THESE DE DOCTORAT

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Etude du rôle de la cytokine thymic stromal lymphopoietin (TSLP) produite par les keratinocytes dans la marche atopique.

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Table of contents

Table of contents	
Table of contents	i-ii
Index of tables and figures	ii
Mouse lines	iii
Abbreviations	iv-vii
Resume en français	viii-xi
Abstract	xii
I. Introduction	1-50
1. Overview of immune system	2-12
1.1 Innate immunity	2-8
1.1.1 Pattern-Recognition Receptors	2
1.1.2 Innate immune cells	5
1.2 Adaptive immunity	8-12
1.2.1 Cell mediated immunity	8
1.2.1.1 T cells	9
1.2.1 Humoral immunity	12
1.2.2.1 B cells	12
2. Biology of the skin	13-15
2.1 Epidermis	13-14
2.2 Dermis	14-15
2.2 Skin immunity	15
3. Thymic stromal lymphopoietin (TSLP)	16-22
3.1 TSLP and its receptor	16
3.2 TSLP expression	16-17
3.3 TSLP effects on immune cells	17-22
4. Atopic dermatitis	23-34
4.1 Epidemiology	23-24
4.2 Clinical aspects of atopic dermatitis	24-26
4.3 Genetics	26-27
4.4 Environmental factors	27
4.5 Physiopathology	28-34
5. Asthma	35-39
5.1 Epidemiology	35
5.2 Clinical aspects of asthma	35
5.3 Genetics and environmental factors	36
5.4 Physiopathology	37-38
5.5 TSLP and airway diseases	38-39
5.6 Resemblance with AD	39

Table of contents

Table of contents	
6. Atopic march: from AD to asthma	40-41
6.1 TSLP and atopic march	41
7. Mouse models of Atopic Dermatitis and asthma	42-50
7.1 Mouse models of AD	42-48
7.1.1 Spontaneous mouse models of AD	42
7.1.2 Genetically engineered mouse models	43
7.1.3 Models induced by topical application of chemical compounds	47
7.1.4 Models induced by epicutaneous sensitization with allergens	47
7.2 Mouse models of allergic asthma	48-50
7.2.1 Acute challenge asthma models	48
7.2.2 Chronic allergen exposure asthma model	49
7.3 Inconvenience of mouse models of AD and allergic asthma	49
II. Objectives	50
III. Results	51
III.1 Part 1	52
III.2 Part 2-A	69
III.3 Part 2-B	93
IV. Discussion	118
V. Bibliography	125

Index of tables and figures

Index of tables and figures	
Tables	
Table 1. Pathogen Recognition Receptors and its ligands	4
Table 2. Mouse models of atopic dermatitis	46
Figures	
Figure 1. CD4+ T helper subsets	10
Figure 2. Basic mechanisms used by Treg cells	11
Figure 3. Structure of the epidermis	14
Figure 4. Immune cell responding to TSLP and its effects	18
Figure 5. Clinical aspects of AD skin lesions	24
Figure 6. Histological analysis of normal skin and skin from patients with AD	25
Figure 7. Schematic showing the Immune complexity involved in AD pathogenesis	30
Figure 8. Histological analysis of healthy and asthmatic lungs	36
