Dermatol. *126*, 507–509.

207. Wollenberg, A., Günther, S., Moderer, M., Wetzel, S., Wagner, M., Towarowski, A., Tuma, E., Rothenfusser, S., Endres, S., and Hartmann, G. (2002). Plasmacytoid Dendritic Cells: A New Cutaneous Dendritic Cell Subset with Distinct Role in Inflammatory Skin Diseases. J Invest Dermatol *119*, 1096–1102.

208. Nestle, F.O., Conrad, C., Tun-Kyi, A., Homey, B., Gombert, M., Boyman, O., Burg, G., Liu, Y.-J., and Gilliet, M. (2005). Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. J. Exp. Med. *202*, 135–143.

209. Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S.H., Onai, N., Schraml, B.U., Segura, E., Tussiwand, R., and Yona, S. (2014). Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nature Reviews Immunology *14*, 571–578.

210. Crozat, K., Guiton, R., Guilliams, M., Henri, S., Baranek, T., Schwartz-Cornil, I., Malissen, B., and Dalod, M. (2010). Comparative genomics as a tool to reveal functional equivalences between human and mouse dendritic cell subsets. Immunol. Rev. *234*, 177–198.

211. Guilliams, M., Henri, S., Tamoutounour, S., Ardouin, L., Schwartz-Cornil, I., Dalod, M., and Malissen, B. (2010). From skin dendritic cells to a simplified classification of human and mouse dendritic cell subsets. European Journal of Immunology *40*, 2089–2094.

212. Alcántara-Hernández, M., Leylek, R., Wagar, L.E., Engleman, E.G., Keler, T., Marinkovich, M.P., Davis, M.M., Nolan, G.P., and Idoyaga, J. (2017). High-Dimensional Phenotypic Mapping of Human Dendritic Cells Reveals Interindividual Variation and Tissue Specialization. Immunity *47*, 1037-1050.e6.

213. Bigley, V., McGovern, N., Milne, P., Dickinson, R., Pagan, S., Cookson, S., Haniffa, M., and Collin, M. (2015). Langerin-expressing dendritic cells in human tissues are related to CD1c+ dendritic cells and distinct from Langerhans cells and CD141high XCR1+ dendritic cells. J. Leukoc. Biol. *97*, 627–634.

214. Carpentier, S., Vu Manh, T.-P., Chelbi, R., Henri, S., Malissen, B., Haniffa, M., Ginhoux, F., and Dalod, M. (2016). Comparative genomics analysis of mononuclear phagocyte subsets confirms homology between lymphoid tissue-resident and dermal XCR1+ DCs in

mouse and human and distinguishes them from Langerhans cells. Journal of Immunological Methods *432*, 35–49.

215. Stojadinovic, O., Yin, N., Lehmann, J., Pastar, I., Kirsner, R.S., and Tomic-Canic, M. (2013). Increased number of Langerhans cells in the epidermis of diabetic foot ulcers correlates with healing outcome. Immunol. Res. *57*, 222–228.

216. Martin, P., D'Souza, D., Martin, J., Grose, R., Cooper, L., Maki, R., and McKercher, S.R. (2003). Wound Healing in the PU.1 Null Mouse—Tissue Repair Is Not Dependent on Inflammatory Cells. Current Biology *13*, 1122–1128.

217. Schuller, E., Teichmann, B., Haberstok, J., Moderer, M., Bieber, T., and Wollenberg, A. (2001). In situ expression of the costimulatory molecules CD80 and CD86 on langerhans cells and inflammatory dendritic epidermal cells (IDEC) in atopic dermatitis. Arch. Dermatol. Res. *293*, 448–454.

218. Leitch, C.S., Natafji, E., Yu, C., Abdul-Ghaffar, S., Madarasingha, N., Venables, Z.C., Chu, R., Fitch, P.M., Muinonen-Martin, A.J., Campbell, L.E., et al. (2016). Filaggrin-null mutations are associated with increased maturation markers on Langerhans cells. J. Allergy Clin. Immunol. *138*, 482-490.e7.

219. Ebner, S., Nguyen, V.A., Forstner, M., Wang, Y.-H., Wolfram, D., Liu, Y.-J., and Romani, N. (2007). Thymic stromal lymphopoietin converts human epidermal Langerhans cells into antigen-presenting cells that induce proallergic T cells. J. Allergy Clin. Immunol. *119*, 982–990.

220. Soumelis, V., Reche, P.A., Kanzler, H., Yuan, W., Edward, G., Homey, B., Gilliet, M., Ho, S., Antonenko, S., Lauerma, A., et al. (2002). Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat. Immunol. *3*, 673–680.

221. Haniffa, M., Shin, A., Bigley, V., McGovern, N., Teo, P., See, P., Wasan, P.S., Wang, X.-N., Malinarich, F., Malleret, B., et al. (2012). Human Tissues Contain CD141hi Cross-Presenting Dendritic Cells with Functional Homology to Mouse CD103+ Nonlymphoid Dendritic Cells. Immunity *37*, 60–73.

222. Schlitzer, A., McGovern, N., Teo, P., Zelante, T., Atarashi, K., Low, D., Ho, A.W.S., See, P., Shin, A., Wasan, P.S., et al. (2013). IRF4 Transcription Factor-Dependent CD11b+ Dendritic Cells in Human and Mouse Control Mucosal IL-17 Cytokine Responses. Immunity *38*, 970–983.

223. Robbins, S.H., Walzer, T., Dembélé, D., Thibault, C., Defays, A., Bessou, G., Xu, H., Vivier, E., Sellars, M., Pierre, P., et al. (2008). Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. Genome Biol. *9*, R17.

224. Villani, A.-C., Satija, R., Reynolds, G., Sarkizova, S., Shekhar, K., Fletcher, J., Griesbeck, M., Butler, A., Zheng, S., Lazo, S., et al. (2017). Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. Science *356*.

225. Yin, X., Yu, H., Jin, X., Li, J., Guo, H., Shi, Q., Yin, Z., Xu, Y., Wang, X., Liu, R., et al. (2017). Human Blood CD1c+ Dendritic Cells Encompass CD5high and CD5low Subsets That Differ Significantly in Phenotype, Gene Expression, and Functions. J. Immunol. *198*, 1553–1564.

226. Froidure, A., Vandenplas, O., D'Alpaos, V., Evrard, G., and Pilette, C. (2015). Defects in Plasmacytoid Dendritic Cell Expression of Inducible Costimulator Ligand and IFN-α Are Associated in Asthma with Disease Persistence. Am. J. Respir. Crit. Care Med. *192*, 392–395.

227. Pritchard, A.L., Carroll, M.L., Burel, J.G., White, O.J., Phipps, S., and Upham, J.W. (2012). Innate IFNs and plasmacytoid dendritic cells constrain Th2 cytokine responses to rhinovirus: a regulatory mechanism with relevance to asthma. J. Immunol. *188*, 5898–5905.

228. Ghirelli, C., Reyal, F., Jeanmougin, M., Zollinger, R., Sirven, P., Michea, P., Caux, C., Bendriss-Vermare, N., Donnadieu, M.-H., Caly, M., et al. (2015). Breast Cancer Cell-Derived GM-CSF Licenses Regulatory Th2 Induction by Plasmacytoid Predendritic Cells in Aggressive Disease Subtypes. Cancer Res. *75*, 2775–2787.

229. McGovern, N., Schlitzer, A., Gunawan, M., Jardine, L., Shin, A., Poyner, E., Green, K., Dickinson, R., Wang, X.-N., Low, D., et al. (2014). Human dermal CD14$^+$ cells are a transient population of monocyte-derived macrophages. Immunity *41*, 465–477.

230. Wollenberg, A., Kraft, S., Hanau, D., and Bieber, T. (1996). Immunomorphological and ultrastructural characterization of Langerhans cells and a novel, inflammatory dendritic epidermal cell (IDEC) population in lesional skin of atopic eczema. J. Invest. Dermatol. *106*, 446–453.

231. Wollenberg, A., Mommaas, M., Oppel, T., Schottdorf, E.-M., Günther, S., and Moderer, M. (2002). Expression and function of the mannose receptor CD206 on epidermal dendritic cells in inflammatory skin diseases. J. Invest. Dermatol. *118*, 327–334.

232. Zaba, L.C., Fuentes-Duculan, J., Eungdamrong, N.J., Abello, M.V., Novitskaya, I., Pierson, K.C., Gonzalez, J., Krueger, J.G., and Lowes, M.A. (2009). Psoriasis is characterized by accumulation of immunostimulatory and Th1/Th17 cell-polarizing myeloid dendritic cells. J. Invest. Dermatol. *129*, 79–88.

233. Jenner, W., Motwani, M., Veighey, K., Newson, J., Audzevich, T., Nicolaou, A., Murphy, S., Macallister, R., and Gilroy, D.W. (2014). Characterisation of leukocytes in a human skin blister model of acute inflammation and resolution. PLoS ONE *9*, e89375.

234. Yasuda, K., Richez, C., Maciaszek, J.W., Agrawal, N., Akira, S., Marshak-Rothstein, A., and Rifkin, I.R. (2007). Murine Dendritic Cell Type I IFN Production Induced by Human IgG-RNA Immune Complexes Is IFN Regulatory Factor (IRF)5 and IRF7 Dependent and Is Required for IL-6 Production. The Journal of Immunology *178*, 6876–6885.

235. Tian, J., Avalos, A.M., Mao, S.-Y., Chen, B., Senthil, K., Wu, H., Parroche, P., Drabic, S., Golenbock, D., Sirois, C., et al. (2007). Toll-like receptor 9–dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. Nature Immunology *8*, 487–496.

236. Vašků, V., Kaňková, K., Vašků, A., Mužík, J., Izakovičová Hollá, L., Semrádová, V., and Vácha, J. (2002). Gene polymorphisms (G82S, 1704G/T, 2184A/G and 2245G/A) of the receptor of advanced glycation end products (RAGE) in plaque psoriasis. Arch Dermatol Res *294*, 127–130.

237. Klarquist, J., Zhou, Z., Shen, N., and Janssen, E.M. (2016). Dendritic Cells in Systemic Lupus Erythematosus: From Pathogenic Players to Therapeutic Tools. Mediators Inflamm *2016*.

238. Wenzel, J., Scheler, M., Proelss, J., Bieber, T., and Tüting, T. (2006). Type I interferon-associated cytotoxic inflammation in lichen planus. Journal of Cutaneous Pathology *33*, 672–678.

239. de Vries, H.J.C., Teunissen, M.B.M., Zorgdrager, F., Picavet, D., and Cornelissen, M. (2007). Lichen planus remission is associated with a decrease of human herpes virus type 7 protein expression in plasmacytoid dendritic cells. Arch Dermatol Res *299*, 213.

240. Santoro, A., Majorana, A., Roversi, L., Gentili, F., Marrelli, S., Vermi, W., Bardellini, E., Sapelli, P., and Facchetti, F. (2005). Recruitment of dendritic cells in oral lichen planus. The Journal of Pathology *205*, 426–434.

241. Bigley, V., Cytlak, U., and Collin, M. (2019). Human dendritic cell immunodeficiencies. Seminars in Cell & Developmental Biology *86*, 50–61.

242. Lagresle-Peyrou, C., Six, E.M., Picard, C., Rieux-Laucat, F., Michel, V., Ditadi, A., Chappedelaine, C.D., Morillon, E., Valensi, F., Simon-Stoos, K.L., et al. (2009). Human adenylate kinase 2 deficiency causes a profound hematopoietic defect associated with sensorineural deafness. Nature Genetics *41*, 106–111.

243. Pannicke, U., Hönig, M., Hess, I., Friesen, C., Holzmann, K., Rump, E.-M., Barth, T.F., Rojewski, M.T., Schulz, A., Boehm, T., et al. (2009). Reticular dysgenesis (aleukocytosis) is caused by mutations in the gene encoding mitochondrial adenylate kinase 2. Nature Genetics *41*, 101–105.

244. Reith, W., and Mach, B. (2001). The Bare Lymphocyte Syndrome and the Regulation of MHC Expression. Annu. Rev. Immunol. *19*, 331–373.

245. Hanna, S., and Etzioni, A. (2014). MHC class I and II deficiencies. Journal of Allergy and Clinical Immunology *134*, 269–275.

246. Malinova, D., Fritzsche, M., Nowosad, C.R., Armer, H., Munro, P.M.G., Blundell, M.P., Charras, G., Tolar, P., Bouma, G., and Thrasher, A.J. (2016). WASp-dependent actin cytoskeleton stability at the dendritic cell immunological synapse is required for extensive, functional T cell contacts. Journal of Leukocyte Biology *99*, 699–710.

247. Cisse, B., Caton, M.L., Lehner, M., Maeda, T., Scheu, S., Locksley, R., Holmberg, D., Zweier, C., den Hollander, N.S., Kant, S.G., et al. (2008). Transcription Factor E2-2 Is an Essential and Specific Regulator of Plasmacytoid Dendritic Cell Development. Cell *135*, 37–48.

248. Bol, K.F., Schreibelt, G., Rabold, K., Wculek, S.K., Schwarze, J.K., Dzionek, A., Teijeira, A., Kandalaft, L.E., Romero, P., Coukos, G., et al. (2019). The clinical application of cancer immunotherapy based on naturally circulating dendritic cells. J Immunother Cancer *7*.

249. Larsen, F.S., and Hanifin, J. (2002). Epidemiology of atopic dermatitis. Immunology and Allergy Clinics of North America *22*, 1–24.

250. Williams, H., Robertson, C., Stewart, A., Aït-Khaled, N., Anabwani, G., Anderson, R., Asher, I., Beasley, R., Björkstén, B., Burr, M., et al. (1999). Worldwide variations in the prevalence of symptoms of atopic eczema in the International Study of Asthma and Allergies in Childhood. J. Allergy Clin. Immunol. *103*, 125–138.

251. Schäfer, T., Krämer, U., Vieluf, D., Abeck, D., Behrendt, H., and Ring, J. (2000). The excess of atopic eczema in East Germany is related to the intrinsic type. Br. J. Dermatol. *143*, 992–998.

252. Taylor, B., Wadsworth, J., Wadsworth, M., and Peckham, C. (1984). Changes in the reported prevalence of childhood eczema since the 1939-45 war. Lancet *2*, 1255–1257.

253. Lee, H.H., Patel, K.R., Singam, V., Rastogi, S., and Silverberg, J.I. (2019). A systematic review and meta-analysis of the prevalence and phenotype of adult-onset atopic dermatitis. J. Am. Acad. Dermatol. *80*, 1526-1532.e7.

254. Ring, J., Zink, A., Arents, B.W.M., Seitz, I.A., Mensing, U., Schielein, M.C., Wettemann, N., de Carlo, G., and Fink-Wagner, A. (2019). Atopic eczema: burden of disease and individual suffering - results from a large EU study in adults. J Eur Acad Dermatol Venereol *33*, 1331–1340.

255. Beck, L.A., and Leung, D.Y.M. (2000). Allergen sensitization through the skin induces systemic allergic responses. Journal of Allergy and Clinical Immunology *106*, S258–S263.

256. Al-Shobaili, H.A., Ahmed, A.A., Alnomair, N., Alobead, Z.A., and Rasheed, Z. (2016). Molecular Genetic of Atopic dermatitis: An Update. Int J Health Sci (Qassim) *10*, 96–120.

257. Wollenberg, A., Kraft, S., Hanau, D., and Bieber, T. (1996). Immunomorphological and ultrastructural characterization of Langerhans cells and a novel, inflammatory dendritic epidermal cell (IDEC) population in lesional skin of atopic eczema. J. Invest. Dermatol. *106*, 446–453.

258. Langeveld-Wildschut, E.G., Bruijnzeel, P.L., Mudde, G.C., Versluis, C., Van Ieperen-Van Dijk, A.G., Bihari, I.C., Knol, E.F., Thepen, T., Bruijnzeel-Koomen, C.A., and van Reijsen, F.C. (2000). Clinical and immunologic variables in skin of patients with atopic eczema and either positive or negative atopy patch test reactions. J. Allergy Clin. Immunol. *105*, 1008–1016.

259. Beck, L.A., Thaçi, D., Hamilton, J.D., Graham, N.M., Bieber, T., Rocklin, R., Ming, J.E., Ren, H., Kao, R., Simpson, E., et al. (2014). Dupilumab Treatment in Adults with Moderate-to-Severe Atopic Dermatitis. New England Journal of Medicine 371, 130–139.

260. Frankland, A.W. (1955). High and low dosage pollen extract treatment in summer hay fever and asthma. Acta Allergol 9, 183–187.

261. Nakajima, S., Igyártó, B.Z., Honda, T., Egawa, G., Otsuka, A., Hara-Chikuma, M., Watanabe, N., Ziegler, S.F., Tomura, M., Inaba, K., et al. (2012). Langerhans cells are critical in epicutaneous sensitization with protein antigen via thymic stromal lymphopoietin receptor signaling. Journal of Allergy and Clinical Immunology 129, 1048-1055.e6.

262. Prue, R.L., Vari, F., Radford, K.J., Tong, H., Hardy, M.Y., D'Rozario, R., Waterhouse, N.J., Rossetti, T., Coleman, R., Tracey, C., et al. (2015). A phase I clinical trial of CD1c (BDCA-1)+ dendritic cells pulsed with HLA-A*0201 peptides for immunotherapy of metastatic hormone refractory prostate cancer. J. Immunother. *38*, 71–76.

263. Schreibelt, G., Bol, K.F., Westdorp, H., Wimmers, F., Aarntzen, E.H.J.G., Duiveman-de Boer, T., van de Rakt, M.W.M.M., Scharenborg, N.M., de Boer, A.J., Pots, J.M., et al. (2016). Effective Clinical Responses in Metastatic Melanoma Patients after Vaccination with Primary Myeloid Dendritic Cells. Clin. Cancer Res. *22*, 2155–2166.

264. Tel, J., Aarntzen, E.H.J.G., Baba, T., Schreibelt, G., Schulte, B.M., Benitez-Ribas, D., Boerman, O.C., Croockewit, S., Oyen, W.J.G., van Rossum, M., et al. (2013). Natural human plasmacytoid dendritic cells induce antigen-specific T-cell responses in melanoma patients. Cancer Res. *73*, 1063–1075.

265. Sabado, R.L., Balan, S., and Bhardwaj, N. (2017). Dendritic cell-based immunotherapy. Cell Research *27*, 74–95.

266. Krause, P., Singer, E., Darley, P.I., Klebensberger, J., Groettrup, M., and Legler, D.F. (2007). Prostaglandin E2 is a key factor for monocyte-derived dendritic cell maturation: enhanced T cell stimulatory capacity despite IDO. J. Leukoc. Biol. *82*, 1106–1114.

267. Lee, A.W., Truong, T., Bickham, K., Fonteneau, J.-F., Larsson, M., Da Silva, I., Somersan, S., Thomas, E.K., and Bhardwaj, N. (2002). A clinical grade cocktail of cytokines and PGE2 results in uniform maturation of human monocyte-derived dendritic cells: implications for immunotherapy. Vaccine *20*, A8–A22.

268. Kim, Y.-C., and Prausnitz, M.R. (2011). Enabling skin vaccination using new delivery technologies. Drug Deliv. and Transl. Res. *1*, 7–12.

269.	Arya, J., and Prausnitz, M.R. (2016). Microneedle patches for vaccination in developing countries. Journal of Controlled Release *240*, 135–141.

270.	Prausnitz, M.R. (2017). Engineering Microneedle Patches for Vaccination and Drug Delivery to Skin. Annual Review of Chemical and Biomolecular Engineering *8*, 177–200.

271.	Leone, M., Mönkäre, J., Bouwstra, J.A., and Kersten, G. (2017). Dissolving Microneedle Patches for Dermal Vaccination. Pharm Res *34*, 2223–2240.

272.	Scheiblhofer, S., Strobl, A., Hoepflinger, V., Thalhamer, T., Steiner, M., Thalhamer, J., and Weiss, R. (2017). Skin vaccination via fractional infrared laser ablation - Optimization of laser-parameters and adjuvantation. Vaccine *35*, 1802–1809.

273.	Hervé, P.-L., Dhelft, V., Plaquet, C., Rousseaux, A., Bouzereau, A., Gaulme, L., Tilleul, S., Ligouis, M., Donne, N., Lambert, P.-H., et al. (2019). Epidermal micro-perforation potentiates the efficacy of epicutaneous vaccination. Journal of Controlled Release *298*, 12–26.

274.	Probst, H.C., Lagnel, J., Kollias, G., and van den Broek, M. (2003). Inducible transgenic mice reveal resting dendritic cells as potent inducers of CD8+ T cell tolerance. Immunity 18, 713–720.

275.	Wang, J., Li, B., and Wu, M.X. (2015). Effective and lesion-free cutaneous influenza vaccination. PNAS *112*, 5005–5010.

276.	Stranges, P.B., Watson, J., Cooper, C.J., Choisy-Rossi, C.-M., Stonebraker, A.C., Beighton, R.A., Hartig, H., Sundberg, J.P., Servick, S., Kaufmann, G., et al. (2007). Elimination of Antigen-Presenting Cells and Autoreactive T Cells by Fas Contributes to Prevention of Autoimmunity. Immunity *26*, 629–641.

277.	Loschko, J., Schreiber, H.A., Rieke, G.J., Esterházy, D., Meredith, M.M., Pedicord, V.A., Yao, K.-H., Caballero, S., Pamer, E.G., Mucida, D., et al. (2016). Absence of MHC class II on cDCs results in microbial-dependent intestinal inflammation. Journal of Experimental Medicine *213*, 517–534.

278.	Zahner, S.P., Kel, J.M., Martina, C.A.E., Brouwers-Haspels, I., Roon, M.A. van, and Clausen, B.E. (2011). Conditional Deletion of TGF-βR1 Using Langerin-Cre Mice Results in Langerhans Cell Deficiency and Reduced Contact Hypersensitivity. The Journal of Immunology *187*, 5069–5076.

279.	Schraml, B.U., van Blijswijk, J., Zelenay, S., Whitney, P.G., Filby, A., Acton, S.E., Rogers, N.C., Moncaut, N., Carvajal, J.J., and Reis e Sousa, C. (2013). Genetic Tracing via DNGR-1 Expression History Defines Dendritic Cells as a Hematopoietic Lineage. Cell *154*, 843–858.

280.	Ohta, T., Sugiyama, M., Hemmi, H., Yamazaki, C., Okura, S., Sasaki, I., Fukuda, Y., Orimo, T., Ishii, K.J., Hoshino, K., et al. (2016). Crucial roles of XCR1-expressing dendritic cells and the XCR1-XCL1 chemokine axis in intestinal immune homeostasis. Scientific Reports *6*, 23505.

281.	Mattiuz, R., Wohn, C., Ghilas, S., Ambrosini, M., Alexandre, Y.O., Sanchez, C., Fries, A., Vu Manh, T.-P., Malissen, B., Dalod, M., et al. (2018). Novel Cre-Expressing Mouse Strains Permitting to Selectively Track and Edit Type 1 Conventional Dendritic Cells Facilitate Disentangling Their Complexity in vivo. Front Immunol *9*.

282.	Puttur, F., Arnold-Schrauf, C., Lahl, K., Solmaz, G., Lindenberg, M., Mayer, C.T., Gohmert, M., Swallow, M., Helt, C. van, Schmitt, H., et al. (2013). Absence of Siglec-H in MCMV Infection Elevates Interferon Alpha Production but Does Not Enhance Viral Clearance. PLOS Pathogens *9*, e1003648.

283. Aliberti, J., Schulz, O., Pennington, D.J., Tsujimura, H., Sousa, C.R. e, Ozato, K., and Sher, A. (2003). Essential role for ICSBP in the in vivo development of murine CD8α+ dendritic cells. Blood *101*, 305–310.

284. Schiavoni, G., Mattei, F., Sestili, P., Borghi, P., Venditti, M., Morse, H.C., Belardelli, F., and Gabriele, L. (2002). ICSBP Is Essential for the Development of Mouse Type I Interferon-producing Cells and for the Generation and Activation of CD8α+ Dendritic Cells. Journal of Experimental Medicine *196*, 1415–1425.

285. Sun, S.-C. (2011). Non-canonical NF-κB signaling pathway. Cell Res *21*, 71–85.

286. Kabashima, K., Banks, T.A., Ansel, K.M., Lu, T.T., Ware, C.F., and Cyster, J.G. (2005). Intrinsic Lymphotoxin-β Receptor Requirement for Homeostasis of Lymphoid Tissue Dendritic Cells. Immunity *22*, 439–450.

287. Hofmann, J., Mair, F., Greter, M., Schmidt-Supprian, M., and Becher, B. (2011). NIK signaling in dendritic cells but not in T cells is required for the development of effector T cells and cell-mediated immune responses. Journal of Experimental Medicine *208*, 1917–1929.

288. Briseño, C.G., Gargaro, M., Durai, V., Davidson, J.T., Theisen, D.J., Anderson, D.A., Novack, D.V., Murphy, T.L., and Murphy, K.M. (2017). Deficiency of transcription factor RelB perturbs myeloid and DC development by hematopoietic-extrinsic mechanisms. Proc Natl Acad Sci U S A *114*, 3957–3962.

289. Satpathy, A.T., Briseño, C.G., Lee, J.S., Ng, D., Manieri, N.A., Kc, W., Wu, X., Thomas, S.R., Lee, W.-L., Turkoz, M., et al. (2013). Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. Nature Immunology *14*, 937–948.

290. Bajaña, S., Turner, S., Paul, J., Ainsua-Enrich, E., and Kovats, S. (2016). IRF4 and IRF8 Act in CD11c+ Cells To Regulate Terminal Differentiation of Lung Tissue Dendritic Cells. The Journal of Immunology *196*, 1666–1677.

291. Gao, Y., Nish, S.A., Jiang, R., Hou, L., Licona-Limón, P., Weinstein, J.S., Zhao, H., and Medzhitov, R. (2013). Control of T Helper 2 Responses by Transcription Factor IRF4-Dependent Dendritic Cells. Immunity *39*, 722–732.

292. Cisse, B., Caton, M.L., Lehner, M., Maeda, T., Scheu, S., Locksley, R., Holmberg, D., Zweier, C., den Hollander, N.S., Kant, S.G., et al. (2008). Transcription Factor E2-2 Is an Essential and Specific Regulator of Plasmacytoid Dendritic Cell Development. Cell *135*, 37–48.

293. Scott, C.L., Soen, B., Martens, L., Skrypek, N., Saelens, W., Taminau, J., Blancke, G., Isterdael, G.V., Huylebroeck, D., Haigh, J., et al. (2016). The transcription factor Zeb2 regulates development of conventional and plasmacytoid DCs by repressing Id2. Journal of Experimental Medicine *213*, 897–911.

294. Onodera, K., Fujiwara, T., Onishi, Y., Itoh-Nakadai, A., Okitsu, Y., Fukuhara, N., Ishizawa, K., Shimizu, R., Yamamoto, M., and Harigae, H. (2016). GATA2 regulates dendritic cell differentiation. Blood *128*, 508–518.

295. Hacker, C., Kirsch, R.D., Ju, X.-S., Hieronymus, T., Gust, T.C., Kuhl, C., Jorgas, T., Kurz, S.M., Rose-John, S., Yokota, Y., et al. (2003). Transcriptional profiling identifies Id2 function in dendritic cell development. Nature Immunology *4*, 380.

296. Kashiwada, M., Pham, N.-L.L., Pewe, L.L., Harty, J.T., and Rothman, P.B. (2011). NFIL3/E4BP4 is a key transcription factor for CD8α+ dendritic cell development. Blood *117*, 6193–6197.

297. Palmiter, R.D., Behringer, R.R., Quaife, C.J., Maxwell, F., Maxwell, I.H., and Brinster, R.L. (1987). Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. Cell *50*, 435–443.

298. Saito, M., Iwawaki, T., Taya, C., Yonekawa, H., Noda, M., Inui, Y., Mekada, E., Kimata, Y., Tsuru, A., and Kohno, K. (2001). Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. Nat. Biotechnol. *19*, 746–750.

299. Jung, S., Unutmaz, D., Wong, P., Sano, G.-I., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., et al. (2002). In Vivo Depletion of CD11c+ Dendritic Cells Abrogates Priming of CD8+ T Cells by Exogenous Cell-Associated Antigens. Immunity *17*, 211–220.

300. Hochweller, K., Striegler, J., Hämmerling, G.J., and Garbi, N. (2008). A novel CD11c.DTR transgenic mouse for depletion of dendritic cells reveals their requirement for homeostatic proliferation of natural killer cells. European Journal of Immunology *38*, 2776–2783.

301. Meredith, M.M., Liu, K., Darrasse-Jeze, G., Kamphorst, A.O., Schreiber, H.A., Guermonprez, P., Idoyaga, J., Cheong, C., Yao, K.-H., Niec, R.E., et al. (2012). Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. Journal of Experimental Medicine *209*, 1153–1165.

302. Loschko, J., Rieke, G.J., Schreiber, H.A., Meredith, M.M., Yao, K.-H., Guermonprez, P., and Nussenzweig, M.C. (2016). Inducible targeting of cDCs and their subsets in vivo. Journal of Immunological Methods *434*, 32–38.

303. Fukaya, T., Murakami, R., Takagi, H., Sato, K., Sato, Y., Otsuka, H., Ohno, M., Hijikata, A., Ohara, O., Hikida, M., et al. (2012). Conditional ablation of CD205+ conventional dendritic cells impacts the regulation of T-cell immunity and homeostasis in vivo. PNAS *109*, 11288–11293.

304. Bennett, C.L., Rijn, E. van, Jung, S., Inaba, K., Steinman, R.M., Kapsenberg, M.L., and Clausen, B.E. (2005). Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. The Journal of Cell Biology *169*, 569–576.

305. Kaplan, D.H., Jenison, M.C., Saeland, S., Shlomchik, W.D., and Shlomchik, M.J. (2005). Epidermal Langerhans Cell-Deficient Mice Develop Enhanced Contact Hypersensitivity. Immunity *23*, 611–620.

306. Welty, N.E., Staley, C., Ghilardi, N., Sadowsky, M.J., Igyártó, B.Z., and Kaplan, D.H. (2013). Intestinal lamina propria dendritic cells maintain T cell homeostasis but do not affect commensalism. Journal of Experimental Medicine *210*, 2011–2024.

307. Bobr, A., Olvera-Gomez, I., Igyarto, B.Z., Haley, K.M., Hogquist, K.A., and Kaplan, D.H. (2010). Acute Ablation of Langerhans Cells Enhances Skin Immune Responses. The Journal of Immunology *185*, 4724–4728.

308. Piva, L., Tetlak, P., Claser, C., Karjalainen, K., Renia, L., and Ruedl, C. (2012). Cutting Edge: Clec9A+ Dendritic Cells Mediate the Development of Experimental Cerebral Malaria. The Journal of Immunology *189*, 1128–1132.

309. Yamazaki, C., Sugiyama, M., Ohta, T., Hemmi, H., Hamada, E., Sasaki, I., Fukuda, Y., Yano, T., Nobuoka, M., Hirashima, T., et al. (2013). Critical Roles of a Dendritic Cell Subset Expressing a Chemokine Receptor, XCR1. The Journal of Immunology *190*, 6071–6082.

310. Alexandre, Y.O., Ghilas, S., Sanchez, C., Bon, A.L., Crozat, K., and Dalod, M. (2016). XCR1+ dendritic cells promote memory CD8+ T cell recall upon secondary infections with Listeria monocytogenes or certain viruses. Journal of Experimental Medicine *213*, 75–92.

311. Muzaki, A.R.B.M., Tetlak, P., Sheng, J., Loh, S.C., Setiagani, Y.A., Poidinger, M., Zolezzi, F., Karjalainen, K., and Ruedl, C. (2016). Intestinal CD103⁺CD11b⁻ dendritic cells restrain colitis via IFN-γ-induced anti-inflammatory response in epithelial cells. Mucosal Immunology *9*, 336–351.

312. Cho, M., Lee, J.-E., Lim, H., Shin, H.-W., Khalmuratova, R., Choi, G., Kim, H.S., Choi, W.S., Park, Y.-J., Shim, I., et al. (2018). Fibrinogen cleavage products and Toll-like receptor 4 promote the generation of programmed cell death 1 ligand 2–positive dendritic cells in allergic asthma. Journal of Allergy and Clinical Immunology *142*, 530-541.e6.

313. Takagi, H., Fukaya, T., Eizumi, K., Sato, Y., Sato, K., Shibazaki, A., Otsuka, H., Hijikata, A., Watanabe, T., Ohara, O., et al. (2011). Plasmacytoid Dendritic Cells Are Crucial for the Initiation of Inflammation and T Cell Immunity In Vivo. Immunity *35*, 958–971.

314. Swiecki, M., Wang, Y., Riboldi, E., Kim, A.H.J., Dzutsev, A., Gilfillan, S., Vermi, W., Ruedl, C., Trinchieri, G., and Colonna, M. (2014). Cell depletion in mice that express diphtheria toxin receptor under the control of SiglecH encompasses more than plasmacytoid dendritic cells. J. Immunol. *192*, 4409–4416.

315. Swiecki, M., Gilfillan, S., Vermi, W., Wang, Y., and Colonna, M. (2010). Plasmacytoid Dendritic Cell Ablation Impacts Early Interferon Responses and Antiviral NK and CD8+ T Cell Accrual. Immunity *33*, 955–966.

RESULTS

PART I
Role of Langerhans cells in TSLP-driven T follicular helper cell differentiation in mouse models of atopic dermatitis

MARSCHALL P.J. et al., (manuscript in preparation)

Introduction

Atopic dermatitis (AD) is a one of the most common chronic inflammatory skin disease which affects up to 20% of children and 3% of adults worldwide, with increasing prevalence in the industrialized countries during the last 30 years (Weidinger and Novak, 2016, Lancet). Patients suffering from AD often present genetic risk factors in the form of mutations affecting the skin barrier structure or the immune system. Onset of AD usually occurs early in life and may lead to allergen sensitization, which can trigger the progression from AD to other allergic reactions, such as athma or food allergy, in a process called atopic march (Dharmage et al., 2014, Allergy; Shaker, 2014, Curr Opin Pediatr).

It has been recognized that Th2 cell response is critically implicated in the pathogenesis of AD. Previous studies from our lab using mouse models have established a central role of the cytokine thymic stromal lymphopoietin (TSLP) expressed by epidermal keratinocytes in promoting Th2 cell response and driving the pathogenesis of AD (Li et al., 2005; Li et al., 2006, P Natl Acad Sci Usa; Li et al., 2009, J Invest Dermatol). In addition to Th2 cell response, humoral immune response is also a key feature of AD, for example, increased serum IgE and IgG1 levels have been found to be associated with AD (Pires et al., 2002, Clin Exp Dermatol; Chapman et al., 1983, J Allergy Clin Immun), but the cellular and molecular mechanisms remain largely unexplained. T follicular helper (Tfh) cells form a recently discovered subset of CD4^{+} T cells critically involved in germinal center (GC) formation, B cell maturation and induction of class-switching, as well as antibody affinity maturation which are required for an efficient humoral response (Crotty, 2014, Immunity). The Tfh cell differentiation process is believed to begin with an initial dendritic cell (DC) priming of a naive CD4^{+} T cell, which undergoes a cell-fate decision with the acquisition of master transcription factor Bcl6 expression and chemokine receptor CXCR5 expressed on cell surface to become early Tfh cell, while the full differentiation and maintenance of Tfh cells implicate the Tfh cell-B cell interaction. GC Tfh cells are phenotypically defined by their high expression of chemokine receptor CXCR5 and programmed death 1 (PD-1), of which CXCR5 promotes their migration from T cell zone to the B cell follicles (Haynes et al., 2007, J Immunol; Hardtke, 2005, Blood). It has been reported that the circulating number of Tfh cells in AD children were correlated with severity of the disease (Szabo et al., 2017, Immunol Lett), and allergen-specific immunotherapy modulated the

balance of circulating Tfh and T follicular regulatory (Tfr) cells (Schulten et al., 2018, J Allergy Clin Immun), suggesting that Tfh cells may play an important role in AD pathogenesis. However, whether TSLP promotes Tfh/GC response in AD and how remains unknown.

In this study, we employed two mouse models of AD, one triggered by the overexpression of TSLP in mouse skin through topical application of MC903 (Li et al., 2006; Li et al., 2009, P Natl Acad Sci Usa), and the other one with epicutaneous allergen sensitization on barrier disrupted skin to investigate the function of TSLP in humoral immune response during AD pathogenesis. Our data demonstrate a crucial role of TSLP in promoting Tfh cell differentiation and GC response in both AD models. Interestingly, we also uncovered a seemingly contradictory role of Langerhans cells, a subset of DCs found in the epidermis, in TSLP-promoted Tfh cell differentiation and GC response.

Material and methods

Mice

Balb/c mice and *Rag1*^{-/-} mice were purchased from Charles River Laboratory. *Tslp*^{-/-} mice were generated by the lab as previously described (Li M., et al., 2009 ; J Invest Dermatol). Mice bearing the 4C13R transgene (Roediger B., et al., 2013 ; Nat Immunol) were provided by Dr. W.E. Paul (NIH, USA). Langerin[DTR] and Langerin[GFP] (Kissenpfennig A., et al., 2005 ; Immunity) were provided by Dr. B. Malissen (Marseille, France). Human Langerin[DTR] (Bobr A., et al., 2010 ; J Immunol) were provided by Dr. D. Kaplan (Pittsburgh, USA). All these mouse lines were initially generated or obtained in C57Bl/6J background and backcrossed in Balb/c background. Breeding and maintenance were performed under institutional guidelines, and all of the animal experiments were approved by the animal care and ethics committee of animal experimentation of the IGBMC.

Laser-assisted microporation (LMP) on mouse ears

Laser-assisted skin microporation (LMP) was performed using P.L.E.A.S.E® research system (Pantec Biosolutions) on the dorsal side of mouse ears. Three sets of parameters were used. For the depth of 30µm (LMP_30µm): 2 pulses per pore, with fluence of 7.5 J/cm2, pulse length of 75 µs, RepRate of 500 Hz and power of 1.0 W; for the depth of 91µm (LMP_91µm): 2 pulses per pore with fluence of 22.7 J/cm2, pulse length of 175 µs, RepRate of 200 Hz and power of 1.2 W; for the depth of 11µm (LMP_11µm): 1 pulse per pore with fluence of 1,8 J/cm2, pelse length of 50 µs, RepRate of 500 Hz and power of 0,7 W. In all cases, pore array size was 14 mm and pore density was 10%.

MC903 topical application

MC903 (Calcipotriol, Sigma, Cat No. C4369) was dissolved in 100% ethanol and topically applied on mouse ears (2 nmol in 25 µl per ear) as previously described (Leyva-Castillo et al., 2013, Nat Commun).

OVA cutaneous sensitization and airway challenge

To induce ovalbumin (OVA) cutaneous sensitization, 10 µl of sterile PBS solution containing 200 µg of Ovalbumin (ref A5503, Sigma-aldrich) were applied on laser microporated (LMP) skin (on the dorsal side of ear) at D0, D4, D7 and D10 (see figure 4C). In case of airway challenge, 25 µL of saline solution containing 50 µg of OVA was intranasally instilled every day from D9 to D12. Mice were sacrificed at D13 for analyses.

Langerin⁺ cells and Langerhans cells depletion

For depletion of langerin⁺ cells or Langerhans cells, Lang^{DTR} or human Lang^{DTR} mice respectively were intraperitoneally injected with 1µg of diphtheria toxin (DT; ref D-0564, Sigma-Aldrich) as indicated in the experimental scheme.

Cell preparation for flow cytometry

For ear-draining lymph nodes (LN) preparation, LNs were dissociated with piston, passed through a 70µm strainer (Falcon) and resuspended in PBS containing 0.5% BSA and 2mM EDTA. LN cells were then centrifuged at 350g and resuspended in PBS containing 1% FCS and 2mM EDTA, counted and used for FACS staining. In case of preparation of LN cells for DC staining, ear-draining LNs were cut in two pieces and incubated 30 minutes at 37°C in 2mg/mL collagenase D (Roche), 0,25mg/mL DNase I (Sigma) and 2.5% FCS (Thermofisher) in PBS prior passing through the strainer as described above.

For preparation of dermal cells, ears were split into ventral and dorsal halves and floated 1h at 37°C on a PBS solution containing 4mg/ml Dispase (Gibco). Dermis was subsequently separated from epidermis and incubated 1h at 37°C with 1mg/ml collagenase D (Roche), 0.25mg/ml DNase I (Sigma) and 2.5% of foetal calf serum (FCS, ThermoFisher) in PBS. Cells were passed through a 70µm cell strainer and resuspended in 5 mL of PBS solution containing 0.5% BSA and 2mM of EDTA, centrifuged at 350g before proceeding for surface staining.

Surface staining for flow cytometry

To perform flow cytometry analysis, 2 million cells were used for antibody (Ab) staining. Cells were first incubated with anti-CD16/CD32 antibody to block unspecific binding, followed by surface markers staining with fluorochrome-conjugated antibodies (Table 1) in a 25µl of FACS buffer (1% of FCS + 2mM EDTA in PBS) for 10 minutes at 4°C. Viability staining was performed

by adding propidium iodide to a final concentration of 1 µg/mL just prior passing the cells to the cytometer.

Table 3: antibodies used for flow cytometry experiments

Marker	Fluorochrome	Clone	Reference	Company	Dilution
CD16/CD32		93		eBioscience	0.5 : 25
Streptavidin	BV605			BD Biosciences	0.5 : 25
Streptavidin	APC			eBioscience	0.5 : 25
CD4	BV421	GK1.5		BD Biosciences	0.5 : 25
CD4	Alexa Fluor 700	RM-5		BD Biosciences	1 : 25
CD8a	PerCP-Cy5.5	53-6.7		eBioscience	0.5 : 25
CXCR5	Biotin	2G8		BD Biosciences	1.5 : 25
PD-1	PE-Cy7	RMP1-30		Biolegend	2 : 25
ST2	FITC	DJ8		MDBioproducts	0.5 : 25
CD19	FITC	1D3		BD Biosciences	1 : 25
B220	APC	RA3-6B2		eBioscience	1.2 : 25
GL-7	PE	GL-7		eBioscience	1.2 : 25
CD95	PE-Cy7	Jo2		BD Biosciences	1 : 25
IgG1	PerCP-Cy5.5	RMG1-1		Biolegend	1 : 25
IgE	Biotin	R35-72		BD Biosciences	0.5 : 25
CD11c	Biotin	HL3		BD Biosciences	0.5 : 25
I-A/I-E	PE	M5/114.15.2		eBioscience	0.01 : 25

Fluorescence-assisted cell sorting of dendritic cells and RNA extraction

Ear-draining lymph node cells were prepared as described above for DC preparation from LNs. Staining was done at a concentration of 50.10^6 cells in 100 µL of FACS buffer (1% of FCS + 2mM EDTA in PBS). Lymph nodes were combined as shown in table 2 and the indicated number of sorted cells were obtained. Tubes used to recover sorted cells were coated overnight with PBS containing 0.5% of BSA. Sorted cells were centrifuged at 500g for 5 minutes at 8°C before resuspension in RLT buffer of RNeasy Micro kit (Qiagen). Samples were stored at -80°C until

RNA was extracted with the above mentioned kit according to the manufacturer's instructions.

Table 4: Number of sorted dendritic cells

Treatment	Population	Approx. number of cells
Untreated	Migratory GFP$^+$	50.10^4
	Migratory GFP$^-$	75.10^4
MC903	Migratory GFP$^+$	85.10^4
	Migratory GFP$^-$	400.10^4
30µm-LMP/OVA	Migratory GFP$^+$	50.10^4
	Migratory GFP$^-$	175.10^4

BAL cell analyses

BAL (bronchoalveolar lavage) was taken in anaesthetized mice, by instillating and withdrawing 0.5 ml of saline solution (0.9% NaCl, 2.6mM EDTA) in the trachea. After six times lavages, BAL fluid was centrifuged, and BAL cells were counted using a Neubauer hemocytometer. Two hundred µl of BAL fluids with 2.5×10^5 cells/ml were used to prepare cytospin slides, which were then stained with Hemacolor kit (Merck) to identify macrophages, lymphocytes, neutrophils and eosinophils. After counting for each cell type to obtain their frequencies, the number of each cell type was calculated according to the total BAL cell number and the frequency. For gene expression analysis, BAL cells RNA was prepared using NucleoSpin RNA XS kit (Macherey-Nagel).

Enzyme-linked immunosorbent assay (ELISA)

TSLP ELISA. Mouse skin was chopped and homogenized with a Mixer Mill MM301 (Retsch, Dusseldorf, Germany) in lysis buffer (25 mmol/L Tris pH 7.8, 2 mmol/L EDTA, 1 mmol/L dithiothreitol, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche). Protein concentrations of skin extract were quantified by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, Calif). TSLP levels in skin extracts and mouse sera were determined with the DuoSet ELISA Development Kits (R&D Systems, Minneapolis, Minn).

OVA-specific serum IgG1 and IgE ELISA. For OVA-specific immunoglobulins, microtiter plates were coated with OVA and then blocked with BSA. Serum samples were incubated in the coated plates overnight at 4°C followed by incubation with a biotinylated rat anti-mouse IgE (BD Biosciences PharMingen; Cat No. 553419; clone R35-118) or IgG1 (BD Biosciences PharMingen; Cat No. 553441; clone A85-1). Extravidin horseradish peroxidase (Sigma, Cat No. E2886) and TMB (tetramethylbenzidine) Substrate Reagent Set (BD Biosciences, Cat No. 555214) were used for detection. Serum levels of OVA-specific IgG1 and OVA-specific IgE were calculated relevant to a pre-prepared serum pool from HDM-sensitized and challenged Balb/c mice and expressed as arbitrary units.

Quantitative RT-PCR

RNA was reverse transcribed by using random oligonucleotide hexamers and SuperScript IV Reverse Transcriptase (Invitrogen) and amplified by means of quantitative PCR with a LightCycler 480 (Roche Diagnostics, Indianapolis, Ind) and the LightCycler 480 SYBR Green kit (Roche), according to the manufacturer's instructions. Relative RNA levels were calculated with hypoxanthine phosphoribosyl- transferase (HPRT) as an internal control. For analyses of each set of gene expression, an arbitrary unit of 1 was given to the samples with the highest level, and the remaining samples were plotted relative to this value. Sequences of PCR primers are shown in Table 2.

Table 5: primer sequences

Gene name		Sequence 5' to 3'	Length
Hprt	F	TGGATACAGGCCAGACTTTG	161 bp
	R	GATTCAACTTGCGCTCATCTTA	
Cd80	F	CCATGTCCAAGGCTCATTCT	203 bp
	R	TTCCCAGCAATGACAGACAG	
Cd86	F	CACGAGCTTTGACAGGAACA	246 bp
	R	TTAGGTTTCGGGTGACCTTG	
Il6	F	GAGGATACCACTCCCAACAGACC	141 bp
	R	AAGTGCATCATCGTTGTTCATACA	
Tnfsf4	F	TGGAAAACGGATCAAGGCCA	145 bp
	R	TGGATTGGAGGGTCCTTTGC	
Il4	F	GGCATTTTGAACGAGGTCAC	132 bp
	R	AAATATGCGAAGCACCTTGG	
Il5	F	AGCACAGTGGTGAAAGAGACCTT	117 bp
	R	TCCAATGCATAGCTGGTGATTT	
Il13	F	GGAGCTGAGCAACATCACACA	142 bp
	R	GGTCCTGTAGATGGCATTGCA	

Ccr3	F	TAAAGGACTTAGCAAAATTCACCA	150 bp
	R	TGACCCCAGCTCTTTGATTC	
Mcpt8	F	GTGGGAAATCCCAGTGAGAA	160 bp
	R	TCCGAATCCAAGGCATAAAG	

RNA-seq data analysis

Dendritic cells were FACS-sorted from ear-draining lymph nodes as described above. RNA was extracted using RNeasy Micro Kit (Qiagen). RNA-seq was performed in IGBMC high-throughput mRNA sequencing facility. Reads were preprocessed in order to remove adapter, polyA and low-quality sequences (Phred quality score below 20). After this preprocessing, reads shorter than 40 bases were discarded for further analysis. These preprocessing steps were performed using cutadapt [Martin M., 2011, EMB-net.journal] version 1.10. Reads were mapped onto the mm10 assembly of mouse genome using STAR version 2.5.3a [Dobin A. et al., 2013, Bioinformatics]. Read counts have been normalized across samples with the median-of-ratios method proposed by Anders and Huber [Anders and Huber, 2010, Genome Biol.], to make these counts comparable between samples. Comparisons of interest were performed using the method proposed by Love et al. [Love M., 2014, Genome Biol.] and implemented in the DESeq2 Bioconductor library version 1.16.1. P-values were adjustes for multiple testing using the Benjamini and Hochberg method [Benjamini Y. and Hochberg Y., 1995, J R Statistic Soc]. Gene expression quantification was performed from uniquely aligned reads using htseq-count [Anders S., 2015, Bioinformatics] version 0.6.1p1, with annotations from Ensembl version 96 and "union" mode.

Histopathology

Mouse ears and lungs were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. 5µm sections were stained with hematoxylin & eosin (H&E). For periodic Acid Schiff (PAS) staining, slides were incubated with 0.5% aqueous periodic acid (Alfa Aesar), washed with water and incubated 15 minutes in Schiff's reagent (Merck). Slides were counterstained with hematoxylin and differentiated with acid alcohol (1ml of 37% HCl in 100ml of 70% ethanol).

Immunohistochemistry

For immunohistochemistry (IHC) staining of major basic protein (MBP) and mast cell protease 8 (MCPT8), 5µm paraffin sections were treated with 0.6% H2O2 (Sigma, Cat No. H3410) to block endogenous peroxidase activity before antigen retrieval with either Pepsin (for IHC of MBP; Life technologies, Ref. 003009) or citric buffer (10 mmol/L citric acid, pH 6; for IHC of MCPT8). Slides were then blocked with normal rabbit serum (Vector Laboratories) and incubated overnight with primary antibody (see Table 3). Slides were then incubated with biotinylated rabbit anti-rat IgG (dilution: 1/300) and treated with AB complex (Vector Laboratories). Staining was finally visualized with AEC high-sensitivity substrate chromogen solution (Dako, Ref. K3469) and counter-stained with hematoxylin.

Table 4: antibodies used for immunohistochemistry

Antibody	Provider	Species
MBP	Provided by Dr. James J. Lee, Mayo Clinic, Rochester	Rat anti-mouse mAb
MCPT8	Biolegend (Cat No. 647402)	Rat anti-mouse TUG8

Statistics

Data were analysed using using GraphPrad Prism 6. Comparison of two samples was performed either by Student's two-tailed unpaired t-test with Welch's correction or the Mann–Whitney rank sum nonparametric test depending on results from the Kolmogorov–Smirnov test for normality. Comparison of more than two samples was performed by ordinary one-way ANOVA followed by Tukey's post-doc test.

Results

Overproduction of TSLP in skin induces T follicular helper (Tfh) differentiation and germinal center (GC) response

To induce TSLP overexpression (TSLP[over]) in skin atopic dermatitis (AD) pathogenesis, Balb/c wildtype (WT) mouse ears were topically treated every other day from day (D)0 to D10 with MC903, a low calcemic analog of vitamin D3 (Li et al., 2006, Proc Natl Acad Sci Usa). To examine Tfh and GC responses, ear-draining lymph nodes (EDLN) were analyzed at D0, D7 and D11 (Figure 1A). Results showed that frequency and absolute number of Tfh cells were both increased in MC903-treated Balb/c WT mice at D7 and further augmented at D11 (Figure 1B, compare WT_D0, WT_D7 and WT_D11). We next examined the expression of IL-4, a key signal provided by Tfh cells to sustain B cell maturation (Crotty, 2014, Immunity), taking use of *Il4/Il13* dual reporter 4C13R$^{Tg/0}$ mice (backcrossed to >99.9% Balb/c background), in which AmCyan and DsRed are expressed under the control of IL-4 and IL-13 regulatory elements, respectively (Roediger et al., 2013, Nat Immunol). In agreement with what was previously reported (Liang et al., 2011, Nat Immunol)), MC903-induced CXCR5$^+$ PD-1$^+$ Tfh cells express IL-4 (AmCyan; Figure 1C) but not IL-13 (DsRed; Figure S1). The expression of IL-4 (AmCyan) by Tfh cells was augmented in MC903-treated 4C13R$^{Tg/0}$ mice, both at D7 and D11 (Figure 1C). Therefore, MC903 treatment induces not only Tfh cell differentiation but also IL-4 production by Tfh cells.

To examine whether such induction of Tfh cells and their expression of IL-4 in MC903 model are triggered by TSLP, 4C13R$^{Tg/0}$ mice were crossed with *Tslp$^{-/-}$* (Li et al., 2009, J Invest Dermatol) (backcrossed to >99.9% Balb/c background) to generate *Tslp$^{-/-}$*/4C13R$^{Tg/0}$ mice. When subjected to MC903 treatment, these mice exhibited highly diminished CXCR5$^+$ PD-1$^+$ Tfh cell frequency and number compared to *Tslp$^{+/+}$*/4C13R$^{Tg/0}$ mice (Figure 1B); and moreover, IL-4 increase in Tfh cells was abrogated (Figure 1C). These results thus indicate that, in MC903-induced AD model, the overproduction of TSLP triggers Tfh cell differentiation and the IL-4 expression by these cells.

We next examined the GC response in MC903-treated Balb/c WT mice. The number of GC B cells, gated as GL-7$^+$ CD95$^+$ B cells, exhibited an increase in MC903-treated Balb/c WT

mice at D11, but not at D7 (Figure 1D). The increase of GC B cells was abrogated in MC903-treated *Tslp*^{-/-} mice (Figure 1D). This was further confirmed by immunofluorescence (IF) staining for GCs (Figure S2A; the GC were identified as PNA[+] clusters in the IgD[+] B cell zone), showing an increased GC size in EDLN from MC903-treated WT mice, which was largely diminished in the absence of TSLP. In addition, we examined IgG1^{+} and IgE^{+} B cells, both of which exhibited an increase in MC903-treated WT mice at D11 that was abrogated in MC903-treated *Tslp*^{-/-} mice (Figure 1E). Interestingly, we observed that most of the IgG1^{+} B cells were GL-7^{+} CD95^{+}, suggesting that these cells harbor a GC phenotype; however, this was not the case for IgE^{+} B cells (Figure 1F).

Taken together, these data indicate that the overproduction of TSLP in skin triggers Tfh cell differentiation and GC response in MC903-induced AD mouse model.

Depletion of Langerin^{+} cells diminishes the TSLP[over]-triggered Tfh/GC response

The role of Langerhans cells (LCs) in skin immunity and Tfh response has remained controversial and incompletely understood (Levin et al., 2017, J Invest Dermatol; Vardam et al., 2017, J Invest Dermatol; Yao et al., 2015, J Allergy Clin Immunol; Zimara et al., 2014, Eur J Immunol). To investigate the role of LCs in Tslp[over]-triggered Tfh/GC response, we employed Langerin-DTR knock-in mice (Lang[DTR]; backcrossed to >99.9% Balb/c background) in which langerin^{+} cells, including LCs and langerin[+] dermal DCs, express the human diphtheria toxin receptor (DTR) and can thus be depleted upon injection of diphtheria toxin (DT) (Kissenpfennig et al., 2005, Immunity). Lang[DTR] mice and their wildtype control littermates were intraperitoneally (i.p.) injected with DT at D-2, D0 and every 4 days to maintain the depletion of langerin^{+} cells (named Lang[DEP] and CT respectively), and were subjected to topical MC903 treatment as shown in Figure 2A. Results showed that the TSLP-triggered Tfh cell differentiation was largely diminished in Lang[DEP] mice (Figure 2B). The expression of IL-4 (AmCyan) by Tfh cells was also reduced (Figure 2C). Accordingly, GC B cell number was lower in the EDLN of MC903-treated Lang[DEP] mice compared with MC903-treated CT mice (Figure 2D, left). Moreover, a decrease in IgG1^{+} but not IgE[+] B cell number was also observed (Figure 2D right).

Together, these results indicate that Langerin⁺ cells play an important role in mediating the TSLP^over-induced Tfh/GC response.

Selective depletion of LCs leads to a diminished Tfh cell differentiation in MC903-induced AD mice

As both LCs and langerin⁺ dermal DCs could be depleted in Lang^DEP mice using the above protocol, we then wondered whether LCs are implicated in TSLP^over-triggered Tfh/GC response. To explore this, we used a previously established protocol to selectively deplete LCs but keep langerin⁺ dermal DCs (dDCs), by taking advantage of the differential recovery time between LCs and langerin⁺ dDCs after DT-induced depletion (Henri et al., 2010, J Exp Med). Lang^DTR mice were i.p. injected with DT at D-2 and D0; at D13, langerin⁺ dDCs already recovered whereas LCs were still missing (Henri et al., 2010, J Exp Med). Ears of these mice were then treated with MC903 from D13 to D19 and EDLN were analyzed at D20 (Figure 3A), which corresponds to D7 of MC903 treatment in Figure 1A. We observed that LCs remained still depleted at D20, however were partially recovered afterwards, e.g. when examined at D24 (data not shown). Analyses of CXCR5⁺ PD-1⁺ Tfh cells showed that they were reduced in Lang^DEP mice compared to CT mice upon MC903 treatment (Figure 3B). Therefore, these results suggest that LCs could be crucially implicated in MC903-induced Tfh cell differentiation.

In another strategy, we took use of human Langerin-DTR mice in which the expression of DTR is controlled by the regulatory elements of the human langerin gene (HuLang^DTR, backcrossed to 98% Balb/c background) and DT injection efficiently depleted LCs whereas langerin⁺ dDCs were not affected (Figure S3 and Bobr et al., 2010, J Immunol). Upon DT injection and MC903 treatment (Figure 3C), HuLang^DEP mice exhibited a decrease in frequency and cell number of Tfh cells (Figure 3D), supporting the conclusion that LCs are crucially implicated in Tfh cell differentiation triggered by TSLP^over.

Ovalbumin sensitization through laser-microporated skin induces a Tfh/GC response which is crucially dependent on TSLP

We have previously reported that TSLP is crucial for promoting skin sensitization to allergens. Indeed, ovalbumin (OVA) treatment on barrier-disrupted skin (via tape-stripping) leads to an allergic AD-like skin inflammation, accompanied by the production of OVA-specific IgG1 (OVA-IgG1) and OVA-IgE, and an asthmatic lung inflammation upon challenge with OVA in airways (Leyva-Castillo et al., 2013, J Invest Dermatol). We then asked whether TSLP was crucial for epicutaneous OVA sensitization-induced Tfh/GC response. Because the barrier disruption by tape-stripping is hard to control, and different severity of barrier disruption leads to varied immune responses, we developed a novel epicutaneous OVA sensitization protocol by using the Precise Laser Epidermal System (P.L.E.A.S.E.®) to disrupt skin barrier and generate patterned micropores at precise depths in mouse epidermis. This method has been successfully used to deliver biomolecules transdermally or intradermally in a controlled manner (Scheiblhofer et al., 2013, Expert Opin Drug Del). By optimizing laser-parameters, we were able to generate micropores on wildtype Balb/c (WT) mouse ears at a depth of 30μm (30μm-LMP), which disrupts the suprabasal layer of ear epidermis (Figure 4A). ELISA analyses of 30μm-LMP skin showed that the protein level of TSLP increased at 48 hours after treatment (Figure 4B), reaching a level (around 200 pg/mg of total proteins) which is comparable to that in tape-stripped WT mouse skin as previously reported (Leyva-Castillo et al., 2013, J Invest Dermatol), although such level of TSLP was much lower compared to that of MC903-treated WT skin (Figure 4B). The additional OVA treatment on 30μm-LMP skin did not further induce TSLP production (Figure 4B).

We then established a new experimental protocol (Figure 4C) in which epicutaneous treatment with OVA on 30μm-LMP ears (LMP/OVA) led to an allergic AD-like skin inflammation and a subsequent asthma (see Figure 7). Examination of EDLNs revealed that both frequency and number of CXCR5$^+$ PD-1$^+$ Tfh cells were increased in LMP/OVA-compared to LMP/PBS-treated WT mice (Figure 4D). Such increase was largely diminished in *Tslp$^{-/-}$* mice (Figure 4D). Moreover, IL-4 (AmCyan) production by Tfh cells was augmented in LMP/OVA-treated WT mice but not *Tslp$^{-/-}$* mice (Figure 4E). GC B cell number analyzed by flow cytometry (Figure 4F) and GC size analyzed by immunofluorescence (Figure S2B)

both showed an increase in LMP/OVA-treated WT mice, and this increase was abrogated in the absence of TSLP (Figure 4F). IgG1^{+} and IgE[+] B cell numbers were also increased in LMP/OVA-treated WT mice, and they were much lower in LMP/OVA-treated *Tslp*^{-/-} mice (Figure 4F). Accordingly, serum levels of OVA-IgG1 and OVA-IgE were both decreased in *Tslp*^{-/-} mice compared to WT mice upon LMP/OVA treatment (Figure 4G). Taken together, these results indicate that TSLP is crucially requried for OVA epicutaneous sensitization-induced Tfh/GC response.

Depletion of Langerin+ cells does not reduce but rather tend to augment 30um-LMP/OVA-induced Tfh cell differentiation

We next examined whether langerin[+] DCs are crucially implicated in OVA-induced Tfh/GC response, by subjecting Lang[DEP] mice to 30μm-LMP/OVA treatment (Figure 5A). To our surprise, CXCR5^{+} PD-1^{+} Tfh cell frequency and number were not reduced in LMP/OVA-treated Lang[DEP] mice; instead, they tended to be higher compared to WT mice (Figure 5B). Also, IL-4 (AmCyan) expression was significantly higher in Tfh cells in EDLN from LMP/OVA-treated Lang[DEP] mice (Figure 5C). In agreement with these data, GC B cell, IgG1^{+} and IgE[+] B cell number were not reduced in LMP/OVA-treated Lang[DEP] mice (Figure 5D), and serum OVA-IgG1 and OVA-IgE exhibited a tendency to be higher in LMP/OVA-treated Lang[DEP] mice (Figure 5E). Together, these data indicate that langerin[+] DCs are not required for the Tfh/GC response in LMP/OVA-induced AD model, and they rather appear to limit the Tfh cell differentiation.

Depletion of Langerin^{+} cells or LCs enhances 11um-LMP/OVA-induced Tfh/GC response

How do we explain the discrepancy on the role of langerin^{+} cells in LMP/OVA and MC903 model? We suspected that the implication of Langerin^{+} cells in promoting Tfh/GC response might be dependent on the depth of the skin barrier impairment. In other words, langerin^{+} cells may only be required for epicutaneous OVA-sensitization when the skin barrier impairment targets rather the epidermis instead of the dermis. To examine this hypothesis,

we first performed laser microporations at a depth of 91 μm (91μm-LMP) which generated a deeper disruption of the skin barrier (Figure S4A). Similar with what was observed in 30μm-LMP/OVA-treated mice, the Tfh/GC response was not reduced, but rather increased in Lang^{DEP} mice treated with 91μm-LMP/OVA (Figure S4B). We next performed laser microporations at a depth of 11μm, which impairs only the cornified layer of the epidermis (Figure 6A). We observed that the 11μm-LMP induced a lower production of TSLP compared to 30μm-LMP (Figure 6B). Treatment of wildtype control (CT) ears with 11μm-LMP/OVA (as shown in Figure 4C) induced a milder but significant increase of CXCR5$^+$ PD-1$^+$ Tfh cell frequency as well as GC B cell number, which were all abolished in *Tslp$^{-/-}$* mice (Figure 6C), indicating that, despite a low expression of TSLP, the Tfh/GC response induced by 11μm-LMP/OVA is still crucially dependent on TSLP. However, when Lang^{DEP} mice were subjected to 11μm-LMP/OVA treatment (as shown in figure 5A), they exhibited a significant increase in frequency of Tfh cells (Figure 6D), IL-4 (AmCyan) expression by Tfh cells (Figure 6E) as well as GC B cell, IgG1$^+$ and IgE$^+$ B cell numbers (Figure 6F). In addition, serum levels of OVA-IgG1 and OVA-IgE were significantly augmented in 11μm-LMP/OVA-treated Lang^{DEP} mice (Figure 6G). Together, these data indicate that, in contrast to what was observed in MC903-induced AD model, the depletion of langerin$^+$ cells does not reduce but rather enhance the Tfh/GC response in LMP/OVA-induced allergic AD.

Furthermore, by subjecting HuLang^{DEP} mice in which LCs were selectively depleted to 11μm-LMP/OVA treatment (Figure 6H), we found that these mice also exhibited an increased Tfh cell frequency, GC B cell, IgG1$^+$ and IgE$^+$ B cell numbers (Figure 6I), similar to what was observed in Lang^{DEP} mice. These results suggest that LCs do not promote but rather limit the Tfh/GC response induced by epicutaneous OVA sensitization irrespectively of the extent of the disruption of the tissue barrier of the skin.

Depletion of langerin$^+$ cells or LCs enhances the allergic skin inflammation and the subsequent asthma

Epicutaneous OVA sensitization through 30μm-LMP skin of wildtype control (CT) mice induced an allergic AD-like skin inflammation, shown as inflammatory infiltration in the

dermis (HE staining, Figure 7A) including eosinophils and basophils (Figure 7B). Note that different with MC903-induced AD where skin inflammation is partially dependent on adaptive immunity (Li et al., 2006, Proc Natl Acad Sci Usa), the LMP/OVA-induced skin inflammation is totally dependent on adaptive immunity, since it is abrogated in *Rag1*^{-/-} mice (Backcrossed to > 99.9% Balb/c background; Figure S5). Analyses of skin from 30μm-LMP/OVA-treated Lang[DEP] mice showed an elevated infiltration of eosinophils and basophils (Figure 7B). In addition, we observed a higher expression of IL-4 (AmCyan) and IL-13 (DsRed) in dermal TCRβ^{+} cells in Lang[DEP]/4C13R[Tg/0] mice (Figure 7C), suggesting that the depletion of langerin^{+} cells leads to an enhanced Th2 inflammation in the skin. Similar observations were also obtained from Lang[DEP] and HuLang[DEP] mice treated with 11μm-OVA/LMP (Figure S6). All these results indicate that LCs limit the allergic skin inflammation induced by epicutaneous OVA sensitization.

We further analyzed the asthmatic phenotype induced by the intranasal challenge with OVA in the epicutaneous OVA-sensitized mice (Figure 7D). Results showed that 30μm-LMP/OVA-sensitized Lang[DEP] mice developed a much stronger asthmatic inflammation compared with CT mice, showing an increase in the total cell number of bronchioalveolar lavage fluid (BAL; Figure 7D), particularly in the number of eosinophils in BAL (Figure 7E). RT-qPCR analyses of BAL cells showed an increase in RNA levels of Th2 cytokines IL-4, IL-5 and IL-13, as well as CCR3 and MCPT8, which are indicators for eosinophils and basophils, respectively (Figure 7F).

Taken together, these data suggest that LCs not only limit epicutaneous OVA-induced Tfh/GC response, but also counteract the allergic skin inflammation and the subsequent asthmatic inflammation.

Transcriptomic analyses of Langerin[+] migratory DCs in skin-draining LNs in TSLP[over] and LMP/OVA mice

To obtain molecular insights underlying the role of langerin[+] DCs in promoting or limiting the Tfh/GC response in MC903 model or LMP/OVA model, we took use of Lang[GFP] mice in which GFP reports the expression of langerin (Kissenpfennig et al., 2005, Immunity), which

facilitates the sorting of Lang$^+$ (GFP^{pos}) migratory (mig) DCs and Lang$^-$ (GFP^{neg}) migDCs in EDLNs. Lang^{GFP} mice (in C57Bl/6 background) were treated with MC903 (at D0, D2 and D4) or LMP/OVA (at D0 and D3) and GFP-positive (GFP^{pos}) and GFP-negative (GFP^{neg}) migDCs were sorted at D5. Note that, despite of a reduced frequency of CXCR5$^+$ PD-1$^+$ Tfh cells (Figure S7A) and GC B cell number (Figure S7B) at D11 in MC903-treated C57Bl/6 mice compared Balb/c mice, Tfh cell differentiation appeared to be initiated, as shown by an increase of CXCR5$^+$ PD-1$^-$ cells at D7 in MC903-treated C57Bl/6 mice (Figure S7C). The gating strategy for cell sorting is shown in Figure 8A. RNA was prepared from the sorted cells and processed for mRNAseq analysis.

We first performed principal component (PC) analyses of mRNA sequencing data. PC1 accounts for almost 49.47% of the variability and segregates GFP^{pos} from GFP^{neg} migDCs, thus rather representing the variation between different cell populations. Interestingly, PCA analysis suggest a similar effect of MC903 treatment of both GFP^{pos} (MC_Pos) and GFP^{neg} (MC_Neg) migDCs, which largely accounts for PC2 (12.88% of the variability) and segregates those cells apart from migDCs from untreated and LMP/OVA-treated mice. Finally, PC3 (6.6% of the variability) shows that LMP/OVA treatment induces different transcriptional changes in GFP^{pos} (OVA_Pos) and GFP^{neg} (OVA_Neg) migDCs. Indeed, OVA_Pos cells were clother to GFP^{pos} cells from untreated mice (NT_Pos) than MC_Pos, whereas OVA_Neg cells were segregated farther than MC_Neg from GFP^{neg} cells from untreated mice (NT_Neg). Unsupervised hierarchical clustering using normalized levels of all detected genes showed that, among the GFP^{pos} groups, OVA_Pos clustered closely to NT_Pos but not to MC_Pos (Figure 8C). Interestingly, among the GFP^{neg} groups, migDCs from LMP/OVA-treated mice (OVA_Neg) clustered closely to that from MC903-treated mice (MC_Neg) (Figure 8C). These results indicate that langerin$^+$ migDCs from MC903-treated mice undergo major transcriptional changes, whereas langerin$^+$ migDCs from LMP/OVA-treated mice were very similar to those from non-treated mice. This is in contrast to Langerin$^-$ migDCs, which exhibited major transcriptional changes in mice treated with MC903 or LMP/OVA, both of which were very different from untreated mice (Figure 8C).

We next analyzed the differentially regulated genes in MC_Pos compared to NT_Pos, and identified 382 upregulated and 367 downregulated genes (Figure 8D). In contrast,

comparison of OVA_Pos with NT_Pos revealed only 28 upregulated and 8 downregulated genes (Figure 8D), further supporting that langerin[+] migDCs from mice treated with LMP/OVA present very minor changes in gene expression compared to those from non-treated mice.

We then used gene ontology (GO) annotations to filter genes associated with "T cell costimulation" (Figure 8E), "Cytokine activity" (Figure 8F) or "Transcription analysis" (Figure 8G) that were significantly upregulated or downregulated in MC_Pos compared to NT_Pos. Of these genes, we noted that the expression of costimulatory molecules CD80 and CD86 was increased in MC_Pos but not in OVA_Pos when compared with NT_Pos, suggesting that MC903 but not LMP/OVA induces the maturation and activation of langerin[+] DCs. Moreover, searching for candidate genes with the potential to induce Tfh cell differentiation, we found that IL-6, which has been shown to be crucial for Tfh cell differentiation (Eto D., 2011, PLoS One), exhibited an increase in MC_Pos but not OVA_Pos. To validate these data, we performed RT-qPCR analyses with the sorted GFP[pos] and GFP[neg] migDCs. Results confirmed that, CD80 and CD86 RNA levels were increased in MC_Pos but not in OVA_Pos (Figure 8H). Interestingly, among the GFP[neg] migDCs, CD80 and CD86 were increased in both MC_Neg and OVA_Neg (although to a lesser extent in OVA_Neg; Figure 8H). RT-qPCR analyses of IL-6 also confirmed mRNAseq data, showing that IL-6 RNA level was increased in MC_Pos, but not in OVA_Pos. In addition, an increase in IL-6 was also observed in MC_Neg (Figure 8G). Finally, to verify these results in mice on Balb/c background, we had started to backcross Lang[GFP] mice to Balb/c background (reaching 96.8%), and performed RT-qPCR analyses of the sorted cells. Results confirmed that the expression of CD80, CD86 and IL-6 in GFP^{pos} cells was induced in these mice upon MC903 treatment (Figure S8A).

Taken together, these transcriptomic analyses suggest that MC903, but not LMP/OVA treatment, differentially polarize langerin[+] migDCs compared to steady state migDCs of untreated mice, with the upregulation of CD80, CD86 and IL-6 which could be potentially implicated in promoting the Tfh cell differentiation.

Discussion and perspectives

In this study, we uncover a seemingly contradictory role of LCs in the Tfh and GC response in two mouse models of AD. On the one hand, LCs exert a "pro-Tfh" role by promoting Tfh cell differentiation and GC response in MC903-induced AD model (MC903-AD). On the other hand, LCs exert an "anti-Tfh" role by limiting the development of Tfh cells and GC response in a model of epicutaneous OVA sensitization-induced allergic AD (OVA-AD). Interestingly, both models are crucially dependent on TSLP: in MC903-AD, overexpression of TSLP (TSLP^{over}) is sufficient to drive Tfh/GC response without the adding of exogenous allergen; in epicutaneous OVA-AD model, where TSLP is expressed in a much lower level, both TSLP and the allergen (OVA) are required for the development of Tfh/GC response. The discrepancies of these roles of LCs in AD and the underlying mechanisms are discussed below.

1) TSLP^{over} engages LCs to promote the Tfh/GC response

Our transcriptomic data show that langerin$^+$ migratory (mig) DCs in ear-draining lymph nodes (EDLN) of TSLP^{over} mice present substantial transcriptional changes, including an activated mature phenotype with a strong induction of costimulatory signals CD80 and CD86, when compared to untreated or LMP/OVA-treated mice (Figure 8E), suggesting that LCs are activated in TSLP^{over} skin and undergo subsequent migration to the draining LN. It will be interesting to confirm these data at the protein level since both CD80 and CD86 are recognized to interact with CD28 on the surface of naïve CD4$^+$ T cells in order to stabilize and strengthen TCR signaling. Interestingly, it has been previously shown that Tfh cell differentiation is favored by a high strength of TCR/peptide:MHCII interaction (Fazilleau et al, 2009, Nat Immunol) and a prolonged interaction between the T cell and the DC (Tubo et al., 2013, Cell). Therefore, the induction of CD80 and CD86 could grant LCs the capability to induce Tfh cell differentiation. In addition, we noticed that langerin$^+$ migDCs in MC903-AD model also exhibited an upregulation of CCL17 (TARC) and CCL22 (MDC) (Figure 8F). These two chemokines are reported to be upregulated in AD patients (Shimada et al., 2004, J Dermatol Sci). Indeed, they are not only important for CCR4-mediated chemotaxis of Th2 cells, but also implicated for LC emigration from the skin (Stutte et al., 2010, P Natl Acad Sci Usa), further supporting the activation and migration of LC to the draining LNs. In agreement with these

data, it has been previously reported that LCs emigrate from human skin explants in the presence of TSLP (Ebner et al, 2007, J Allergy Clin Immunol), and TSLP can act directly on humans DCs to promote cell migration (Fernandez et al., 2011, Blood).

Among several DC-derived Tfh-promoting signals which have been described (reviewed in Krishnaswamy et al., 2018, Front Immunol and Ritvo, 2019, Front. Immunol.), such as transmembrane costimulatory signals (CD40L, OX40L, ICOSL, Notch ligands) and cytokines (IL-1 family members, IL-2, IL-6, IL-12, IL-21 (Vogelzang et al., 2008, Immunity) and type I interferons), we found IL-6 but none of the others in the list of upregulated genes in langerin[+] migDCs from MC903-treated mice, raising a hypothesis that IL-6 mediates the "pro-Tfh" role of LCs in TSLP[over]-driven AD. To explore this hypothesis, we can employ *in vivo* and *in vitro* strategies. First, the requirement of IL-6 in TSLP[over]-driven Tfh cell differentiation can be tested by administrating IL-6 neutralizing antibody (Eto, 2011, PLoS One). Second, we can develop an *in vitro* LC-T cell coculture assay: after MC903 treatment, mouse ears will be floated on culture medium, and LCs that emigrate into the culture medium will be sorted and cocultured with CD4^{+} T cells to test whether these LCs induce Tfh cell differentiation. If yes, this validates that LCs from TSLP[over] mice drive Tfh cell differentiation. Further, we can perform RT-qPCR analysis or intracellular staining to examine the expression of IL-6, and if yes, anti-IL-6 neutralizing antibody will be added to the coculture to determine whether LC-derived IL-6 promotes Tfh cell differentiation.

It remains to be examined whether TSLP signals through its receptor (TSLPR/IL7Rα complex) on LCs to drive their migration and activation, as well as the Tfh/GC response. We are generating mice with the selective and inducible ablation of TSLPR in LCs (TSLPR[iLC-/-]) by breeding mice bearing a *Crlf2* floxed allele with human langerin-CRE[ERT2] mouse line (Bobr et al., 2012, P Natl Acad Sci Usa). These mice will eventually allow us to determine whether TSLPR expression by LCs is required for their activation, migration and their "pro-Tfh" role.

Several other studies have also reported a "pro-Tfh" role of LCs. One earliest report showed that in a model of Leishmania parasite infection, LCs catalyze the interaction between T cells and B cells and thus the GC formation and immunoglobulin production (Zimara, 2014, EJI). Later, it was shown that selective antibody-mediated allergen targeting to LCs induced the

antigen-specific Tfh cell differentiation (Yao et al., 2015, J Allergy Clin Immun). More recently, it was reported that LCs and other dermal DCs promote Tfh cell differentiation and GC response after intradermal immunization with HIV p24-coated nanoparticles (P24-NPs; Levin et al., 2017, J Invest Dermatol). Despite these reports, how LCs induce Tfh response remains poorly understood. It was shown that IL-1β, IL-6 and IL-21 were upregulated in migratory DCs from skin-draining LN after interdermal administration of p24-NPs, but their role for Tfh cell induction was not explored (Levin et al., 2017, J Invest Dermatol). Moreover, it remains unclear whether TSLP or other signals are implicated in licensing LCs for their "pro-Tfh" role in these abovementioned studies. Notably, TSLP production may be triggered by cutaneous parasites infestation (Bergheme et al., 2012, Cytokines).

2) LCs counteracts Tfh/GC response and allergic inflammation in OVA epicutaneous sensitization model

Using OVA-AD model, we showed that LCs limit the epicutaneous OVA-induced Tfh cell differentiation, GC B cell formation and OVA-specific IgG1 and IgE production. Importantly, our LMP experimental system allowed us to examine the role of LCs when skin barrier is disrupted at different depths, leading us to conclude that in all tested depths ($11 - 30 - 91\mu m$-LMP), LCs exert an "anti-Tfh" role. Interestingly, such role of LCs is more clearly observed when OVA sensitization occurs in skin with superficial barrier impairment ($11\mu m$-LMP).

Our mRNAseq data show that langerin[+] migDCs in EDLN from OVA-AD mice present very minor transcriptional changes, in contrast to those from MC903-AD mice. It suggests that either langerin^{+} cells do not migrate in EDLN or even if they do, they are not activated. We may eventually compare the migration of LCs using an *ex vivo* system: ears from untreated, MC903- or LMP/OVA-treated mice will be floated with dermal side down in the culture medium, and LCs emigrated into the medium can then be collected and analyzed by flow cytometry.

One possible explanation for the "anti-Tfh" role of LCs is that they induce Treg in EDLNs from OVA-AD mice. However, langerin[+] migDCs do not exhibit any change for Treg-inducing signals, including costimulatory molecules CD80 and CD86 (Guo et al., 2008, J Immunol), or classical Treg-inducing cytokines IL-10 and TGFβ, or RALDH2, an enzyme involved in the metabolic pathway leading to the formation of retinoic acid (Manicassamy et al., 2009, Seminars in

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Immunology) (Figure 8E and S9). This suggests that, even if langerin^{+} DCs migrate to EDLNs, they would rather induce the anergy of OVA-specific CD4^{+} T cells due to the absence of costimulatory signals, than the differentiation of Tregs. Nevertheless, it will be interesting to examine the differentiation and expansion of Tregs and T follicular regulatory (Tfr, CXCR5^{+} Tregs) cells in EDLNs from LMP/OVA-treated wildtype and Lang[DEP] mice or HuLang[DEP] mice.

Our data show that LCs not only limit the Tfh cell response, but also the skin allergic inflammation in OVA-AD model (Figure 7). It has been shown that, in steady state, human LCs induce the activation and expansion of skin resident Tregs (Seneschal et al., 2012, Immunity). It is thus possible that LCs limit the allergic inflammation through their local effects on resident Tregs in OVA-AD mice. We can address this possibility by analyzing skin Tregs in LMP/OVA-treated control and HuLang[DEP] mice. If this is indeed the case, we may further investigate what signals (e.g. IL-10) from LCs are important for exerting their Treg-promoting role or for repressing the pro-inflammatory role of dermal DCs.

Finally, we observed that epicutaneous OVA sensitized Lang[DEP] mice developed an aggravated asthma upon OVA challenge in the airway, suggesting that langerin^{+} cells limit the development of allergen sensitization, thus preventing the atopic march (the progression from AD to asthma). It will be necessary to further determine whether it is LCs that exert such a role, by performing the experiments as shown in figure 7D using HuLang[DEP] mice. Moreover, it remains to be clarified whether this asthma-preventing role is mediated by skin but not airway langerin^{+} cells. To this aim, we may subject HuLang[DEP] mice with an intraperitoneal OVA sensitization followed by airway challenge. If in this case, HuLang[DEP] mice do not develop an exacerbated asthma inflammation, we will be able to conclude that skin LCs limit the skin allergen-sensitization and prevent the atopic march.

3) Can TSLP quantity be responsible for the switch from anti-Tfh to pro-Tfh role of LCs?

We show that MC903 treatment induces a high production of TSLP (Figure 4B) which is sufficient to induce not only skin inflammation (Li et al., 2006, P Natl Acad Sci Usa), but also Tfh cell and GC B cell response, without the need of experimental allergen. Unpublished data from the lab showed that MC903-treated DO11.10 mice in which the majority of T cells harbor an OVA-specific TCR developed a much lower Tfh cell response, suggesting that Tfh cell

differentiation in TSLP[over] mice is antigen dependent eventhough the nature of the antigen triggering T cell differentiation in MC903 model remains undetermined.

On the other hand, skin barrier disruption with LMP induces TSLP to a much lower extent (Figure 4B). This is not sufficient to induce Tfh cell differentiation in absence of an experimental allergen, but LMP plus OVA together promote Tfh/GC response (Figure 4D, compared LMP/OVA with LMP). Indeed, even though TSLP production increases with the depth of LMP (i.e. 30μm-LMP or 91μm-LMP induces a higher TSLP level than 11μm-LMP), such TSLP levels are still by far lower compared to MC903 treatment (Figure 4B).

We suspect that TSLP quantity is an important switch for LCs from an "anti-Tfh" player to become a "pro-Tfh" player. To further explore how quantitative TSLP signaling may define the role of LCs, by performing *in vivo* or *ex vivo* dose-dependent experiments and mathematic modeling to integrate several parameters including TSLP quantity and allergen concentration.

4) Clinical relevance of our findings

It has been recognized that TSLP is overproduced in AD lesional skin (Soumelis, 2002, Nat Immunol), however, its expression varies from high to low, which may be dependant on the cause (e.g. genetic mutation of spink5 which induces high TSLP (Briot et al., 2009, J Exp Med) versus skin barrier impairment which induces lower TSLP (Angelova-Fischer et al., 2010, J Invest Dermatol)), age (Yao et al., 2013, Immunity), or the nature of disease (e.g. intrinsic versus extrinsic AD)). Our study demonstrate a crucial role for TSLP in promoting Tfh/GC response in AD pathogenesis in the two mouse models either with high or low TSLP expression. This adds new evidences that blocking or reducing TSLP level should be helpful for regulating Tfh cell differentiation and B cell response in AD. Moreover, the discovery of the quite opposite role of LCs in these two models may indicate the different implication of these cells in AD pathogenesis and immunotherapy. To further explore our findings in AD patients, we need to study the correlation of the emigration of LCs from the epidermis, the number of LCs in the dermis and the quantity of TSLP in the local skin; examine whether TSLP level correlates with a the activation state of LCs, for example CD80 and CD86; and examine the potential "pro-Tfh" mediators (for example IL-6) expressed by LCs. Once we acquire a better understanding of what signals define the role of LCs either as "pro-Tfh" or as "anti-Tfh", we

may expect to revert LCs back to its regulatory role. That could help not only to limit the skin inflammation, but also limit the allergen sensitization of these patients through the skin, and thus prevent the progression from AD to asthma.

In addition, the potential of LCs to induce Tfh cell differentiation and GC response and the subsequent induction of antigen-specific antibodies has been of interest for skin vaccination (Romani et al., 2010, Immunol Cell Biol). A better understanding of the switch triggering the change of LCs from an anti-Tfh to a pro-Tfh player as well as the signaling cascade used by LCs to promote Tfh/GC response should also provide important insights into the design of LC-based skin vaccination.

References

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. Genome Biology *11*, R106.

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics *31*, 166–169.

Angelova-Fischer, I., Fernandez, I.M., Donnadieu, M.-H., Bulfone-Paus, S., Zillikens, D., Fischer, T.W., and Soumelis, V. (2010). Injury to the stratum corneum induces in vivo expression of human thymic stromal lymphopoietin in the epidermis. J. Invest. Dermatol. *130*, 2505–2507.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological) *57*, 289–300.

Bergheme, G., Carvalho, A.M., Costa, R., Passos, S., Carvalho, E.M., and Carvalho, L.P. (2012). P015 TSLP enhances type 1 immune response in cutaneous leishmaniasis patients. Cytokine *59*, 523.

Bobr, A., Olvera-Gomez, I., Igyarto, B.Z., Haley, K.M., Hogquist, K.A., and Kaplan, D.H. (2010). Acute Ablation of Langerhans Cells Enhances Skin Immune Responses. The Journal of Immunology 1001802.

Bobr, A., Igyarto, B.Z., Haley, K.M., Li, M.O., Flavell, R.A., and Kaplan, D.H. (2012). Autocrine/paracrine TGF-β1 inhibits Langerhans cell migration. PNAS *109*, 10492–10497.

Briot, A., Deraison, C., Lacroix, M., Bonnart, C., Robin, A., Besson, C., Dubus, P., and Hovnanian, A. (2009). Kallikrein 5 induces atopic dermatitis–like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. Journal of Experimental Medicine *206*, 1135–1147.

Chapman, M.D., Rowntree, S., Mitchell, E.B., Fuenmajor, M.C.D.P. de, and Platts-Mills, T. a. E. (1983). Quantitative assessments of IgG and IgE antibodies to inhalant allergens in patients with atopic dermatitis. Journal of Allergy and Clinical Immunology *72*, 27–33.

Crotty, S. (2014). T follicular helper cell differentiation, function, and roles in disease. Immunity *41*, 529–542.

Dharmage, S.C., Lowe, A.J., Matheson, M.C., Burgess, J.A., Allen, K.J., and Abramson, M.J. (2014). Atopic dermatitis and the atopic march revisited. Allergy *69*, 17–27.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15–21.

Ebner, S., Nguyen, V.A., Forstner, M., Wang, Y.-H., Wolfram, D., Liu, Y.-J., and Romani, N. (2007). Thymic stromal lymphopoietin converts human epidermal Langerhans cells into antigen-presenting cells that induce proallergic T cells. J. Allergy Clin. Immunol. *119*, 982–990.

Eto, D., Lao, C., DiToro, D., Barnett, B., Escobar, T.C., Kageyama, R., Yusuf, I., and Crotty, S. (2011). IL-21 and IL-6 Are Critical for Different Aspects of B Cell Immunity and Redundantly Induce Optimal Follicular Helper CD4 T Cell (Tfh) Differentiation. PLOS ONE *6*, e17739.

Fazilleau, N., McHeyzer-Williams, L.J., Rosen, H., and McHeyzer-Williams, M.G. (2009). The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nat Immunol *10*, 375–384.

Fernandez, M.-I., Heuzé, M.L., Martinez-Cingolani, C., Volpe, E., Donnadieu, M.-H., Piel, M., Homey, B., Lennon-Duménil, A.-M., and Soumelis, V. (2011). The human cytokine TSLP triggers a cell-autonomous dendritic cell migration in confined environments. Blood *118*, 3862–3869.

Guo, F., Iclozan, C., Suh, W.-K., Anasetti, C., and Yu, X.-Z. (2008). CD28 Controls Differentiation of Regulatory T Cells from Naive CD4 T Cells. The Journal of Immunology *181*, 2285–2291.

Hardtke, S., Ohl, L., and Förster, R. (2005). Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help. Blood *106*, 1924–1931.

Haynes, N.M., Allen, C.D.C., Lesley, R., Ansel, K.M., Killeen, N., and Cyster, J.G. (2007). Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. J. Immunol. *179*, 5099–5108.

Kissenpfennig, A., Henri, S., Dubois, B., Laplace-Builhé, C., Perrin, P., Romani, N., Tripp, C.H., Douillard, P., Leserman, L., Kaiserlian, D., et al. (2005). Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity *22*, 643–654.

Krishnaswamy, J.K., Alsén, S., Yrlid, U., Eisenbarth, S.C., and Williams, A. (2018). Determination of T Follicular Helper Cell Fate by Dendritic Cells. Front Immunol *9*, 2169.

Levin, C., Bonduelle, O., Nuttens, C., Primard, C., Verrier, B., Boissonnas, A., and Combadière, B. (2017). Critical Role for Skin-Derived Migratory DCs and Langerhans Cells in TFH and GC Responses after Intradermal Immunization. J. Invest. Dermatol. *137*, 1905–1913.

Leyva-Castillo, J.M., Hener, P., Michea, P., Karasuyama, H., Chan, S., Soumelis, V., and Li, M. (2013). Skin thymic stromal lymphopoietin initiates Th2 responses through an orchestrated immune cascade. Nature Communications *4*, 2847.

Li, M., Messaddeq, N., Teletin, M., Pasquali, J.-L., Metzger, D., and Chambon, P. (2005). Retinoid X receptor ablation in adult mouse keratinocytes generates an atopic dermatitis triggered by thymic stromal lymphopoietin. PNAS *102*, 14795–14800.

Li, M., Hener, P., Zhang, Z., Kato, S., Metzger, D., and Chambon, P. (2006). Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. PNAS *103*, 11736–11741.

Li, M., Hener, P., Zhang, Z., Ganti, K.P., Metzger, D., and Chambon, P. (2009). Induction of thymic stromal lymphopoietin expression in keratinocytes is necessary for generating an

atopic dermatitis upon application of the active vitamin D3 analogue MC903 on mouse skin. J. Invest. Dermatol. *129*, 498–502.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550.

Manicassamy, S., and Pulendran, B. (2009). Retinoic acid-dependent regulation of immune responses by dendritic cells and macrophages. Semin. Immunol. *21*, 22–27.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.Journal *17*, 10–12.

Pires, M.C., Calux, M.J.F., Mori, J.C., and Montealegre, F. (2002). Reactivity of anti-Blomia tropicalis IgG and IgE in patients with atopic dermatitis. Clin. Exp. Dermatol. *27*, 309–313.

Ritvo, P.-G., and Klatzmann, D. (2019). Interleukin-1 in the Response of Follicular Helper and Follicular Regulatory T Cells. Front Immunol *10*, 250.

Roediger, B., Kyle, R., Yip, K.H., Sumaria, N., Guy, T.V., Kim, B.S., Mitchell, A.J., Tay, S.S., Jain, R., Forbes-Blom, E., et al. (2013). Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. Nat. Immunol. *14*, 564–573.

Romani, N., Thurnher, M., Idoyaga, J., Steinman, R.M., and Flacher, V. (2010). Targeting of antigens to skin dendritic cells: possibilities to enhance vaccine efficacy. Immunol. Cell Biol. *88*, 424–430.

Schulten, V., Tripple, V., Seumois, G., Qian, Y., Scheuermann, R.H., Fu, Z., Locci, M., Rosales, S., Vijayanand, P., Sette, A., et al. (2018). Allergen-specific immunotherapy modulates the balance of circulating Tfh and Tfr cells. Journal of Allergy and Clinical Immunology *141*, 775-777.e6.

Seneschal, J., Clark, R.A., Gehad, A., Baecher-Allan, C.M., and Kupper, T.S. (2012). Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells. Immunity *36*, 873–884.

Shaker, M. (2014). New insights into the allergic march. Curr. Opin. Pediatr. *26*, 516–520.

Shimada, Y., Takehara, K., and Sato, S. (2004). Both Th2 and Th1 chemokines (TARC/CCL17, MDC/CCL22, and Mig/CXCL9) are elevated in sera from patients with atopic dermatitis. J. Dermatol. Sci. *34*, 201–208.

Soumelis, V., Reche, P.A., Kanzler, H., Yuan, W., Edward, G., Homey, B., Gilliet, M., Ho, S., Antonenko, S., Lauerma, A., et al. (2002). Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat. Immunol. *3*, 673–680.

Stutte, S., Quast, T., Gerbitzki, N., Savinko, T., Novak, N., Reifenberger, J., Homey, B., Kolanus, W., Alenius, H., and Förster, I. (2010). Requirement of CCL17 for CCR7- and CXCR4-dependent migration of cutaneous dendritic cells. PNAS *107*, 8736–8741.

Szabó, K., Gáspár, K., Dajnoki, Z., Papp, G., Fábos, B., Szegedi, A., and Zeher, M. (2017). Expansion of circulating follicular T helper cells associates with disease severity in childhood atopic dermatitis. Immunol Lett *189*, 101–108.

Tubo, N.J., Pagán, A.J., Taylor, J.J., Nelson, R.W., Linehan, J.L., Ertelt, J.M., Huseby, E.S., Way, S.S., and Jenkins, M.K. (2013). Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. Cell *153*, 785–796.

Vogelzang, A., McGuire, H.M., Yu, D., Sprent, J., Mackay, C.R., and King, C. (2008). A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity *29*, 127–137.

Weidinger, S., and Novak, N. (2016). Atopic dermatitis. Lancet *387*, 1109–1122.

Yao, C., Zurawski, S.M., Jarrett, E.S., Chicoine, B., Crabtree, J., Peterson, E.J., Zurawski, G., Kaplan, D.H., and Igyártó, B.Z. (2015). Skin dendritic cells induce follicular helper T cells and protective humoral immune responses. Journal of Allergy and Clinical Immunology *136*, 1387-1397.e7.

Yao, W., Zhang, Y., Jabeen, R., Nguyen, E.T., Wilkes, D.S., Tepper, R.S., Kaplan, M.H., and Zhou, B. (2013). Interleukin-9 is Required for Allergic Airway Inflammation Mediated by the Cytokine Thymic Stromal Lymphopoietin. Immunity *38*, 360–372.

Zimara, N., Florian, C., Schmid, M., Malissen, B., Kissenpfennig, A., Männel, D.N., Edinger, M., Hutchinson, J.A., Hoffmann, P., and Ritter, U. (2014). Langerhans cells promote early germinal center formation in response to Leishmania-derived cutaneous antigens. European Journal of Immunology *44*, 2955–2967.
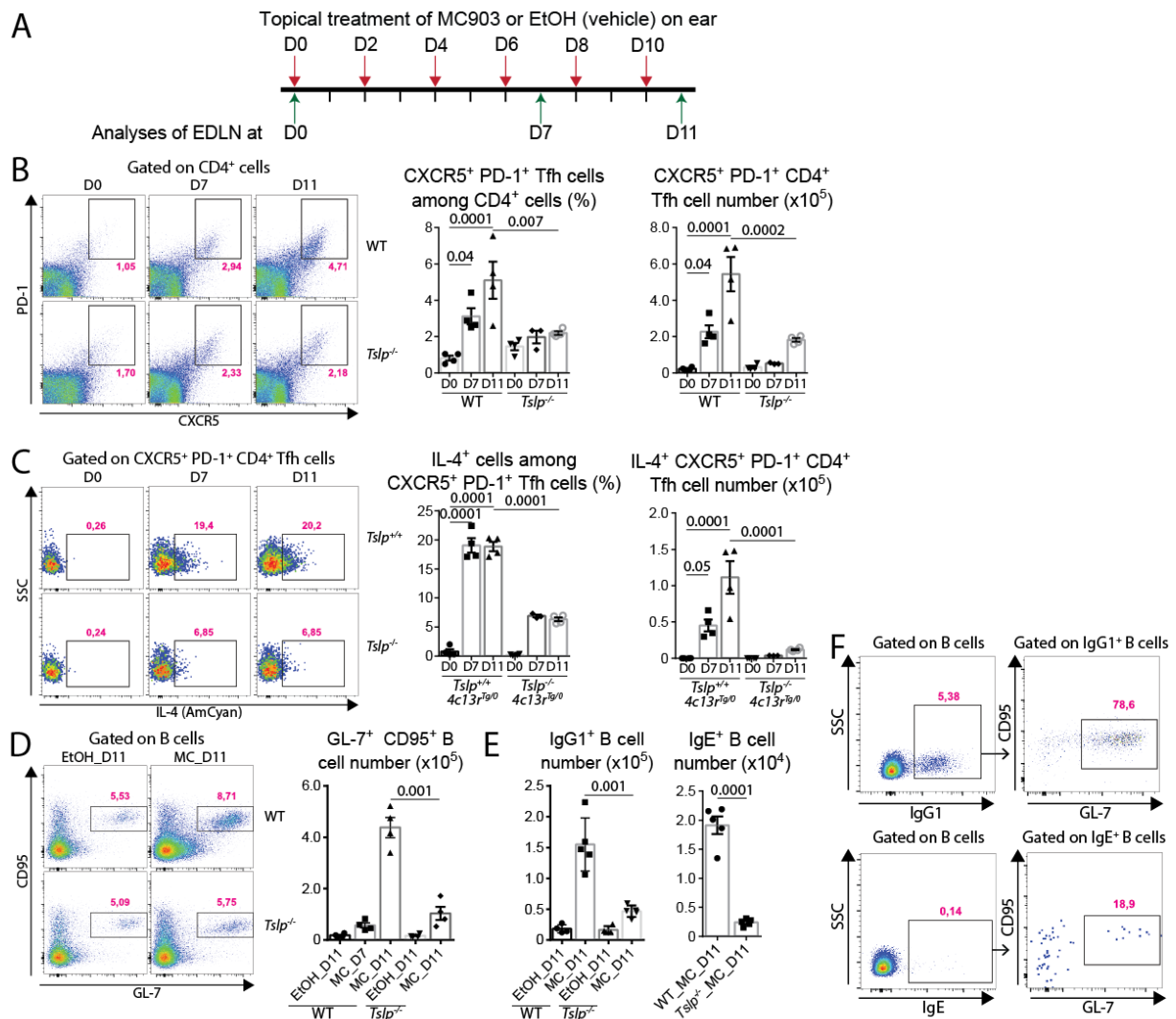
Figure 1: Overproduction of TSLP in skin triggers Tfh differentiation and GC reponse in MC903-induced atopic dermatitis (AD) mice.

(A) Experimental protocol. Mouse ears were topically treated with MC903 or ethanol (EtOH; as vehicle control) every other day from day (D) 0 to D11 and ear-draining lymph nodes (EDLN) were analyzed at D0, D7 and 11. (B) Frequency and number of CXCR5^{+} PD-1^{+} Tfh cells in EDLN from EtOH- or MC903-treated Balb/c wildtype (WT) and *Tslp*^{-/-} mice. (C) Frequency and number of IL-4 (AmCyan)[+] cells in EDLN from *Tslp*^{+/+}/4C13R[Tg/0] and *Tslp*^{-/-}/4C13R[Tg/0] mice. (D) Number of CD95^{+} GL-7^{+} GC B cells. (E) IgG1^{+} and IgE^{+} B cells number in EDLN of EtOH- or MC903-treated WT and *Tslp*^{-/-} mice. (F) The majority of IgG1^{+} but not IgE^{+} cells are GL-7^{+} CD95^{+}.
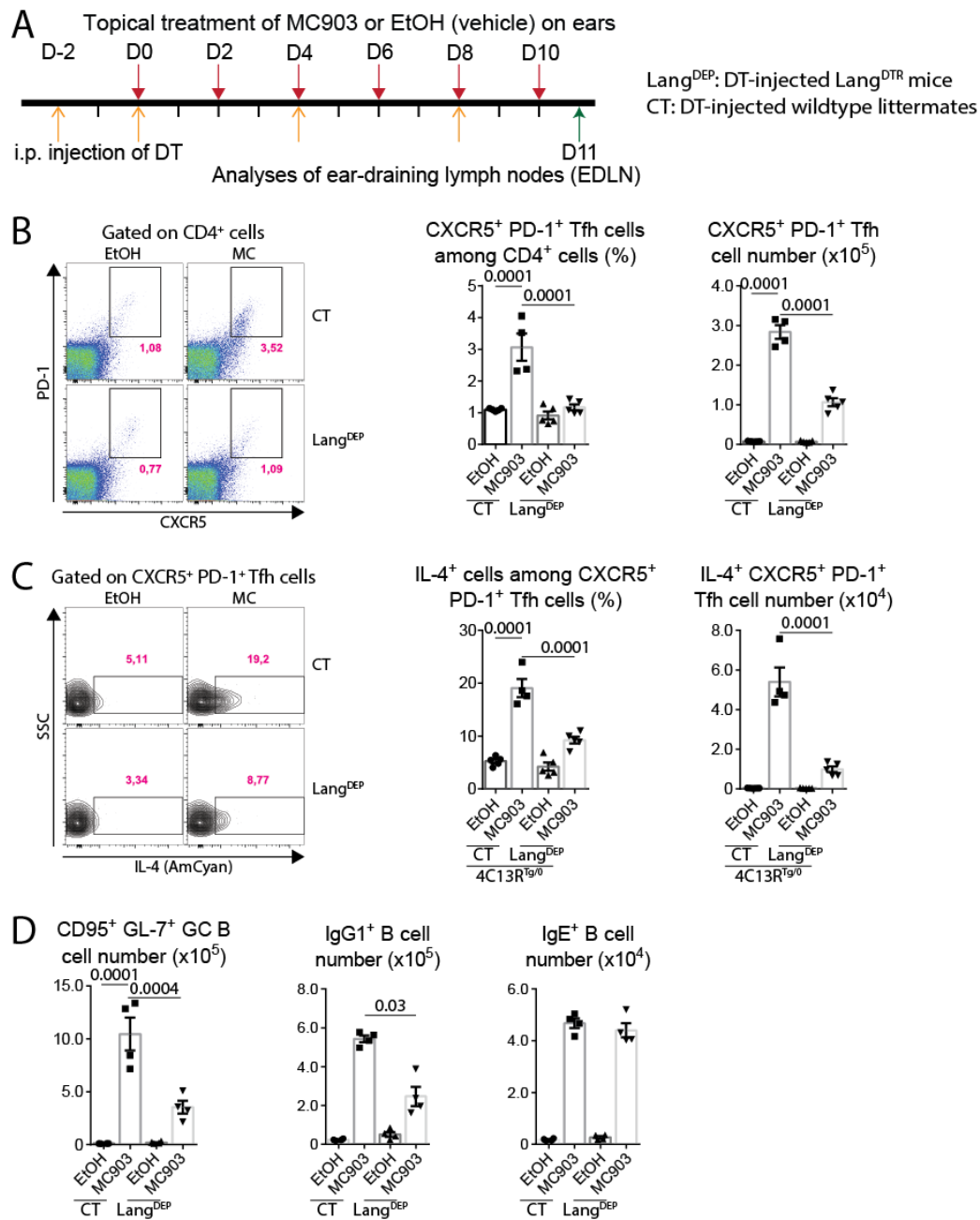
Figure 2: Depletion of Langerin+ cells diminishes the TSLP^over^-induced Tfh/GC response.

(A) Experimental protocol. Lang^DTR mice and wildtype littermates (CT) were intraperitoneally (i.p.) injected with diphteria toxin (DT) at D-2 and D0 and then every 4 days. Mouse ears were topically treated with MC903 or EtOH every other day from D0 to D10 and ear-draining lymph nodes (EDLN) were analyzed at D11. (B) Frequency and number of CXCR5+ PD-1+ Tfh cells. (C) IL-4 (AmCyan) expression by Tfh cells. (D) Total number of CD95+ GL-7+ GC B cells, IgG1+ and IgE+ B cells.
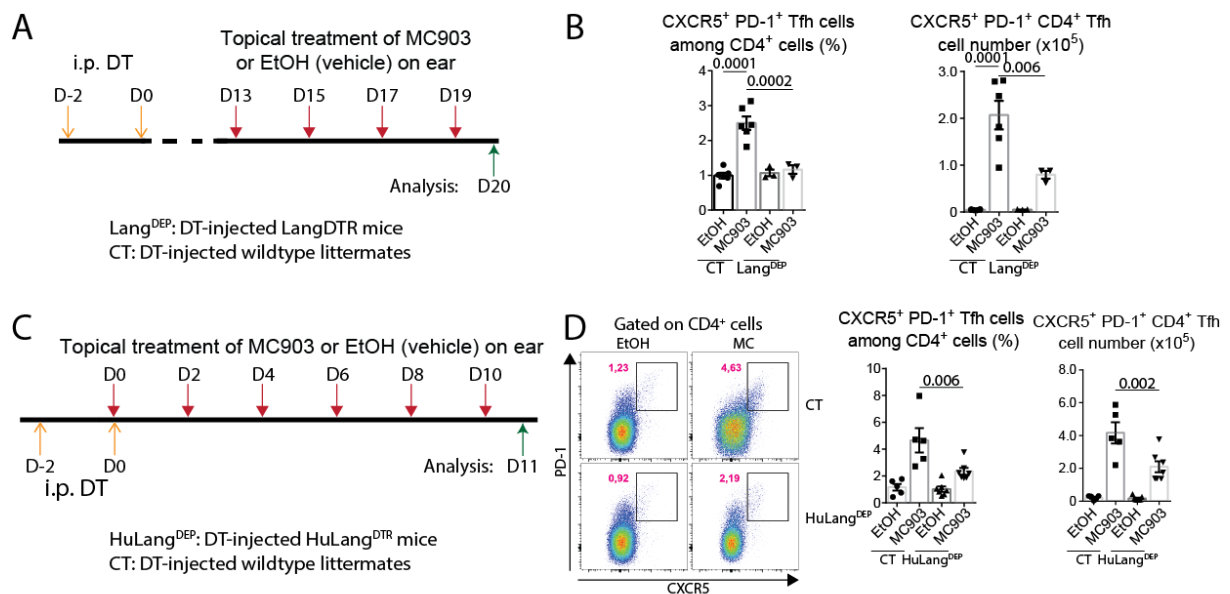
Figure 3: Selective depletion of LCs leads to a diminished Tfh cell differentiation.
(A) Experimental protocol. Lang^DTR mice and wildtype littermates (CT) were intraperitoneally (i.p.) injected with diphteria toxin (DT) at D-2 and D0. Mouse were then topically treated with MC903 or EtOH every other day from D13 to D19 and ear draining lymph nodes (EDLN) were analyzed at D20. (B) Frequency and number of CXCR5^+ PD-1^+ Tfh cells in Lang^DTR mice and CT at D20. (C) Experimental protocol. HuLang^DTR mice and wildtype littermates (CT) were intraperitoneally i.p. injected with DT at D-2 and D0. Mouse were then topically treated with MC903 or EtOH every other day from D0 to D10 and EDLN were analyzed at D20. (D) Frequency and number of CXCR5^+ PD-1^+ Tfh cells in HuLang^DEP mice and CT at D11.
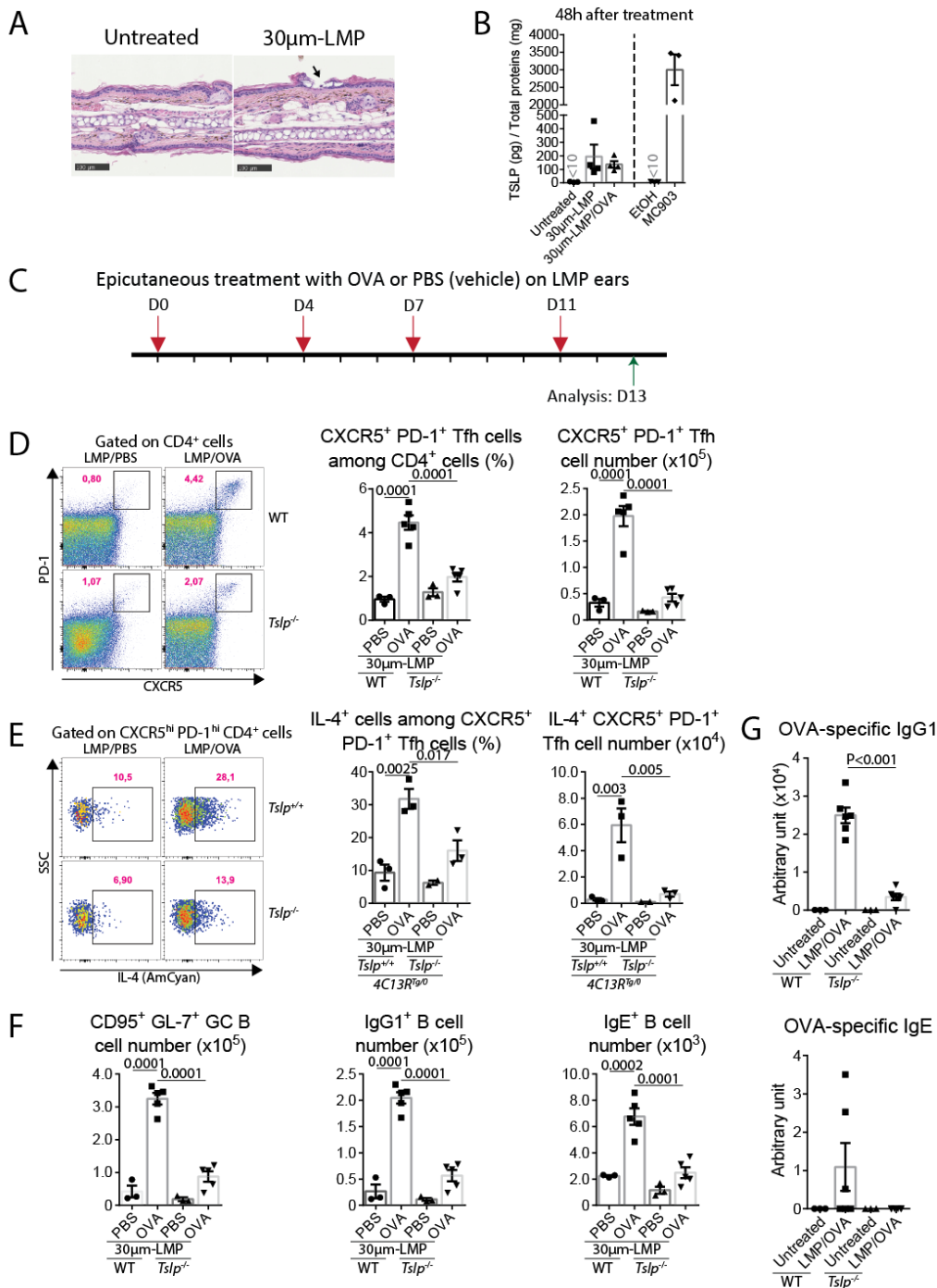
Figure 4: Ovalbumin sensitization through laser-microporated (LMP) skin induces TSLP-dependant Tfh/GC response

(A) Hematoxylin and eosin staining (HE) of untreated or 30µm-LMP ears of Balb/c wildtype (WT) mice. The arrow points to a micropore with disruption of the suprabasal layaer of epidermis. (B) TSLP protein levels in ears of WT mice at 48h after indicated treatment. (C) Experimental protocol for OVA epicutaneous sensitization through LMP ears. OVA or PBS (vehicle) were topically applied on LMP ears at D0, D4, D7 and D11 and ear-draining lymph nodes (EDLN) were analyzed at D13. (D-E) Comparison of the frequency and cell number of CXCR5+ PD-1+ Tfh cells (D) and IL-4 (AmCyan) producing Tfh cells (E) in EDLN of 30µm-LMP/OVA-treated WT and *Tslp-/-* mice. (F) Comparison of GC B cell, IgG1+ and IgE+ B cell number in EDLN of 30µm-LMP/OVA-treated WT and *Tslp-/-* mice. (G) Comparison of serum levels of OVA-IgG1 and OVA-IgE in 30µm-LMP/OVA-treated WT and *Tslp-/-* mice.

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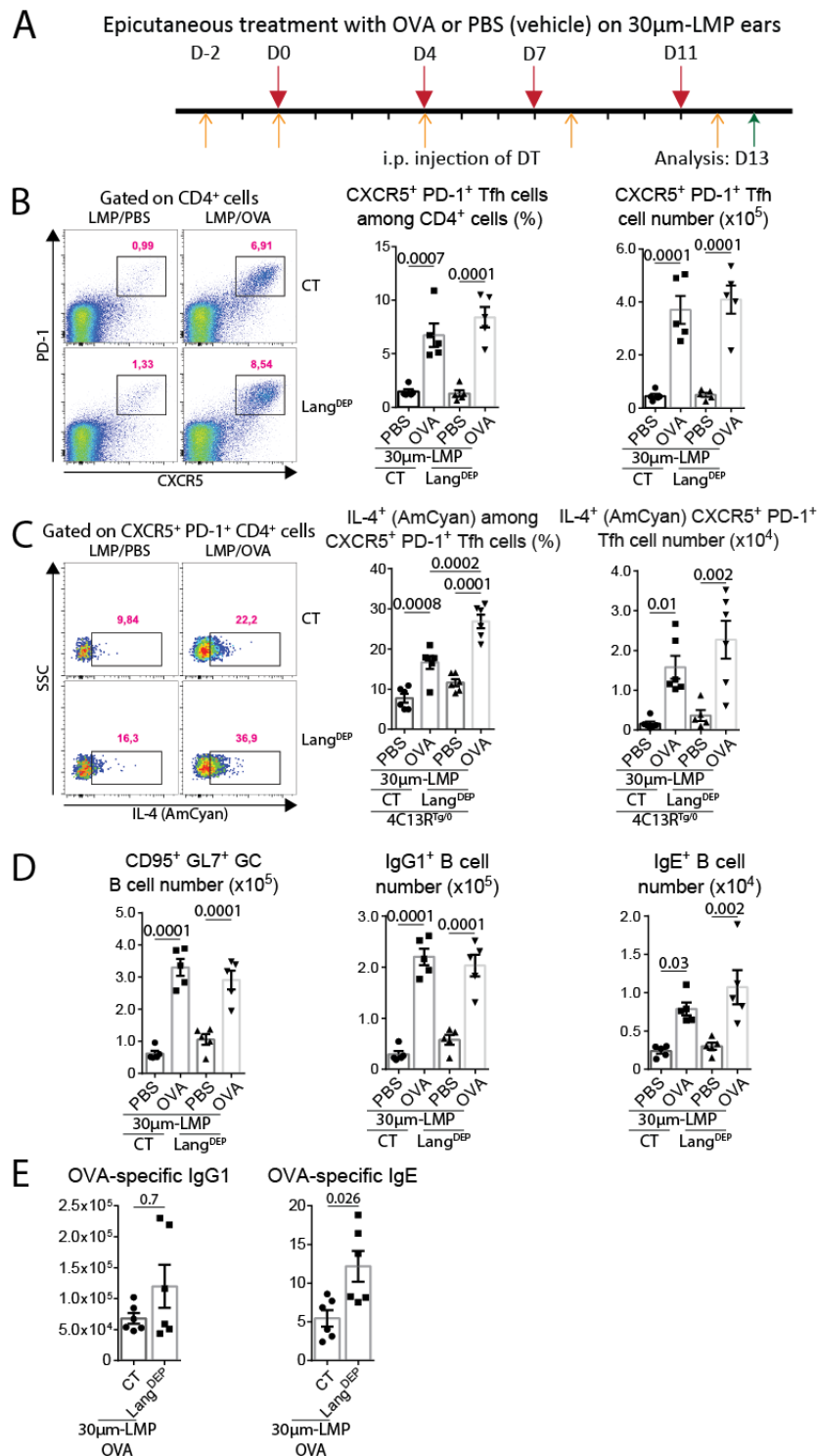
Figure 5: Depletion of Langerin+ cells does not reduce but rather tend to augment 30µm-LMP/OVA-induced Tfh/GC response.

(A) Experimental protocol. Lang[DTR] mice and wildtype (WT) littermates were injected intraperitoneally (i.p.) with diphteria toxin (DT) at D-2, D0 and then every 4 days. Mouse ears were treated by 30µm-LMP/OVA or 30µm-LMP/PBS at D0, D4, D7 and D11 and ear-draining lymph nodes (EDLN) were analyzed at D13. (B) Frequency and number of Tfh cells. (C) IL-4 (AmCyan) expression by Tfh cells. (D) Total number of CD95^{+} GL-7^{+} GC B cells, IgG1^{+} and IgE^{+} B cells. (E) Serum levels of OVA-IgG1 and OVA-IgE after 30µm-LMP/OVA treatment in Lang[DEP] or WT mice.
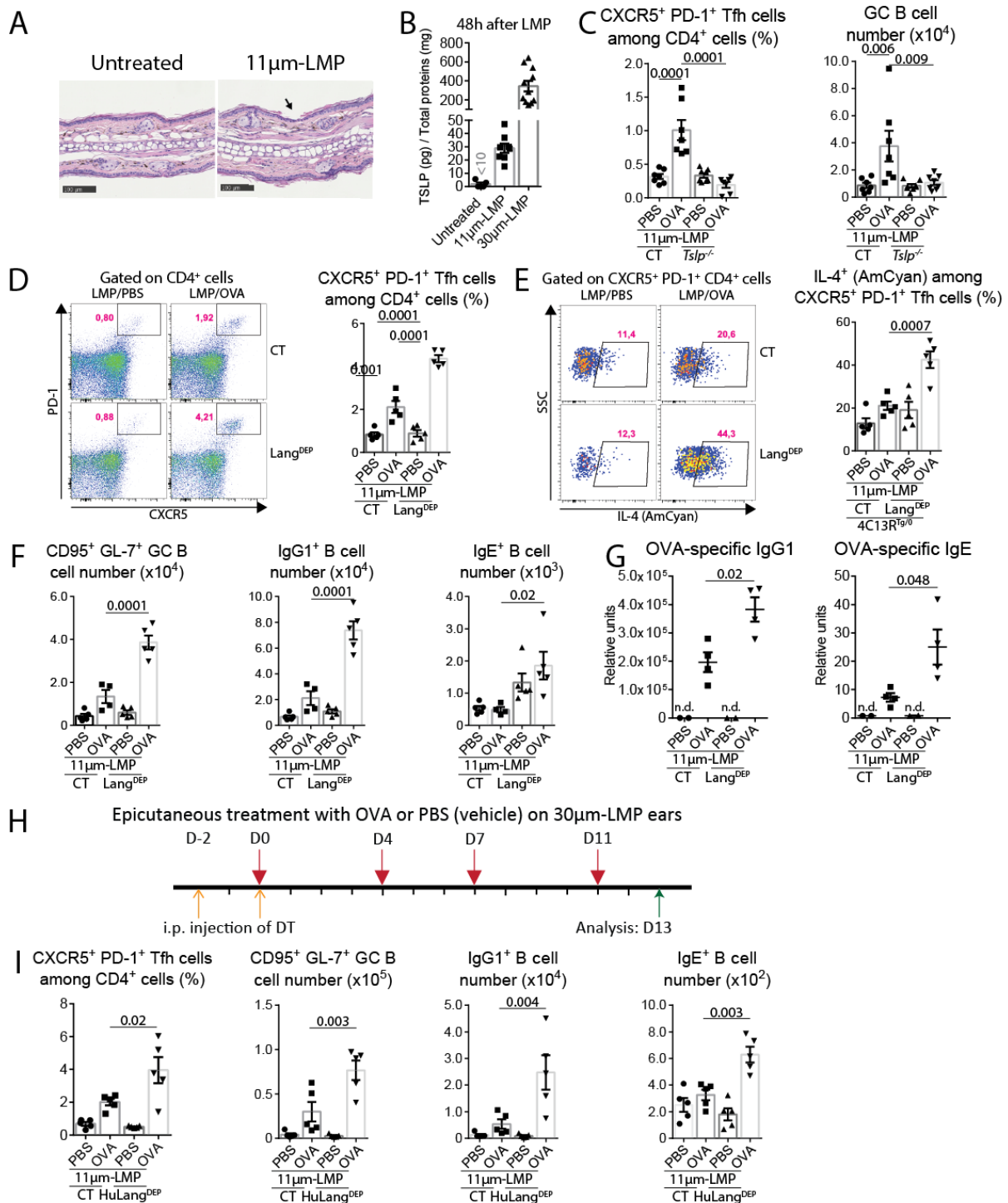
Figure 6: Depletion of Langerin+ cells or LCs significantly enhances 11μm-LM-P/OVA-induced Tfh/GC response.

(A) Hematoxylin and eosin (HE) staining of untreated of 11μm-LMP ears of Balb/c wildtype (WT) mice. The arrow points to a micropore with the impairment of cornified layer. (B) TSLP protein levels in ears of WT mice at 48h after indicated treatment. (C) Frequency of CXCR5+ PD-1+ Tfh cells (left) and number of CD95+ GL-7+ GC B cells (right) in ear-draining lymph nodes (EDLN) of WT or $Tslp^{-/-}$ mice after 11μm-LMP/OVA. (D) Frequency of Tfh cells in CT and Lang^{DTR} mice after 11μm-LMP/OVA. (E) IL-4 (AmCyan) expression by Tfh cells. (F) Number of GC B cells (left), IgG1+ and IgE+ B cells (right) in EDLN. (G) ELISA of serum OVA-IgG1 and OVA-IgE in CT or Lang^{DTR} mice after 11μm-LMP/OVA. (H) Experimental protocol. Lang^{DTR} mice and wildtype (CT) littermates were injected intraperitoneally (i.p.) with diphteria toxin (DT) at D-2 and D0. Mouse ears were treated by 30μm-LMP/OVA or 30μm-LM-P/PBS at D0, D4, D7 and D11 and ear-draining lymph nodes (EDLN) were analyzed at D13. (I) Frequency of Tfh cells and number of GC B cells, IgG1+ and IgE+ B cells in 11μm-LMP/OVA-treated CT and HuLang^{DTR} mice.
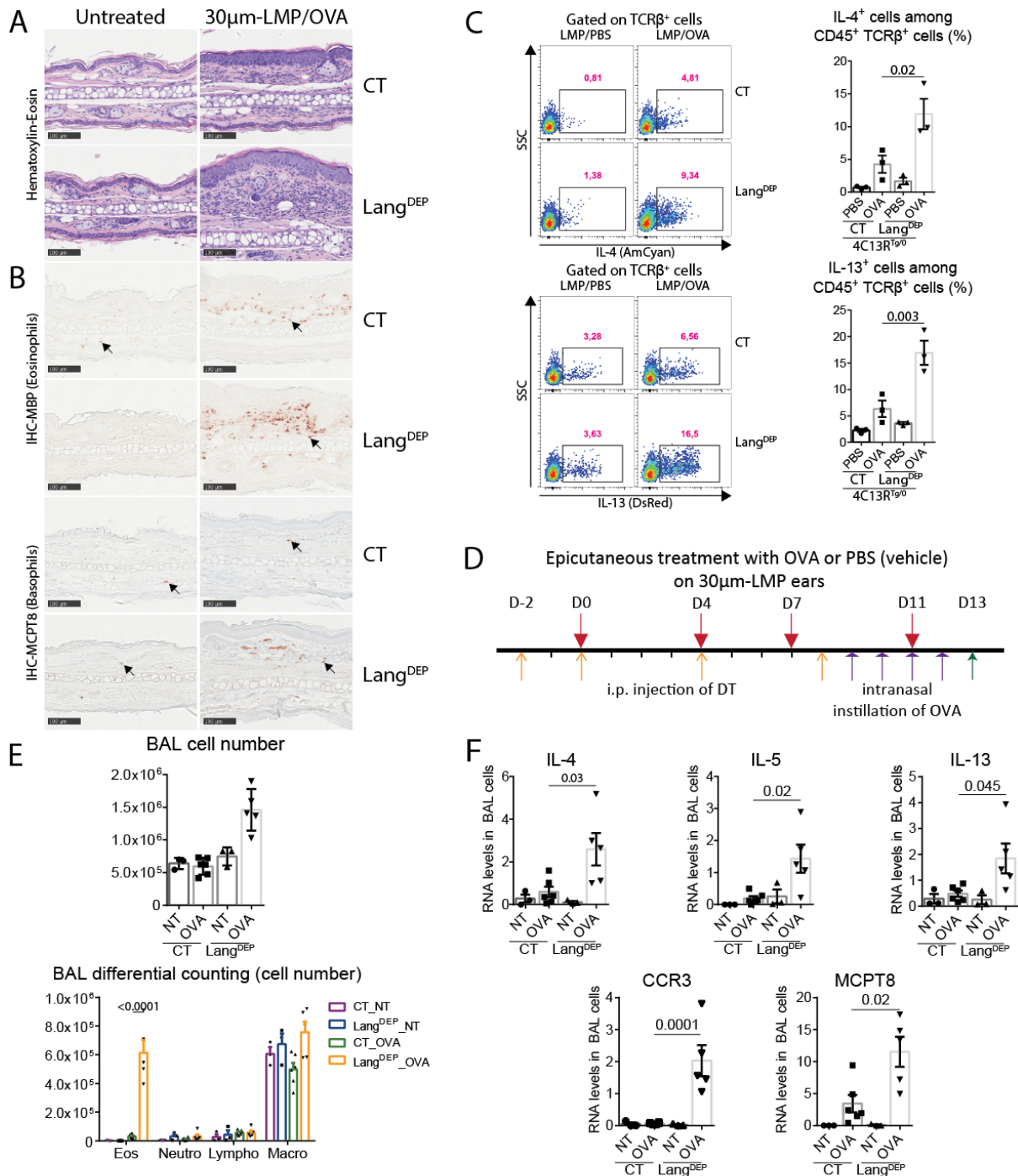
Figure 7: Langerin+ cells counteracts LMP/OVA sensitization-induced skin inflammation and the subsequent asthmatic response.

(A) Hematoxylin and eosin (HE) staining of mouse ears. (B) Immunohistochemistry staining of mouse ears with anti-MBP (for eosinophils) or anti-MCPT8 (for basophils). Arrows point to positive signals. (C) IL-4 (AmCyan) and IL-13 (DsRed) expression in TCRβ+ dermal cells. Scale bar, 100µm. (D) Experimental protocol for OVA epicutaneous sensitization and airway challenge. Mice were intraperitoneally injected with diphteria toxin (DT) at D-2, D0 and then every 4 days. OVA or PBS (vehicle) were topically applied on LMP ears at D0, D4, D7 and D11 and mice were subjected to intranasal instillation with OVA from D9 to D12. Ears, ear-draining lymph nodes (EDLN) and lungs were analyzed at D13. (E) Total bronchioalveolar laval fluid (BAL) cell number and differential counting of eosinophils (Eos), neutrophils (Neutro), lymphocytes (Lympho) and macrophages (Macro). (F) RNA levels of indicated genes in BAL cells by RT-qPCR.

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A

B

NT_Neg: Untreated GFP^neg migratory DCs
NT_Pos: Untreated GFP^pos migratory DCs
MC_Neg: MC-treated GFP^neg migratory DCs
MC_Pos: MC-treated GFP^pos migratory DCs
OVA_Neg: LMP/OVA-treated GFP^neg migratory DCs
OVA_Pos: LMP/OVA-treated GFP^pos migratory DCs

C

D

MC_Pos vs NT_Pos   MC_Neg vs NT_Neg

257   125   320
206   161   401

upregulated genes
downregulated genes

OVA_Pos vs NT_Pos   OVA_Neg vs NT_Neg

13   15   202
7   1   211

MC_Pos vs NT_Pos   OVA_Pos vs NT_Pos

360   22   6
365   2   6

upregulated genes
downregulated genes

MC_Neg vs NT_Neg   OVA_Neg vs NT_Neg

317   128   89
456   106   106

E

GO Term: T cell costimulation

F

GO Term: Cytokine activity

G

GO Term: Transcription factor

Figure 8: Differential gene expression of langerin⁺ cells in skin-draining lymph nodes after TSLP^over and LMP/OVA.

Lang^GFP mice were treated with MC903 at D0, D2 and D4 or 30µm-LMP/OVA on D0 and D3; ear-draining lymph nodes (EDLN) were collected at D5 for cell sorting and RNAseq analyses or RT-qPCR analyse. (A) Gating strategy used to sort resident (res) and migratory (mig) GFP^pos and GFP^neg DCs. (B) Principal component (PC) analysis plot displaying PC1 and PC2 of messenger RNA (mRNA) expressed mig GFP^pos and GFP^neg DCs sorted from MC903-, LMP/OVA-treated or untreated mice at D5. The number in the brackets indicates the percentage of explained variance associated with this axis. Each dot represent one sample and each color represent a group as indicated. (C) Unsupervised hierarchical clustering of samples using the entire mRNA-seq dataset. (D) Venn diagram showing the number of upregulated and downregulated genes (fold change > 2; p < 0.05) and the number of commonly upregulated or downregulated genes between the comparisons, as indicated. (E-G) Heatmaps showing the z-score of the expression level of the differentially regulated genes in MC903-treated Lang^GFP+ cells compared with non-treated Lang^GFP+ cells (FC > 2; p < 0.05; raw read > 200 in at least one sample) corresponding to GO terms "T cell costimulation" (E), "Cytokine activity" (F) or "Transcription factor" (G). (H) RT-qPCR analyses of RNA levels of the indicated gene in GFP^pos or GFP^neg migratory DCs.
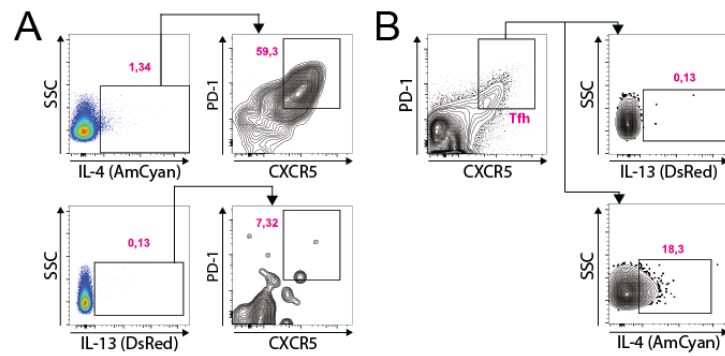
Figure S1: Tfh cells produce IL-4 but not IL-13 in ear-draining lymph nodes of MC903-treated wildtype Balb/c mice.

(A) IL-4 (AmCyan), but not IL-13 (DsRed) expressing cells contain CXCR5+ PD-1+Tfh cells. (B) CXCR5+ PD-1+ Tfh cells produce IL-4 (AmCyan) but not IL-13 (DsRed).
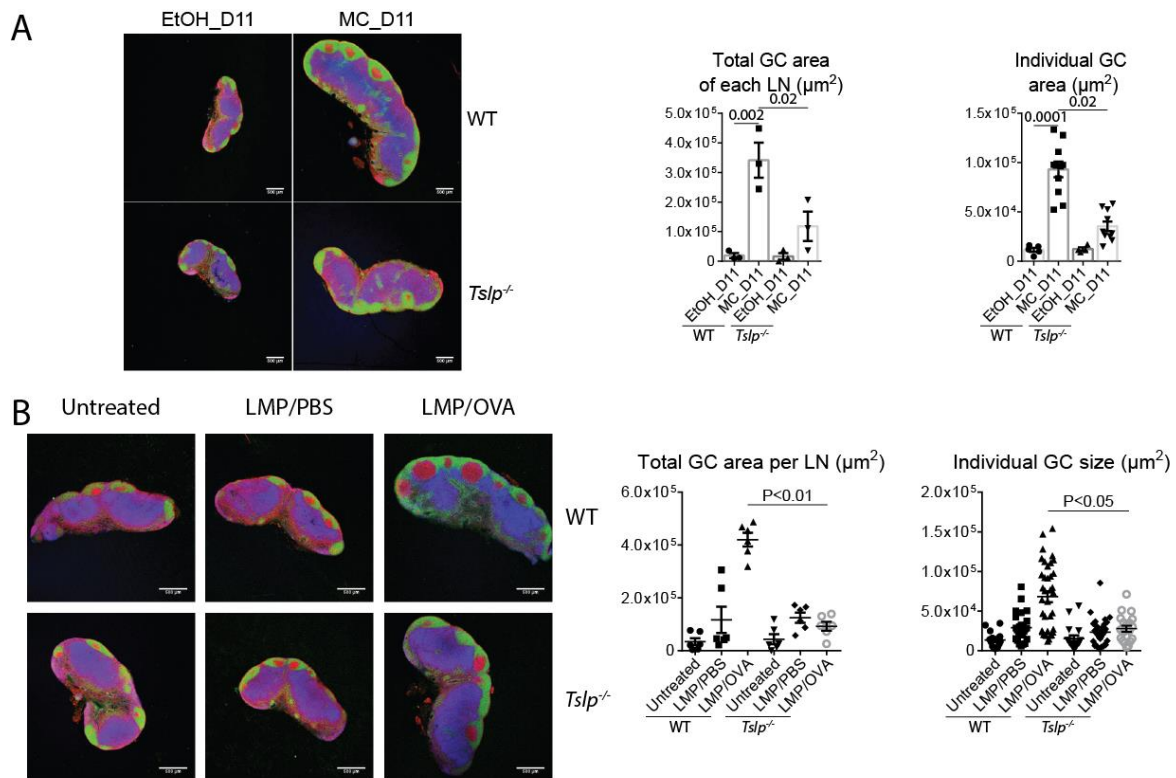
Figure S2: germinal center staining of MC903- and OVA-treated WT Balb/c and Tslp^{-/-} mice
Wildtype (WT) and Tslp-/- mice were treated with MC903 (A) or subjected to OVA-sensitization (B) as shown in figure 1A and 4B respectively. EDLN were collected and fixed overnight with 4% PFA at 4°C. After 2 times 30 minutes of wash in PBS at room temperature (RT), samples were included in 4% low melting point agarose in PBS. Vibratome sections of 100μm were blocked with 5% normal donkey serum (NDS), 0.1% Triton X-100 in PBS and then stained overnight at 4°C with anti CD4-AlexaFluor 647 (RM4-5, biolegend, d=1/100), anti IgD-FITC (11-26c.2a, BD Biosciences, d=1/50) and biotinylated PNA (réf B-1075, Vectorlabs, d=1/250) diluted in 5% NDS, 0.1% Triton X-100 in PBS. Sections were subsequently incubated 1h at RT with Neutravidin-Dylight550 (réf 84606, Thermofisher, d=1/200) diluted in PBS. After 2 washing of 30 minutes with PBS at RT, sections were kept at 4°C in PBS containing 1 ug/mL Hoechst 33342 (ref. B-2261, Sigma Aldrich) and images were acquired using Leica LSI confocal macroscope. Measurements were performed with ImageJ software (NIH).
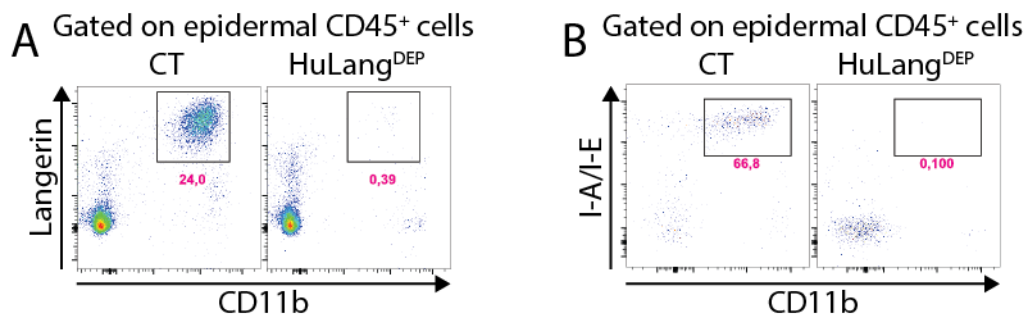
Figure S3: Langerhans cells are efficiently depleted in DT-injected HuLang^DTR mice.

Diphteria toxin (DT) was intraperitonally injected at D-2 and D0 and ear epidermal cells were analyzed at D2. Langerhans cells, gated either as langerin+ CD11b+ cells (A) or MHCII+ CD11b+ cells (B) among epidermal CD45+ cells, are efficiently depleted in DT-injected HuLang^DTR mice (HuLang^DEP). CT, DT-injected wildtype littermate controls.
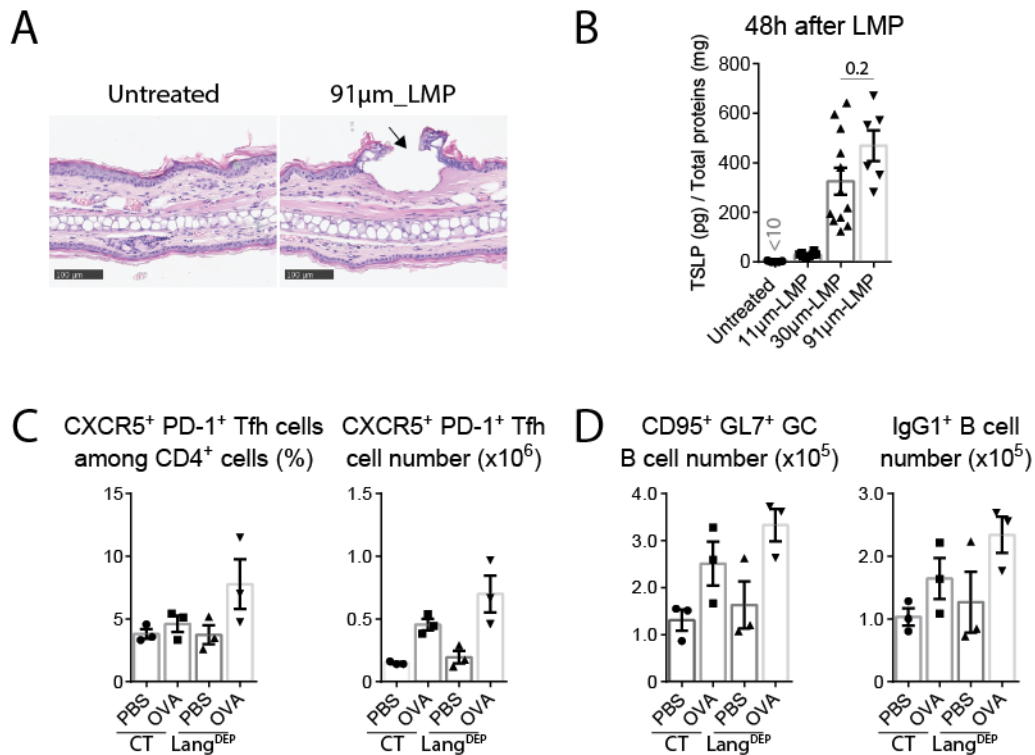
Figure S4: Tfh/GC response tends to be increased in Lang^DEP mice upon 91μm-LMP/OVA treatment compared with wildtype (WT) mice.

(A) Hematoxylin and eosin staining (HE) of untreated of 91μm-LMP/OVA ears of Balb/c WT mice. The arrow points to a micropore with the disruption of epidermis and partially dermis. Scale bar, 100μm. (B) TSLP protein levels in ears of WT mice at 48h after indicated treatment. (C) Frequency and number of CXCR5+ PD-1+ Tfh cells. (D) Number of CD95+ GL-7+ GC B cells and IgG1+ B cells in 91μm-LMP/PBS or 91μm-LMP/OVA-treated Lang^DEP or WT mice.
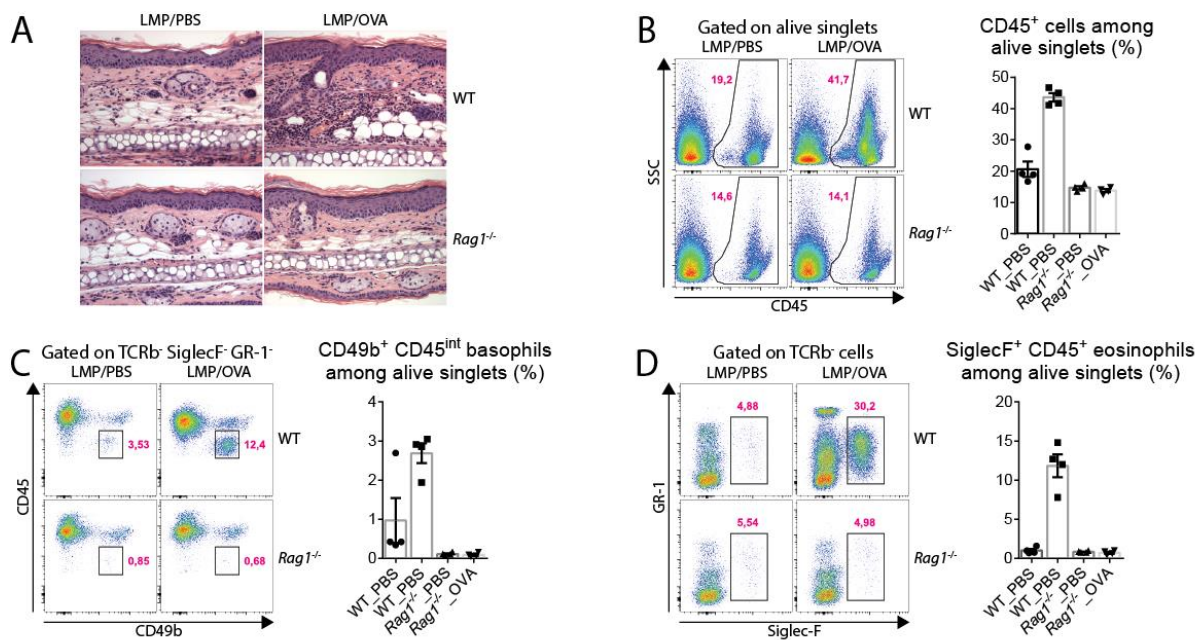
Figure S5: LMP/OVA-induced skin inflammation is dependant on adaptive immunity.
Balb/c wildtype (WT) or Rag1^{-/-} mice were treated with 30µm-LMP/PBS or 30µm-LMP/OVA as described in figure 4C. Ears were analyzed at D13. (A) Hematoxylin and eosin staining (HE) of 30µm-LMP/PBS or 30µm-LMP/OVA ears of Balb/c wildtype (WT) or Rag1^{-/-} mice. (B-D) Frequency of CD45^{+} cells (B), Basophils (C) and eosinophils (D) in dermal cells.
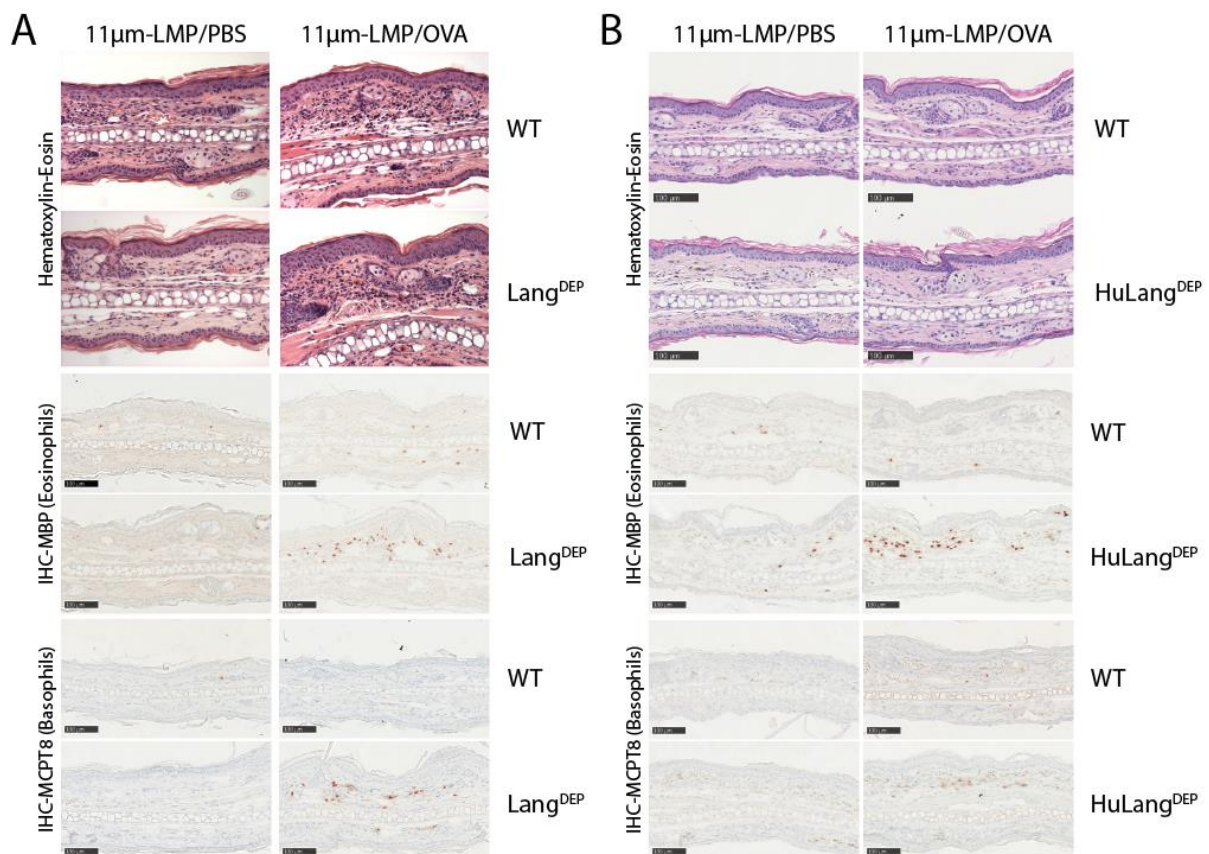
Figure S6: 11µm-LMP/OVA treatement induces a mild skin inflammation which is stronger after depletion of LCs.
Hematoxylin and eosin staining of ears from Lang^DEP (A top) and HuLang^DEP (B top) mice after 11µm-LMP/OVA treatment. Immunohistochimstry for MBP (eosinophils) and MCPT8 (basophils) of ears from Lang^DEP (A bottom) and HuLang^DEP (B bottom) mice after 11µm-LMP/OVA treatment. Scale bar, 100µm.
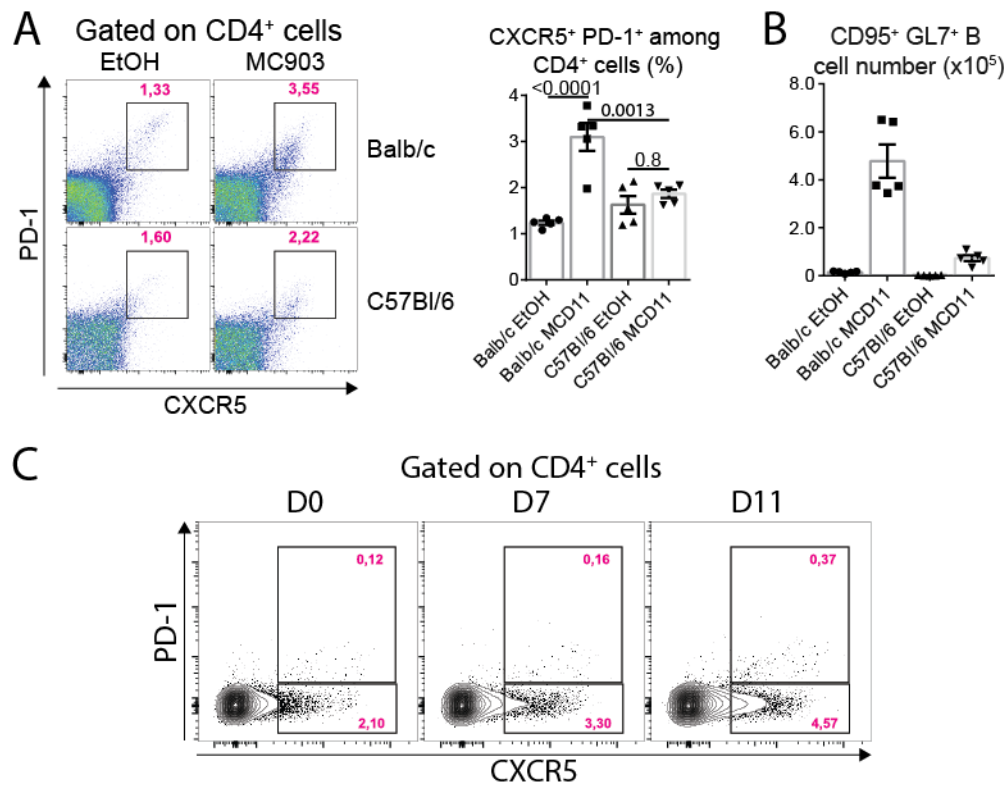
Figure S7: GC Tfh cell differentiation is defective in MC903-treated C57Bl/6 mice.

(A-B) Comparison of the frequency of CXCR5+ PD-1+ GC Tfh (A) and GC B cell number (B) at D11 upon MC903 treatment in Balb/c or C57Bl/6 mice. (C) Representative flow cytometry plot of CXCR5 and PD-1 staining in CD4+ cells from ear-draining lymph nodes of C57Bl/6 wildtype mice at D0, D7 or D11 uppon MC903 treatment as indicated in Figure 1A. Initial induction of CXCR5+ Tfh cells occurs in C57Bl/6 mice but the development of CXCR5+ PD-1+ GC Tfh is defective in MC903-treated C57Bl/6 mice.
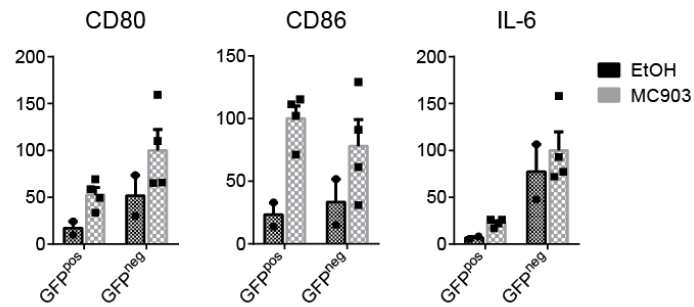
Figure S8: Expression of CD80, CD86 and IL-6 is induced in GFP^pos cells in EDLNs from MC903-treated Lang^GFP mice (in 96.8% Balb/c background).

Relative RNA levels of CD80, CD86 and IL-6 in migratory GFP^pos and GFP^neg DCs of EtOH-, MC903- or LMP/OVA-treated Lang^GFP mice are shown.
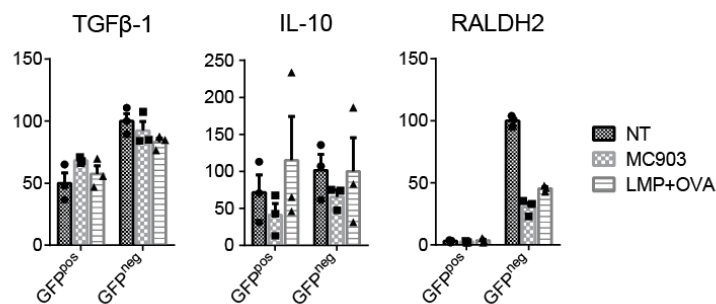
Figure S9: Expression of the regulatory genes is not upregulated in migratory GFP^pos cells in EDLNs from LMP/OVA-treated Lang^GFP mice.

Relative RNA levels of genes involved in regulatory pathways in migratory GFP^pos and GFP^neg migratory DCs from non-treated (NT), MC903- or LMP/OVA-treated Lang^GFP mice (on C57Bl/6J background).

PART II
Study of the TSLP-TSLPR-OX40L signaling axis in T helper cell differentiation in a mouse model of atopic dermatitis

MARSCHALL P.J. et al., (manuscript in preparation)

Introduction

With the initial aim to elucidate the requirement of the TSLP receptor (TSLPR) expressed by dendritic cells (DCs) in TSLP-driven Th2 cell differentiation, we generated a mouse line in which *Crlf2*, the TSLPR encoding gene, is selectively ablated in DCs (*Crlf2$^{DC-/-}$*). Unexpectedly, we found that, in addition to promote Th2 cell differentiation, TSLP signals through TSLPR expressed by DCs (TSLPR^{DC}) to induce the differentiation of ST2$^+$ Tregs in skin-draining lymph nodes (LNs). Interestingly, the differentiation of these cells was also dramatically diminished in our newly generated mice with the ablation of OX40L, a costimulatory molecule expressed in a subset of migratory DCs, thus suggesting that TSLP-TSLPR^{DC}-OX40L axis plays a previously unrecognized role in the induction of ST2$^+$ Tregs in skin-draining LNs.

Material and methods

Mice

C57Bl/6J mice were purchased from Charles River Laboratory. *Crlf2^{L-/L-}*, *Crlf2^{L2/L2}* and *Tnfsf4^{KI/+}* mice were all generated in C57Bl/6J background. *Tslp^{-/-}* mice (in C57Bl/6J background) were as previously reported (Li M., et al., 2009 ; J. *Invest. Dermatol.*). CD11c-Cre^{Tg/0} mice (in C57Bl/6J background) were purchased from Jax (ref 008068). Breeding and maintenance were performed under institutional guidelines, and all of the animal experiments were approved by the animal care and ethics committee of animal experimentation of the IGBMC.

MC903 topical application

MC903 (Calcipotriol, Sigma, Cat No. C4369) was dissolved in 100% ethanol and topically applied on mouse ears (2 nmol in 25 µl per ear) as previously described (Leyva-Castillo et al., 2013, Nat. Commun.).

Lymph node (LN) cells preparation for flow cytometry

For ear-draining lymph nodes (LN) preparation, LNs were dissociated with piston, passed through a 70µm strainer (Falcon) and resuspended in PBS containing 0.5% BSA and 2mM EDTA. LN cells were then centrifuged at 350g and resuspended in PBS containing 1% FCS and 2mM EDTA, counted and used for FACS staining. In case of preparation of LN cells for DC staining, ear-draining LNs were cut in two pieces and incubated 30 minutes at 37°C in 2mg/mL collagenase D (Roche), 0,25mg/mL DNase I (Sigma) and 2.5% FCS (Thermofisher) in PBS prior passing through the strainer as described above.

Surface staining for flow cytometry

To perform flow cytometry analysis, 2 million cells were used for antibody (Ab) staining. Cells were first incubated with anti-CD16/CD32 antibody to block unspecific binding, followed by surface markers staining with fluorochrome-conjugated antibodies (Table 1) in a 25µl of FACS buffer (1% of FCS + 2mM EDTA in PBS) for 10 minutes at 4°C. Viability staining was performed by adding propidium iodide to a final concentration of 1 µg/mL just prior passing the cells to the cytometer.

Intracellular staining

In case of intracellular staining for cytokines, 2 to 3 million of cells were stimulated *in vitro* at 37°C for 4 hours in IMDM containing PMA/Ionomycine (eBioscience, 500X stimulation cocktail #00-4970), GolgiSTOP (BD, #554714) and 10% FCS. Cells were then proceeded for surface staining as described above before intracellular staining.

For intracellular staining of FoxP3, GATA3 and cytokines, the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used according to the manufacturer's procedure. Briefly, after surface staining, cells were fixed and permeabilized by 30 minutes of incubation of ice in Foxp3 Fixation/Permeabilization working solution. After two washes with 1X Permeabilization Buffer using centrifugation for 5 minutes at 500g, cells were resuspended in 1X Permeabilization Buffer containing fluorochromes labeled antibodies against intracellular markers and incubated 30 minutes at room temperature. . After two additional washes with 1X Permeabilization Buffer using centrifugation for 5 minutes at 500g, cells were resuspended in PBS containing 1% FCS and 2mL of EDTA. Viability staining was performed by incubating the cells with Fixable Viability Dye 506 (eBioscience) for 30 minutes at 4°C prior to the surface staining.

Tableau 1: antibodies used for flow cytometry experiments

Marker	Fluorochrome	Clone	Reference	Company	Quantity
CD4	BV421	GK1.5		BD Biosciences	0.5 : 25
CD4	Alexa Fluor 700	RM-5		BD Biosciences	1 : 25
CD8a	PerCP-Cy5.5	53-6.7		eBioscience	0.5 : 25
ST2	FITC	DJ8		MDBioproducts	0.5 : 25
B220	APC	RA3-6B2		eBioscience	1.2 : 25
CD11c	Biotin	HL3		BD Biosciences	0.5 : 25
I-A/I-E	PE	M5/114.15.2		eBioscience	0.01 : 25
CD301b	eFluor660	11A10-B7		eBioscience	1.2 : 25
XCR1	FITC	ZET		Biolegend	2 : 25
FoxP3	APC	FJK-16s		eBioscience	1.5 : 100
FoxP3	Alexa Fluor 700	FJK-16s		eBioscience	1.2 : 100
GATA3	PE-Cy7	TWAJ		eBioscience	5 : 100
OX40	APC	OX86		eBioscience	1.2 : 25
IL-4	BV421	11B11		BD Biosciences	1 : 100
IL-13	PE	eBio13A		eBioscience	1.2 : 100

Statistics

Data were analysed using using GraphPrad Prism 6. Comparison of two samples was performed either by Student's two-tailed unpaired t-test with Welch's correction or the Mann–Whitney rank sum nonparametric test depending on results from the Kolmogorov–Smirnov test for normality. Comparison of more than two samples was performed by ordinary one-way ANOVA followed by Tukey's post-doc test.

Results

TSLP induces ST2⁺ CXCR5⁻ CD4⁺ T cells via TSLPR expressed by CD11c⁺ cells in EDLN of MC903-treated mice

To investigate the role of TSLPR expressed by dendritic cells (DC) in TSLP-induced Th2 differentiation, we generated a novel genetic mouse tool (in C57Bl/6J background) in which the exon 3 of *Crlf2*, the TSLPR-encoding gene, is flanked by LoxP sequences, thus allowing for conditional knock-out of Crlf2 gene (Figure 1A). Mice harboring the targeting construct were crossed with Flp-deleter mice (Birling et al., 2012) to generate *Crlf2*$^{L2/+}$ mice, which were either crossed with Cre-deleter line mice (Birling et al., 2011, Technology report) to generate *Crlf2*$^{L-/L-}$ (germline knockout) or with *Itgax*-Cre mice (Caton et al., 2007, J Exp Med) to generate CD11c-Cre$^{Tg/0}$/*Crlf2*$^{L2/L2}$ mice (named *Crlf2*$^{DC-/-}$) in which TSLPR is selectively ablated in CD11c-expressing cells, including dendritic cells (DCs).

We then subjected *Crlf2*$^{L-/L-}$ mice and their wildtype controls (CT) to MC903 treatment, which induces the overexpression of TSLP, every other day from D0 to D10 and ear-draining lymph nodes (EDLN) were analyzed by flow cytometry at D11 (Figure 1B). To identify Th2 cells, we used a gating strategy on the expression of the IL-33 receptor subunit ST2 and the chemokine receptor CXCR5 as previously described (Kobayashi, 2017, J Allergy Clin Immun), based on the fact that ST2 and CXCR5 are recognized as reliable markers for differentiated Th2 cells (Löhning, 1998, P Natl Acad Sci Usa) and Tfh cells (Crotty, 2014, Immunity) respectively. Results showed that MC903 treatment induces an increase of ST2⁺ CXCR5⁻ CD4⁺ cells in EDLN of CT mice at D11 (Figure 1C), which were abrogated in *Crlf2*$^{L-/L-}$ mice (Figure 1C). Similar results were obtained from MC903-treated *Tslp*$^{-/-}$ mice (Figure 1D), indicating that TSLP-TSLPR signaling induces ST2⁺ CXCR5⁻ CD4⁺ cells in MC903-treated mice. We then examined these cells in MC903-treated *Crlf2*$^{DC-/-}$ mice. Again, we observed that the induction of ST2⁺ CXCR5⁻ CD4⁺ cells was abrogated in *Crlf2*$^{DC-/-}$ mice, similar to what was observed in *Crlf2*$^{L-/L-}$ and *Tslp*$^{-/-}$ mice (Figure 1E). Together, these results led us to conclude that TSLPR expressed by CD11c⁺ cells mediates the TSLP-induced ST2⁺ Th2 cell differentiation.

TSLP signaling through TSLPR expressed by CD11c⁺ cells induces ST2⁺ cells that express FoxP3, CD25, GATA3 and OX40, resembling to ST2⁺ Tregs

Even though the above conclusion appears to keep in line with the recognized role of TSLP in driving Th2 cell differentiation, we were frustrated with the observation that the ST2$^+$ CD4$^+$ cells produced no or very little Th2 cytokine IL-13 or IL-4, as assessed by intracellular stainings (Figure 2A). Indeed, results showed that IL-13 and IL-4 were mainly produced by ST2$^-$ CD4$^+$ cells, which calls into question the Th2 nature of the MC903-induced ST2$^+$ CD4$^+$ cells and the use of ST2 as a reliable marker to identify Th2 cells.

Recently, a new subset of T regulatory cells (Tregs) expressing ST2 as well as FoxP3, CD25, GATA3 and OX40 was described in mouse colon (Schiering et al., 2014, Nature). Further studies have shown that these cells exhibit higher regulatory function compared to ST2$^-$ Tregs *in vitro* (Matta et al., 2014, Journal Immunol; Siede et al., 2016, PLoS One). We then sought to examine whether MC903-induced ST2$^+$ CD4$^+$ cells in EDLN could be ST2$^+$ Tregs. Intracellular staining of FoxP3 showed that a majority of ST2$^+$ cells express FoxP3 and that the frequency of ST2$^+$ FoxP3$^+$ cells in CD4$^+$ cells was increased after MC903 treatment in wildtype control (CT) mice (compare CT, EtOH and MC903) (Figure 2B). In contrast, frequencies of ST2$^+$ FoxP3$^-$ cells or ST2$^-$ FoxP3$^+$ cells were not changed (Figure 2B). Moreover, such induction of ST2$^+$ FoxP3$^+$ observed in CT mice was abolished in *Crlf2$^{L-/L-}$* and *Tslp$^{-/-}$* mice, and largely abrogated in *Crlf2$^{DC-/-}$* mice (Figure 2B), suggesting a crucial role of the TSLP-TSLPR^{DC} axis in the induction of ST2$^+$ FoxP3$^+$ cells in EDLNs of MC903-treated mice.

Further characterization of ST2$^+$ FoxP3$^+$ cells showed that they express CD25 in a comparable level to that of ST2$^-$ Foxp3$^+$ cells (Figure 2C), reinforcing the hypothesis that these ST2$^+$ FoxP3$^+$ T cells induced by MC903 treatment are *bona fide* Tregs. In addition, ST2$^+$ FoxP3$^+$ cells express GATA3 and OX40, exhibited the highest median fluorescence intensity (MFI) compared with ST2$^+$ FoxP3$^-$, ST2$^-$ FoxP3$^+$ and ST2$^-$ FoxP3$^-$ cell populations (Figure 2C). Taken together, our analyses denied ST2$^+$ cells as a reliable marker for Th2 cells. Instead, our results indicated that TSLP-TSLPR^{DC} signaling induces ST2$^+$ FoxP3$^+$ CD25$^+$ GATA3$^+$ OX40$^+$ CD4$^+$ cells that resemble ST2$^+$ Tregs reported in the gut.

MC903-treated *Crlf2$^{DC-/-}$* mice exhibit a decrease of GATA3$^+$ FoxP3$^-$ Th2 cells

As the MC903-induced Tregs express both FoxP3 and GATA3, we then wondered whether cells expressing GATA3 but not FoxP3 (GATA3$^+$ FoxP3$^-$) could be the Th2 cells induced in MC903

model. Our analyses showed that in EDLNs of MC903-treated wildtype control (CT) mice, GATA3$^+$ FoxP3$^-$ but not GATA3$^+$ FoxP3$^+$, GATA3$^-$ FoxP3$^+$ or GATA3$^-$ FoxP3$^-$ cells produced IL-13 and IL-4 (Figure 3A), and moreover, they were the main producers of these cytokines (Figure 3B), suggesting that GATA3$^+$ FoxP3$^-$ cells represent the *bona fide* Th2 cells.

We next compared GATA3$^+$ FoxP3$^+$ (ST2$^+$ Tregs) and GATA3$^+$ FoxP3$^-$ (Th2) cells in MC903-treated CT, *Crlf2$^{L-/L-}$*, *Tslp$^{-/-}$* and *Crlf2$^{DC-/-}$* mice. Results showed that both populations were induced in CT mice upon MC903 treatment (Figure 4A), and were abrogated in *Crlf2$^{L-/L-}$* and *Tslp$^{-/-}$* mice and largely diminished in *Crlf2$^{DC-/-}$* mice (Figure 4A), suggesting that TSLP-TSLPR^{DC} promotes both ST2$^+$ Tregs and Th2 cells. Furthermore, we examined the Th2 cytokine expression by intracellular staining for IL-13 and IL-4. Interestingly, results indicated that the expression of IL-13 and IL-4 was abolished in *Crlf2$^{L-/L-}$* and *Tslp$^{-/-}$* mice; however, it was not significantly reduced in *Crlf2$^{DC-/-}$* mice (Figure 4B and 4C). These results thus indicate that, in mice lacking TSLPR in DCs, the MC903-induced differentiation of GATA3$^+$ FoxP3$^-$ Th2 cells is reduced, but the Th2 cytokine expression appears to be maintained.

Generation of a novel genetic mouse tool for studying the expression and function of OX40L

As reported in the gut, ST2$^+$ Tregs induced in EDLN by MC903 expressed a high level of OX40 (Figure 2C, 3A), raising an interesting question on OX40L-OX40 axis in the differentiation and maintenance of these cells. To explore this question, we generated a new mouse tool based on the Eucomm "knock-out first" design (Skarnes et al., 2011, Nature), in which a gene trap cassette encoding the blue fluorescent protein (mTagBFP; hereafter called BFP) was inserted in the intron between exon 2 and 3 of OX40L-encoding gene Tnfsf4 (Figure 5A).The allele containing the targeting construct was named as knock-in (KI). We generated *Tnfsf4$^{KI/+}$* mice which allow us to identify and track OX40L (BFP)-expressing cells, and *Tnfsf4$^{KI/KI}$* mice which serve as knock-out mice lacking OX40L. To validate these mice, splenocytes from *Tnfsf4$^{+/+}$*, *Tnfsf4$^{KI/+}$* and *Tnfsf4$^{KI/KI}$* mice were *in* vitro stimulated with anti-CD40 and anti-IgM to induce a high expression of OX40L (Murata, 2000, J Exp Med) and stained with anti-OX40L antibody. As expected, flow cytometry analysis of B cells from *Tnfsf4$^{+/+}$* mice exhibited OX40L$^+$ signal but no BFP signal, and B cells from *Tnfsf4$^{KI/+}$* mice exhibited OX40L$^+$ BFP$^+$ double positive signal, whereas B cells from *Tnfsf4$^{KI/KI}$* mice exhibited only BFP$^+$ but not OX40L$^+$ signal (Figure 5B).

These analyses thus validated *Tnfsf4$^{KI/+}$* as a faithful OX40L-reporter line and *Tnfsf4$^{KI/KI}$* as an OX40L-ablated line.

MC903 treatment induces OX40L expression in a subset of migratory DCs

In order to identify OX40L-expressing cells, *Tnfsf4$^{KI/+}$* mice were treated with EtOH or MC903, and ear-draining lymph nodes (EDLNs) were analyzed at D5 (Figure 6A). BFP (OX40L) signal was minimally detected in CD3$^+$ T cells, B220$^+$ B cells or CD11c^{hi} I-A/I-E^{int} resident DCs, which all remained unchanged between MC903- and EtOH-treated mice (Figure 6B and 6C). Most of BFP$^+$ cells detected in EDLN from EtOH-treated *Tnfsf4$^{KI/+}$* mice were inside I-A/I-E^{hi} CD11c^{int} migratory (mig) DCs. Importantly, their frequencies as well as median fluorescence intensity (MFI) were further increased upon MC903 treatment (Figure 6B and 6C).

Further analyses revealed that among the BFP$^+$ mig DCs in EDLN from MC903-treated *Tnfsf4$^{KI/+}$* mice, the majority (80%) were CD301b$^-$ XCR1$^-$, expressing neither CD301b (which was reporter as a marker of Th2-promoting DCs; Kumamoto, 2013, Immunity), nor XCR1 (marker of cDC1s; Crozat et al., 2011, Journal Immunol; Guilliams et al., 2016, Immunity) (Figure 6D and 6E). Comparison of BFP$^+$ cells in migDCs in EDLNs from EtOH- or MC903-treated *Tnfsf4$^{KI/+}$* mice showed that their frequencies and MFI were both increased in CD301b$^-$ XCR1$^-$ and CD301b$^+$ cells, but not in XCR1$^+$ DCs (Figure 6F). Of note, RT-qPCR analyses of LangGFP-positive and LangGFP-negative migDCs sorted from EtOH- or MC903-treated Lang^{GFP} mice (Kissenpfennig, 2005, Immunity) showed that OX40L RNA was expressed by langerin$^-$ migDCs and its level was further induced by MC903 treatment, but it was not expressed nor induced in langerin$^+$ cells (including LCs and XCR1$^+$ cells) (Figure 6G). Together, these data indicate that MC903 treatment induces OX40L expression in a subset of langerin$^-$ migratory DCs.

Mice lacking OX40L exhibit diminished ST2$^+$ Tregs and elevated GATA3$^+$ FoxP3$^-$ Th2 cells upon MC903 treatment

We next subjected *Tnfsf4$^{KI/KI}$* mice which are deficient for OX40L to MC903 treatment as described in figure 1B. The ST2/CXCR5 staining showed that the induction ST2$^+$ CXCR5$^-$ CD4$^+$ cells by MC903 was abrogated in *Tnfsf4$^{KI/KI}$* mice (Figure 7A); the ST2/FoxP3 staining and the GATA3/FoxP3 staining showed that the induction of ST2$^+$ FoxP3$^+$ and GATA3$^+$ FoxP3$^+$ cells was abrogated in the absence of OX40L, respectively (Figure 7B and 7C). All together, these data

indicate that the development of MC903-induced ST2$^+$ Tregs was abrogated in *Tnfsf4$^{KI/KI}$* mice, similar to what was observed in *Crlf2$^{DC-/-}$* mice, thus suggesting that the TSLP-TSLPR^{DC}-OX40L axis plays a crucial role in the induction of ST2$^+$ Tregs.

On the other hand, we observed that, in contrast to *Crlf2$^{DC-/-}$* mice, MC903-treated *Tnfsf4$^{KI/KI}$* did not exhibit a reduction of GATA3$^+$ FoxP3$^-$ (Th2 cells); instead, the frequency of these cells was rather increased compared to MC903-treated CT mice (Figure 7C). In addition, the expression of IL-13 and IL-4 in CD4$^+$ cells was also increased (Figure 7D). Taken together, these data indicate that the absence of OX40L leads to an abrogation of ST2$^+$ Treg cells accompanied by an enhancement of Th2 cells in MC903-treated mice.

Discussion and Perspectives

In this study, using topical MC903 treatment to induce the overexpression of TSLP in mouse skin epidermal keratinocytes, combined with $Tslp^{-/-}$ and our newly generated $Crlf2^{L-/L-}$ and $Crlf2^{DC-/-}$ mice, we demonstrated that TSLP signals through TSLPR expressed by CD11c$^+$ cells (TSLPR^{DC}) to induce the differentiation of ST2$^+$CXCR5$^-$ CD4$^+$ cells in skin draining LNs. Further characterization showed that these cells were not Th2 cells, in contrary to what was previously reported; instead, they express FoxP3, CD25, GATA3 and OX40, resembling ST2$^+$ Tregs recently described in the mouse gut. Additionally, we identified GATA3$^+$ FoxP3$^-$ as *bona fide* Th2 cells producing Th2 cytokines IL-13 and IL-4, and showed that these cells were also diminished in $Crlf2^{DC-/-}$ mice. Moreover, employing a new OX40L-BFP reporter ($Tnfsf4^{KI/+}$) mouse line, we identified that OX40L was expressed at steady state in a subset of migratory DCs in skin draining LNs and its expression was further induced when TSLP was overexpressed. Finally, by generating $Tnfsf4^{KI/KI}$ mice in which OX40L is ablated, we showed that TSLP-induced ST2$^+$ Tregs were diminished in these mice, accompanied by an increased differentiation of GATA3$^+$ FoxP3$^-$ Th2 cells with a higher expression of IL-13 and IL-4. Together, our data suggest that TSLP-TSLPR^{DC}-OX40L axis plays a crucial role in the induction of ST2$^+$ Tregs in skin-draining LNs, whereas signal axis other than OX40L-OX40 mediate the Th2 differentiation triggered by TSLP.

One limit of our study is that $Tnfsf4^{KI/KI}$ is a germline knock-out mouse line, thus not providing a direct proof on the function of OX40L expressed by DCs in promoting ST2$^+$ Tregs in response to TSLP signaling. To demonstrate the critical importance of TSLP-TSLPR^{DC}-OX40L^{DC} axis for ST2$^+$ Tregs, we are under the way to generate a DC-selective knockout of OX40L ($Tnfsf4^{DC-/-}$) by breeding mice bearing a floxed allele of OX40L with CD11c-Cre mice (Caton et al., 2007, J Exp Med). In addition, to avoid the developmental defects from the constitutive knockout, floxed OX40L mice can be bred with Rosa26-CreER^{T2} mice (Ventura et al., 2007, Nature) (as CD11c-CreER^{T2} are not available), which allows us to generate an inducible knockout of OX40L to study its function at the time of TSLP-induced AD. Moreover, *in vitro* coculture of BFP$^+$ DCs from MC903-treated $Tnfsf4^{KI/+}$ mice (OX40L-sufficient) and $Tnfsf4^{KI/KI}$ (OX40L-deficient) mice with CD4$^+$ T cells will enable us to see whether $Tnfsf4^{KI/+}$ but not $Tnfsf4^{KI/KI}$ migDCs induce the differentiation of ST2$^+$ Tregs.

Our data show that TSLP induces selectively ST2$^+$ Tregs but not ST2$^-$ Tregs. Unlike the ST2$^+$ Tregs, MC903 treatment on wildtype mice does not result in any change in the frequency of ST2$^-$ Tregs; moreover, wildtype, *Tslp$^{-/-}$*, *Crlf2$^{L-/L-}$* or *Crlf2$^{DC-/-}$* mice all present similar frequencies of these cells (Figure 2B). However, *Tnfsf4$^{KI/KI}$* mice exhibit a decrease of ST2$^-$ Tregs in steady state. Indeed, OX40 is constitutively expressed by ST2$^-$ Tregs, although the highest expression is observed in the MC903-induced ST2$^+$ Tregs. Therefore, it is possible that OX40L-expressing DCs in steady state are implicated in the development and/or expansion of ST2$^-$ Tregs, but this is TSLP-independent. On the other hand, the TSLP-induced OX40L^{hi} DCs may be responsible for the differentiation and/or expansion of ST2$^+$ Treg. It will be interesting to further explore the differences of OX40L (BFP)-expressing migratory DCs from steady state and MC903-treated mice by performing single cell mRNA sequencing. This may lead us to identify molecular signatures of OX40L^{hi} DCs for a better understanding of their function in promoting ST2$^+$ Tregs.

Also importantly, our data suggest that TSLP-TSLPR-induced Th2 response does not appear to implicate OX40L, despite previous report suggesting a role of OX40L in the initiation of Th2 cell differentiation and IL-4 production (Flynn et al., 1998, J Exp Med; Ohshima et al., 1998, Blood; So et al., 2006, Proc Natl Acad Sci USA). Indeed, while GATA3$^+$ FoxP3$^-$ Th2 cell differentiation and production of IL-13 and IL-4 are abolished in MC903-treated *Tslp$^{-/-}$* and *Crlf2$^{L-/L-}$* mice, they are found to be rather increased in *Tnfsf4$^{KI/KI}$* mice which lack OX40L. On the other hand, *Crlf2$^{DC-/-}$* mice exhibit a partial reduction of GATA3$^+$ FoxP3$^-$ (Th2 cells; Figure 4A). This suggest that other signals than OX40L-OX40 are likely implicated in Th2 differentiation, but these remain to be identified. Moreover, it is somewhat surprising that *Crlf2$^{DC-/-}$* mice present a reduction of GATA3$^+$ FoxP3$^-$ Th2 cells, but their Th2 cytokine expression appears to be maintained (Figure 4). This suggests that, either the expression of IL-13 and IL-4 in Th2 cells could be mediated by TSLPR on other cells than DCs (e.g. basophils, ILCs), or such observation reflects the output of decreased Th2 and decreased ST2$^+$ Tregs (which may repress the production of Th2 cytokines).

Previous studies have described a strong suppressive capacity of ST2$^+$ Tregs, even superior than that of ST2$^-$ Tregs (Siede et al., 2016, PLoS One). In MC903-treated *Tnfsf4$^{KI/KI}$* mice, we observed higher GATA3$^+$ FoxP3$^-$ Th2 cells and cytokine production. This could be due to the

reduced Tregs, but as both ST2^{+} Tregs and ST2^{-} Tregs were lower in these mice, it remains to be demonstrated whether the decrease in ST2^{+} Tregs is responsible for the observed increase in Th2 reponse. To explore the function of TSLP-induced ST2^{+} Tregs, it will be interesting to perform *in vitro* proliferation suppression assay (Collison, 2011, Methods Mol Biol) to compare the regulatory function of ST2^{+} and ST2^{-} Tregs sorted from skin-draining lymph nodes of MC903-treated mice. In addition, we should examine the expression of IL-10 and TGFβ, which have been recently shown to mediate the suppressive function of ST2^{+} Tregs *in vitro* (Siede et al., 2016, PLoS One).

Another interesting question is about the plasticity of these cells. It has been shown that ST2^{+} Tregs may have the capacity to produce Th2 cytokines upon IL-33 stimulation (Siede et al., 2016, PLoS One). Moreover, it was recently shown that, in patients with systemic sclerosis, ST2^{+} Tregs express IL-13 and IL-4 in skin, but not in the blood (MacDonald, 2015, J Allergy Clin Immun). Our analyses showed that IL-13 and IL-4 were not expressed by ST2^{+} Tregs in skin-draining LNs from MC903-treated mice, but it remains to be investigated whether these cells migrate to the inflamed skin, and whether they could produce Th2 cytokines, particularly when IL-33 is present in the lesional skin.

Finally, it will be important to explore the relevance of our findings, by using an allergic AD mouse model induced by skin allergen sensitization (e.g. LMP/OVA model in the Part I of my thesis). In addition, it will be necessary to validate the TSLP-OX40L induction of ST2^{+} FoxP3^{+} Tregs in human, by performing coculture experiments of human DCs and T cells, and to examine the presence of ST2^{+} Tregs in human AD compared to healthy skin, as well as their correlation with TSLP and OX40L expression.

References

Birling, M.-C., Dierich, A., Jacquot, S., Hérault, Y., and Pavlovic, G. (2012). Highly-efficient, fluorescent, locus directed cre and FlpO deleter mice on a pure C57BL/6N genetic background. Genesis *50*, 482–489.

Caton, M.L., Smith-Raska, M.R., and Reizis, B. (2007). Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. J. Exp. Med. *204*, 1653–1664.

Collison, L.W., and Vignali, D.A.A. (2011). In vitro Treg suppression assays. Methods Mol. Biol. *707*, 21–37.

Crotty, S. (2014). T follicular helper cell differentiation, function, and roles in disease. Immunity *41*, 529–542.

Crozat, K., Tamoutounour, S., Manh, T.-P.V., Fossum, E., Luche, H., Ardouin, L., Guilliams, M., Azukizawa, H., Bogen, B., Malissen, B., et al. (2011). Cutting Edge: Expression of XCR1 Defines Mouse Lymphoid-Tissue Resident and Migratory Dendritic Cells of the CD8α+ Type. The Journal of Immunology *187*, 4411–4415.

Flynn, S., Toellner, K.M., Raykundalia, C., Goodall, M., and Lane, P. (1998). CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blr-1. J. Exp. Med. *188*, 297–304.

Guilliams, M., Dutertre, C.-A., Scott, C.L., McGovern, N., Sichien, D., Chakarov, S., Van Gassen, S., Chen, J., Poidinger, M., De Prijck, S., et al. (2016). Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. Immunity *45*, 669–684.

Kissenpfennig, A., Henri, S., Dubois, B., Laplace-Builhé, C., Perrin, P., Romani, N., Tripp, C.H., Douillard, P., Leserman, L., Kaiserlian, D., et al. (2005). Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity *22*, 643–654.

Kobayashi, T., Iijima, K., Dent, A.L., and Kita, H. (2017). Follicular helper T cells mediate IgE antibody response to airborne allergens. J. Allergy Clin. Immunol. *139*, 300-313.e7.

Kumamoto, Y., Linehan, M., Weinstein, J.S., Laidlaw, B.J., Craft, J.E., and Iwasaki, A. (2013). CD301b+ dermal dendritic cells drive T helper 2 cell-mediated immunity. Immunity *39*, 733–743.

Leyva-Castillo, J.M., Hener, P., Michea, P., Karasuyama, H., Chan, S., Soumelis, V., and Li, M. (2013). Skin thymic stromal lymphopoietin initiates Th2 responses through an orchestrated immune cascade. Nature Communications *4*, 2847.

Li, M., Hener, P., Zhang, Z., Ganti, K.P., Metzger, D., and Chambon, P. (2009). Induction of thymic stromal lymphopoietin expression in keratinocytes is necessary for generating an atopic dermatitis upon application of the active vitamin D3 analogue MC903 on mouse skin. J. Invest. Dermatol. *129*, 498–502.

Löhning, M., Stroehmann, A., Coyle, A.J., Grogan, J.L., Lin, S., Gutierrez-Ramos, J.-C., Levinson, D., Radbruch, A., and Kamradt, T. (1998). T1/ST2 is preferentially expressed on

murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. PNAS *95*, 6930–6935.

MacDonald, K.G., Dawson, N.A.J., Huang, Q., Dunne, J.V., Levings, M.K., and Broady, R. (2015). Regulatory T cells produce profibrotic cytokines in the skin of patients with systemic sclerosis. J. Allergy Clin. Immunol. *135*, 946-.e9.

Matta, B.M., Lott, J.M., Mathews, L.R., Liu, Q., Rosborough, B.R., Blazar, B.R., and Turnquist, H.R. (2014). IL-33 Is an Unconventional Alarmin That Stimulates IL-2 Secretion by Dendritic Cells To Selectively Expand IL-33R/ST2+ Regulatory T Cells. The Journal of Immunology 1400481.

Murata, K., Ishii, N., Takano, H., Miura, S., Ndhlovu, L.C., Nose, M., Noda, T., and Sugamura, K. (2000). Impairment of Antigen-Presenting Cell Function in Mice Lacking Expression of Ox40 Ligand. Journal of Experimental Medicine *191*, 365–374.

Ohshima, Y., Yang, L.P., Uchiyama, T., Tanaka, Y., Baum, P., Sergerie, M., Hermann, P., and Delespesse, G. (1998). OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. Blood *92*, 3338–3345.

Schiering, C., Krausgruber, T., Chomka, A., Fröhlich, A., Adelmann, K., Wohlfert, E.A., Pott, J., Griseri, T., Bollrath, J., Hegazy, A.N., et al. (2014). The Alarmin IL-33 Promotes Regulatory T Cell Function in the Intestine. Nature *513*, 564–568.

Siede, J., Fröhlich, A., Datsi, A., Hegazy, A.N., Varga, D.V., Holecska, V., Saito, H., Nakae, S., and Löhning, M. (2016). IL-33 Receptor-Expressing Regulatory T Cells Are Highly Activated, Th2 Biased and Suppress CD4 T Cell Proliferation through IL-10 and TGFβ Release. PLOS ONE *11*, e0161507.

Skarnes, W.C., Rosen, B., West, A.P., Koutsourakis, M., Bushell, W., Iyer, V., Mujica, A.O., Thomas, M., Harrow, J., Cox, T., et al. (2011). A conditional knockout resource for the genome-wide study of mouse gene function. Nature *474*, 337–342.

So, T., Song, J., Sugie, K., Altman, A., and Croft, M. (2006). Signals from OX40 regulate nuclear factor of activated T cells c1 and T cell helper 2 lineage commitment. Proc. Natl. Acad. Sci. U.S.A. *103*, 3740–3745.

Ventura, A., Kirsch, D.G., McLaughlin, M.E., Tuveson, D.A., Grimm, J., Lintault, L., Newman, J., Reczek, E.E., Weissleder, R., and Jacks, T. (2007). Restoration of p53 function leads to tumour regression in vivo. Nature *445*, 661–665.
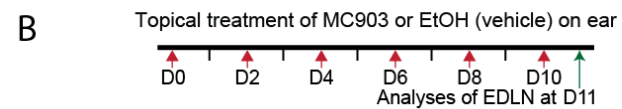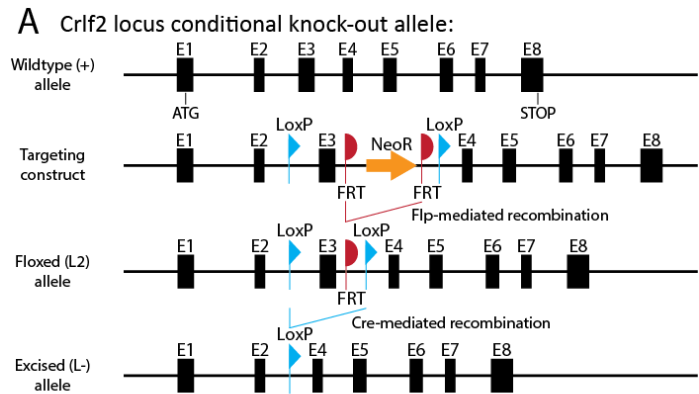
Figure 1: TSLP induces ST2⁺ CXCR5⁻ CD4⁺ T cells via TSLPR expressed by DCs in EDLN of MC903-treated mice.

(A) Schematic drawing of the wild-type allele (+) of the Crlf2 locus coding for TSPLR, the targeting construct (containing the FRT-flanked neomycin resistance gene (NeoR)), the floxed (L2) allele after FLP-mediated excision of Neo, and the excised (L-) allele after Cre-mediated excision of exon 3. Black boxes stand for exons. LoxP and FRT sites are indicated. (B) Experimental protocol. Mouse ears were topically treated with MC903 or ethanol (EtOH; as vehicle control) every other day from day (D) 0 to D10 and ear-draining lymph nodes (EDLN) were analyzed at D11. (C-E) Frequency and number of ST2⁺ CXCR5⁻ CD4⁺ cells in MC903- and EtOH-treated *Crlf2⁻/⁻* (C), *Tslp⁻/⁻* (D) or *Crlf2^DC-/-* (E).
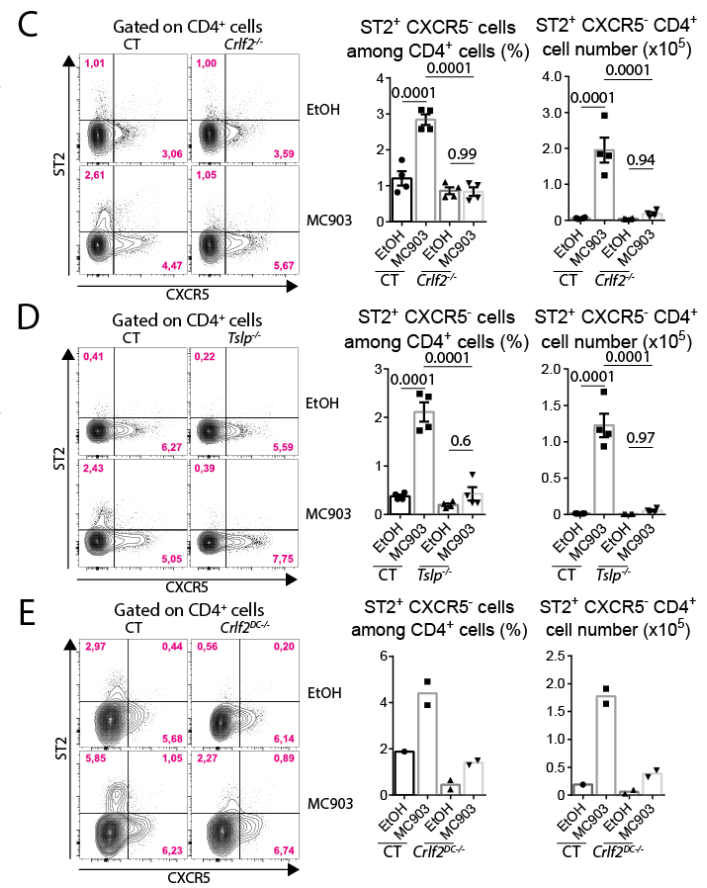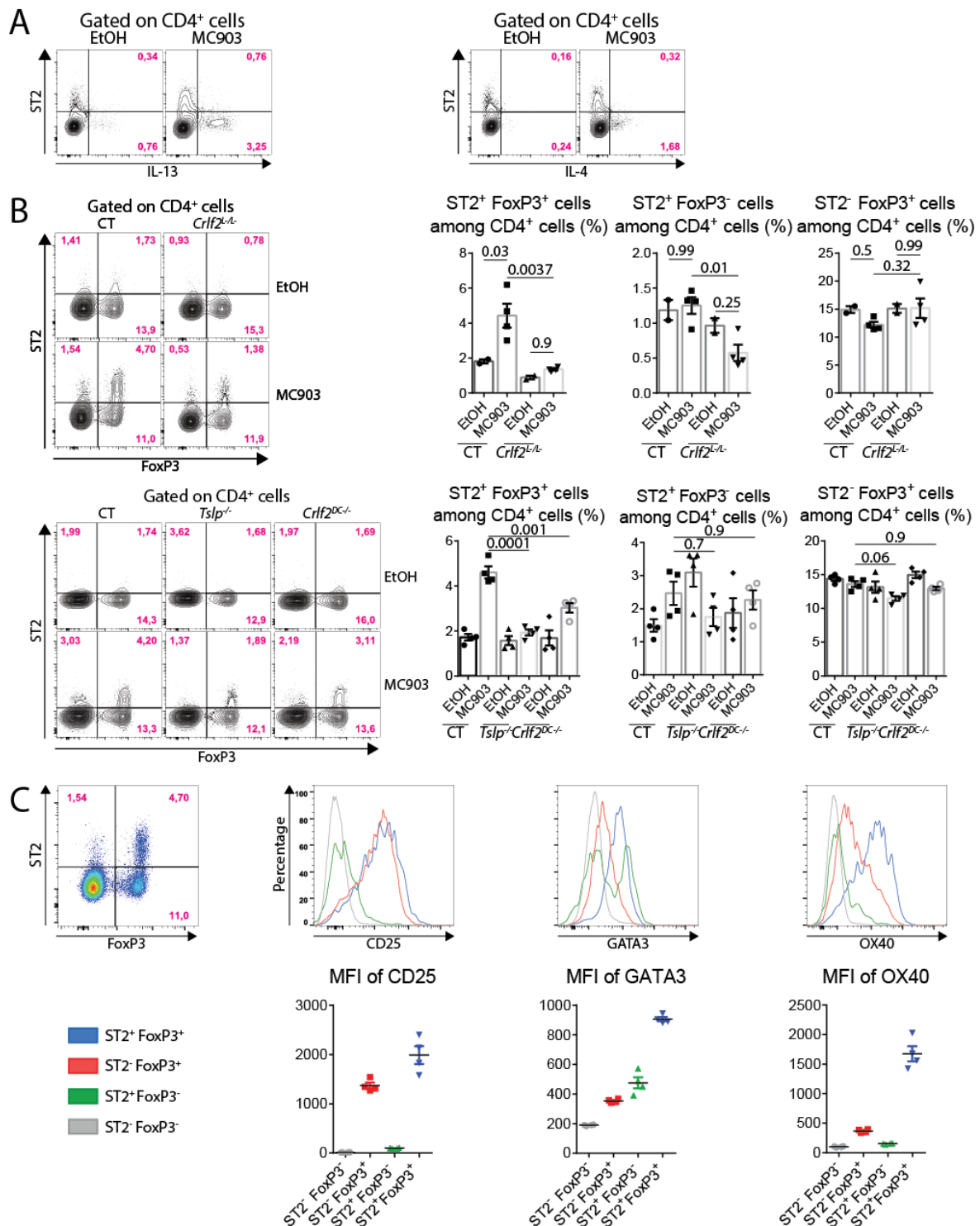
Figure 2: TSLP signaling through TSLPR on DCs induces ST2+ cells that express FOXP3, CD25, GATA3 and OX40, resembling to ST2+ Tregs.
(A) Flow cytometry plots of IL-13 (left) or IL-4 (right) intracellular staining and ST2 surface staining among CD4+ cells in ear-draining lymph nodes from wildtype EtOH- or MC903-treated wildtype C57Bl/6 mice at D11. (B) Frequency of ST2+ FoxP3+, ST2+ FoxP3- and ST2- FoxP3+ cells in CD4+ cells of wildtype (CT), *Crlf2L-/L-*, *Tslp-/-* or *Crlf2DC-/-* mice. (C) Phenotypic analysis of ST2+ FoxP3+, ST2+ FoxP3-, ST2- FoxP3+ and ST2- FoxP3- cells. Representative histograms and the median fluorescence intensity (MFI) for CD25, GATA3 and OX40.
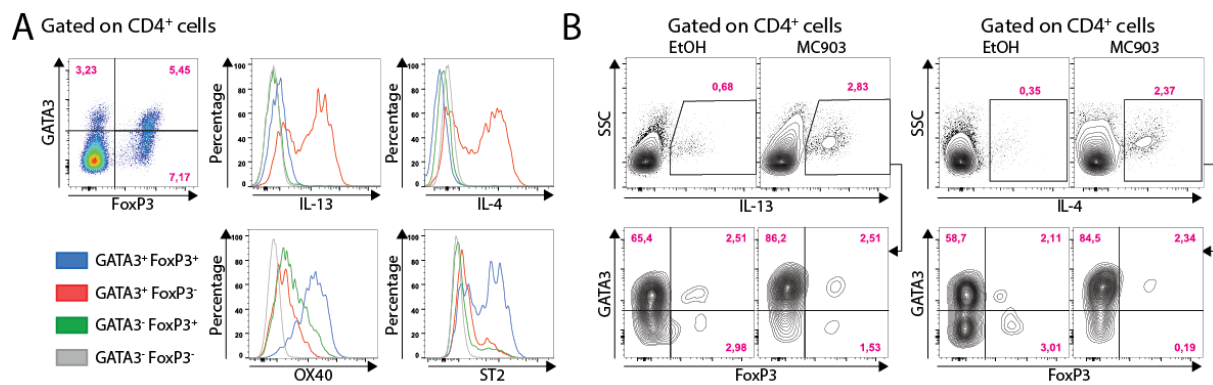
Figure 3: MC903-induced GATA3⁺ FoxP3⁻ cells but not GATA3⁺ FoxP3⁺ cells are Th2 cytokines producers.
(A) Representative histograms of flow cytometry showing the intensity of IL-13, IL-4 and OX40 expression in the indicated population of CD4⁺ cells. (B) Representative flow cytometry plots showing the expression of FoxP3 and GATA in IL-13 (left) or IL-4 (right) expressing CD4⁺ cells.
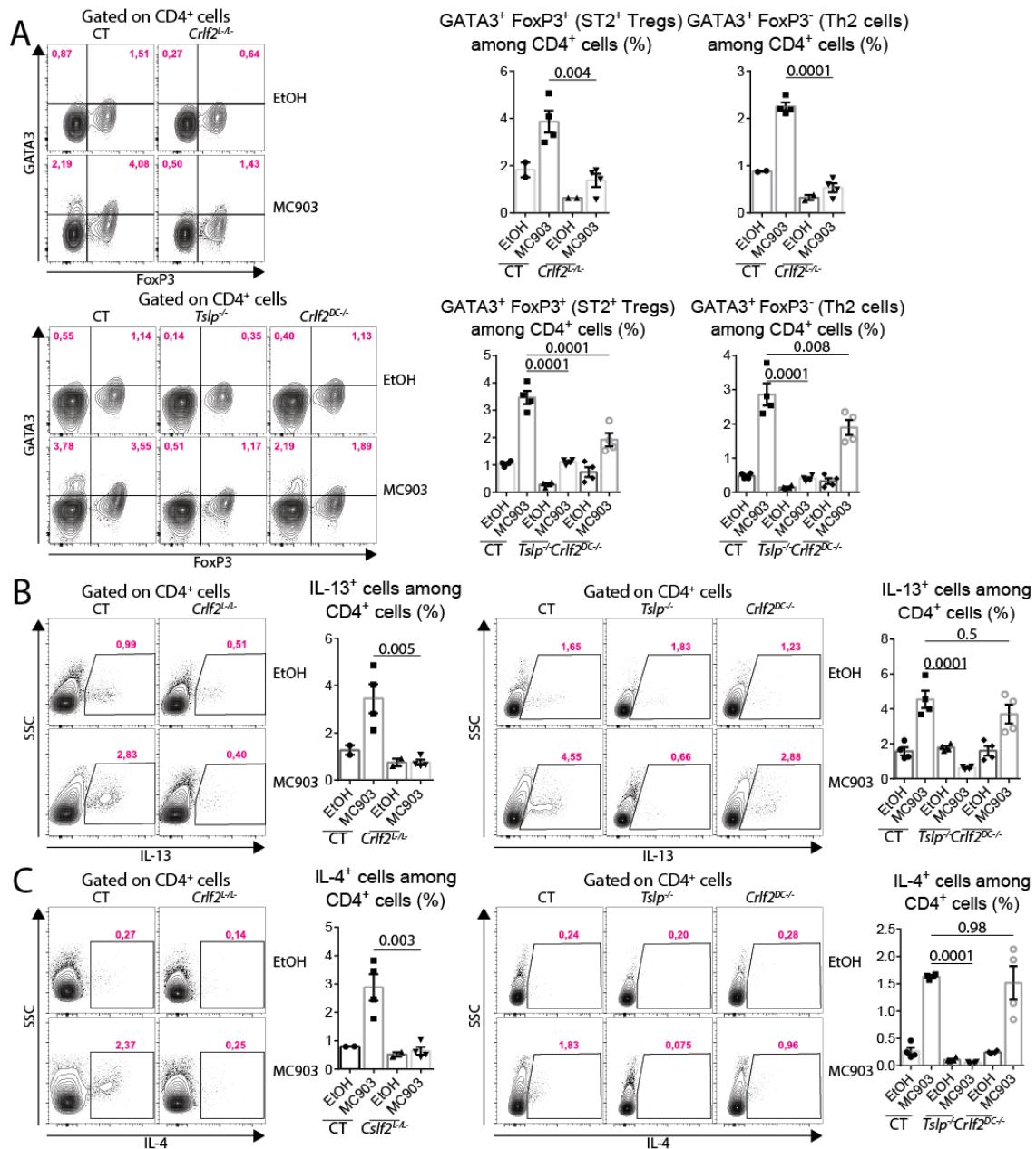
Figure 4: MC903-treated *Crlf2^DC-/-* mice exhibit a decrease in GATA3^+ FoxP3^-
Th2 differentiation but not in the production of Th2 cytokines
(A-C) Comparison of frequencies of FoxP3^+ GATA3^+ cells and FoxP3^- GATA3^+ cells (A), IL-13^+ cells (B)
and IL-4^+ cells (C) in CD4^+ cells of wildtype control (CT), *Crlf2^L-/L-*, *Tslp^-/-* or *Crlf2^DC-/-* mice..
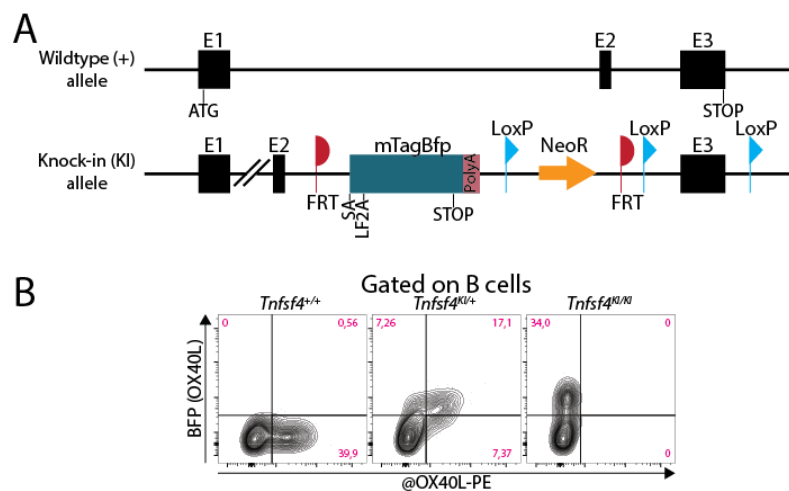
Figure 5: A novel genetic tool to study OX40L expression and function.

(A) Schematic drawing of the wild-type allele (+) of the Tnfsf4 gene locus coding for OX40L and the targeting construct (containing a gene trap cassette encoding the blue fluorescent protein (mTag-BFP). NeoR is a neomycin resistance cassette. Black boxes stand for exons. SA stands for splicing acceptor. LF2A is a ribosome skippin peptide. ATG initiator, STOP codon, LoxP and FRT sites are indicated. (B) Flow cytometry plots of OX40L antibody staining (X axis) and BFP (OX40L reporter; Y axis) gated on splenic B cells from *Tnfsf4*^{+/+}, *Tnfsf4*[KI/+] and *Tnfsf4*[KI/KI] mice stimulated in vitro with anti-CD40 and anti-IgM.
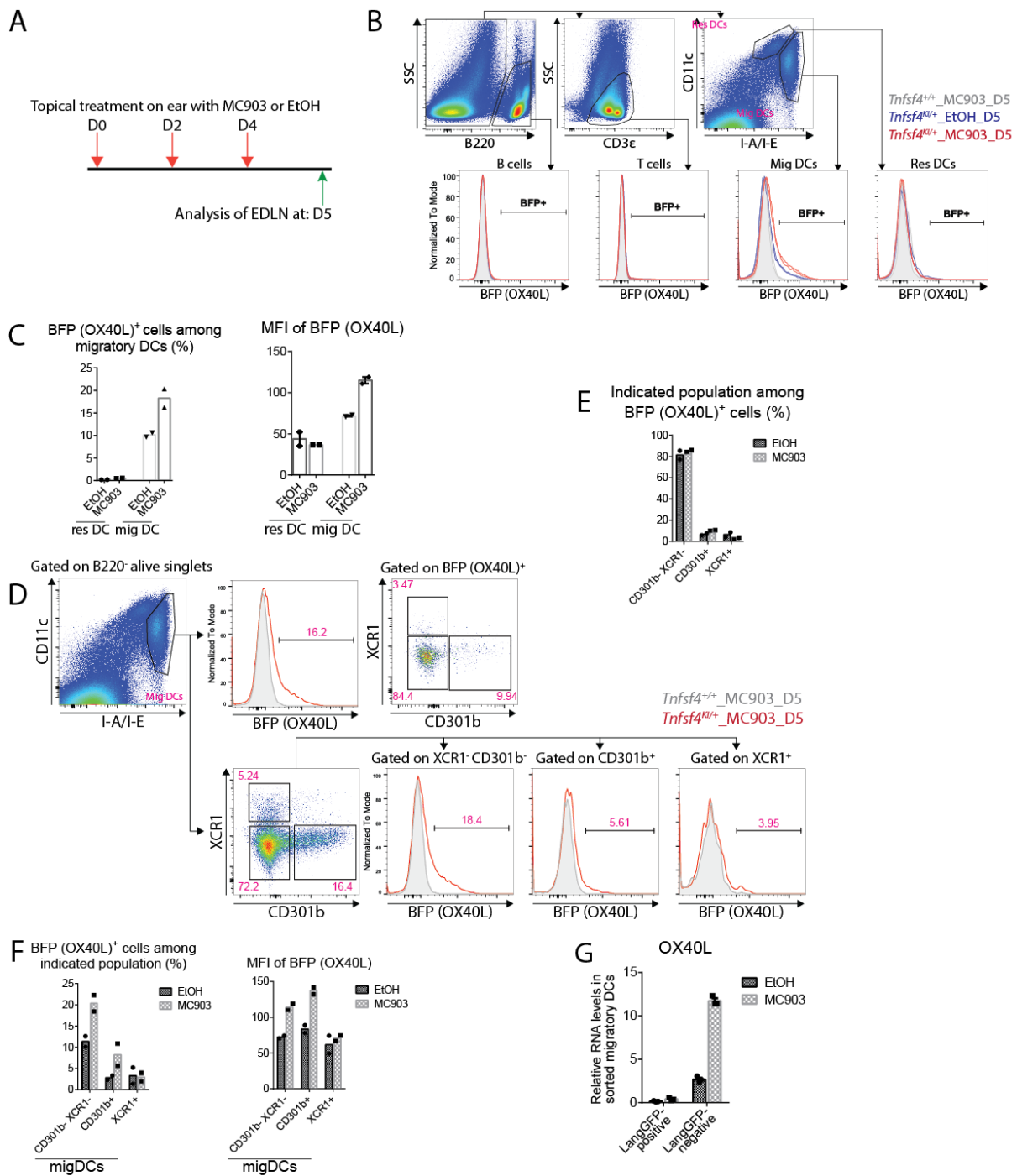
Figure 6: MC903 induces OX40L expression in a subset of migratory DCs.
(A) Experimental protocol. *Tnfsf4*[KI/+] mice were treated with MC903 or EtOH (as vehicle) on ears at Day (D)0, D2 and D4. Ear draning lymph nodes (EDLN) were analyzed at D5. (B) Gating strategy (top) and representative histograms of flow cytometry (bottom) showing the intensity of BFP (OX40L) in the indicated population. (C) Frequency and median fluorescence intensity (MFI) of BFP (OX40L) in resident (res) and migratory (mig) DCs after EtOH or MC903 treatment. (D) Flow cytometry plots of CD301b and XCR1 expression in BFP (OX40L)-expressing cells (top) and BFP (OX40L) expression in the indicated population (bottom). (E) Frequency of the indicated population among BFP^{+} cells. (F) Frequency and MFI of BFP^{+} cells in the indicated population. (G) OX40L mRNA level in sorted LangGFP-positive and LangGFP-negative migratory DCs from EtOH- or MC903-treated Lang[GFP] mice.
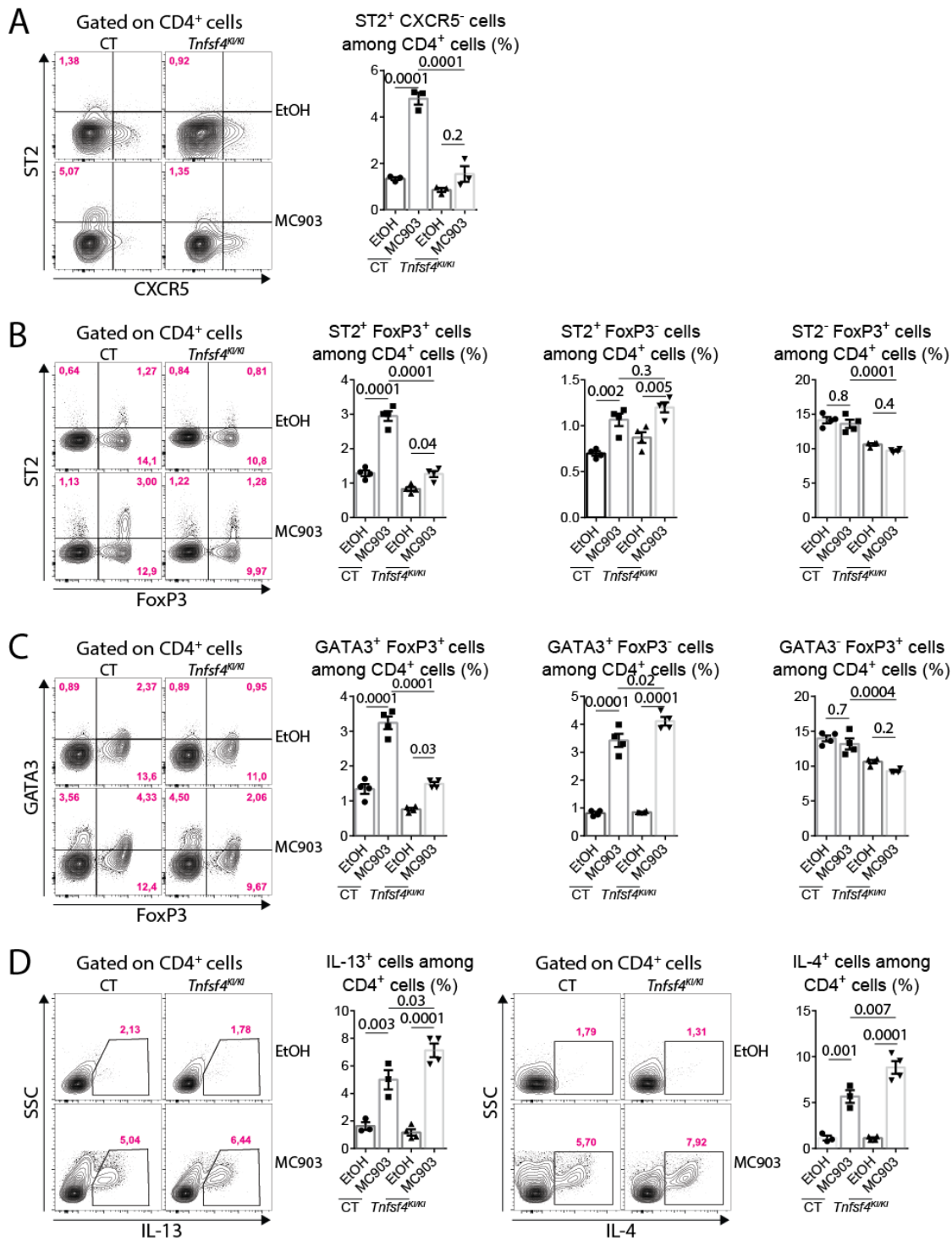
Figure 7: *Tnfsf4^{Kl/Kl}* mice lacking OX40L exhibit diminished ST2+ Tregs, accompanied by elevated GATA3+ FoxP3- Th2 cells, upon MC903 treatment.

(A) Frequency of ST2+ CXCR5- CD4+ cells in EtOH- or MC903-treated wildtype (CT) or *Tnfsf4^{Kl/Kl}* mice. (B-D) Frequencies of FoxP3+ ST2+, FoxP3- ST2+ and FoxP3+ ST2- cells (B), frequencies of FoxP3+ GATA3+, FoxP3- GATA3+ and FoxP3+ GATA3- cells (C), frequencies of IL-13 (left) and IL-4 (right)(D) in CD4+ cells in EtOH- or MC903-treated CT or *Tnfsf4^{Kl/Kl}* mice.

CONCLUSION

To conclude, my PhD thesis was focused on studying the role of DCs and DC-derived signals in the pathogenesis of AD using mouse models. In a first part, I investigated the role of LCs in the TSLP-driven Tfh cell differentiation and in the second part, I explored the expression of OX40L in different DC populations, and its role in T cell response.

In the first part, I uncovered the context-dependant role of LCs in skin TSLP-promoted cell differentiation in two mouse models developed in my lab. In TSLP^over model, TSLP is overproduced in skin keratinocytes therefore inducing the pathogenesis of an AD-like disease. Inb allergen sensitization model, we induced a mechanical disruption of the epidermal barrier using laser microporations (LMP) whose parameters can be finely adjusted to reach a precise anatomical location in the skin, followed by the topical application of the allergen OVA. I demonstrated that, in both models, a critical role of TSLP in the induction of Tfh cell differentiation and germinal center (GC) B cell response. However, LCs appear to play a seemingly contradictory role by either promoting or repressing Tfh/GC response in MC903 or LMP model respectively.

Transcriptomic analyses of Lang+ migDCs in the skin-draining lymph node showed a large number of differentially regulated genes in MC903- but not LMP/OVA-treated mice. This suggest that the initial trigger could differentially polarize LCs toward a pro-Tfh or a rather regulatory phenotype. Because the quantity of TSLP in the skin and the presence of an experimental exogenous antigen are two major differences between the two models, we hypothesize that these could be responsible of the different role of LCs. For instance, TSLP could represent a trigger that switches LCs from a regulatory to a pro-Tfh phenotype when overproduced in AD skin.
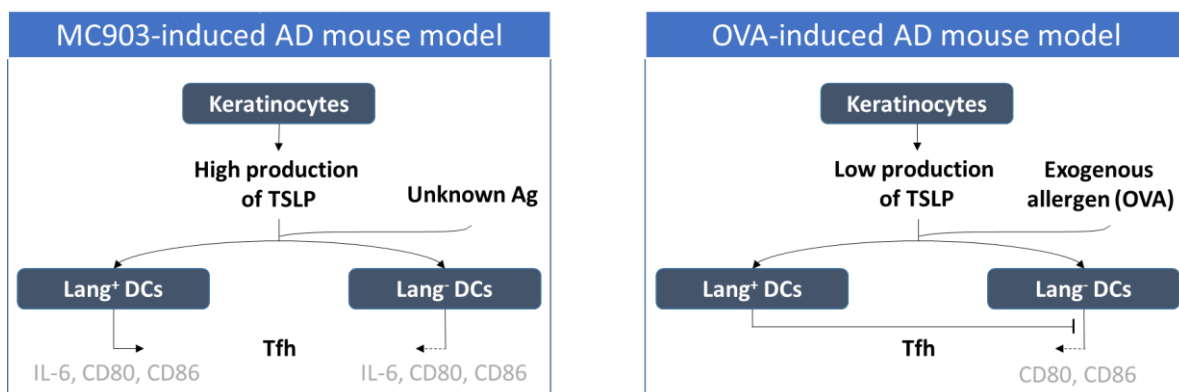
In the second part, using MC903-induced AD mouse model, I describe a pathway implicating OX40L and leading to the generation of cells resembling ST2$^+$ Tregs recently reported in mice (gut and lung) as well as in human (including in skin from multiple sclerosis patient).

Indeed, we show that TSLP induces that accumulation of cells that express the IL-33 receptor ST2 in the skin-draining lymph node of MC903-treated mice. Of note, we demonstrate that most of the ST2$^+$ T cells induced by MC903 do not produce hallmark Th2 cytokines IL-4 and IL-13 despite their expression of GATA3, but rather express the Treg master transcription factor FoxP3. IL-4 and IL-13 expression was limited to a subset of GATA3$^+$ T cells that express only marginaly ST2.

Moreover, we demonstrated that a population of Lang$^-$ XCR1$^-$ CD301b$^-$ DCs in skin-draining lymph node of untreated mice express OX40L and this expression is further increased by TSLP after MC903 treatment. By using OX40L-deficient mice, we showed that this costimulatory molecule is critically required for the induction of ST2$^+$ Tregs, but not Th2 cells. Altogether, we report here a TSLP-TSLPR^{CD11c}-OX40L axis that mediates the accumulation of ST2$^+$ Tregs in the lymph node of mice.
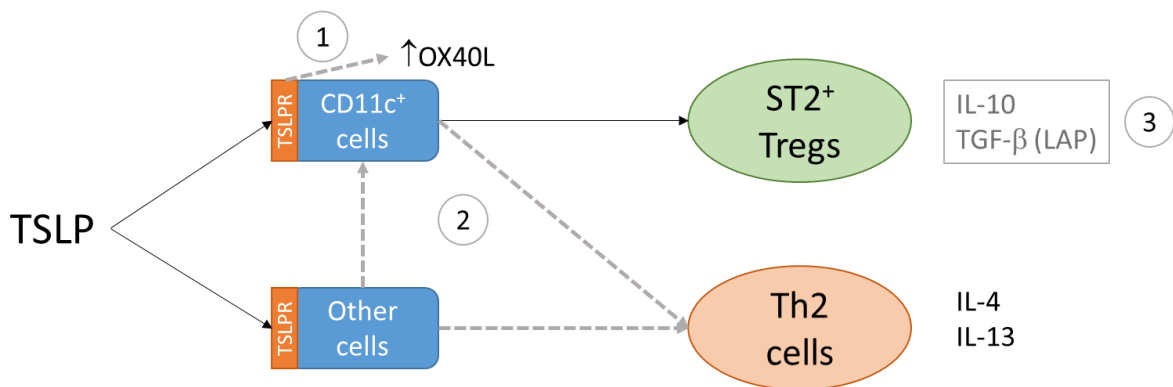
Several interesting points remain to be explored:

1. Is the upregulation of OX40L on migratory DCs after MC903 treatment mediated by the TSLPR on the surface of these DCs? To answer this, I will perform MC903 treatment on TSLPR$^{DC-/-}$ mice. .

2. It is interesting that the accumulation of Th2 cells exhibits only minor, if any, decrease in TSLP$^{DC-/-}$ mice. Does it mean that a) the induction of Th2 cells upon MC903 treatment does not require DCs? b) or that DCs are still required but not the expression of TSLPR by DCs? To answer this question, I will use CD11c^{DTR} mice in which CD11c-expressing cells, including DCs, are depleted upon DT injection. If Th2 cells are lower in DT-injected CD11c^{DTR} mice compared with WT mice upon MC903 treatment, it would suggest that DCs are still required for Th2 cell differentiation.

3. Regarding ST2$^+$ Tregs, what is their potentiential function? Do they exert regulatory function? Can we detect the production of Th2 cytokines under certain conditions? Do they produce hallmark regulatory cytokines IL-10 and TGF-β? Can we detect a suppressive function using *in vitro* or *in vivo* suppression assay?

Overall, my PhD work explored the network of skin dendritic cells involved in the pathogenesis of AD. I believe that it provides novel insights and a better understanding of the high complexity of this network, including the context-dependant role of LCs in inducing Tfh cells and GC response, as well as a new TSLP-TSLPR^{CD11c}-OX40L axis inducing ST2$^+$ Tregs cells in the pathogenesis of AD.

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Étude de la fonction des cellules dendritiques dans la réponse immunitaire cutanée de type 2

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Résumé

La dermatite atopique (AD) est une des maladies inflammatoires chroniques cutanée les plus fréquentes qui affecte jusqu'à 20% des enfants et 3% des adultes dans le monde. Elle se caractérise par une inflammation chronique de la peau et des réponses immunitaires humorale et de type 2. Mon travail de thèse est d'étudier le rôle des cellules dendritiques (DCs) dans la génération des lymphocytes T et la pathogenèse de l'AD. Dans la partie I, à l'aide de deux modèles murins d'AD, l'un déclenché par la surexpression de TSLP dans la peau induite par l'application topique de MC903 et l'autre par sensibilisation à un allergène à travers une peau dont la barrière épidermique est lésée, nous montrons le rôle crucial joué par TSLP dans la différentiation des lymphocytes T auxiliaires folliculaires (Tfh) et le développement des centres germinatifs (GC). Nous établissons le rôle contradictoire des cellules de Langerhans dans la réponse Tfh/GC promue par TSLP. Dans la partie II, nous montrons que, en plus de son implication dans la réponse Th2, TSLP signale par son récepteur TSLPR à la surface des DCs pour induire la différentiation des lymphocytes Tregs ST2$^+$ dans le ganglion drainant. De plus, la différentiation de ces cellules implique OX40L, molécule de costimulation exprimée par certaines DCs migratoires, suggérant que l'axe TSLP-TSLPR^{DC}-OX40L joue un rôle non reconnu dans l'induction des lymphocytes Tregs ST2$^+$ dans le cadre de l'AD.

Mots clés: dermatite atopique, TSLP, cellule dendritique, cellule de Langerhans, lymphocyte Tfh, OX40L, souris.

Résumé en anglais

Atopic dermatitis (AD) is a one of the most common chronic inflammatory skin disease which affects up to 20% of children and 3% of adults worldwide, with increasing prevalence in the industrialized countries during the last 30 years. It is characterized by chronic cutaneous inflammation, humoral and T helper type 2 (Th2) responses. My PhD study is to investigate the role of skin dendritic cells (DCs) in the generation of T helper cells in the pathogenesis of AD. In the Part I, using two mouse models of AD, one triggered by the overexpression of TSLP in mouse skin through topical application of MC903, and the other one with epicutaneous allergen sensitization on barrier-disrupted skin, we demonstrated a crucial role of TSLP in promoting T follicular helper (Tfh) cell differentiation and germinal center (GC) response. We uncovered a seemingly contradictory role of Langerhans cells in TSLP-promoted Tfh/GC response. In the part II, we showed that, in addition to promote Th2 cell differentiation, TSLP signals through TSLPR expressed by DCs to induce the differentiation of ST2$^+$ Tregs in skin-draining lymph nodes. Interestingly, the differentiation of these cells implicates OX40L, a costimulatory molecule expressed in a subset of migratory DCs, suggesting a previously unrecognized role of TSLP-TSLPRDC-OX40L axis in the induction of ST2$^+$ Tregs in AD pathogenesis.

Keywords: atopic dermatitis, TSLP, dendritic cells, Langerhans cells, T follicular helper (Tfh), OX40L, mouse.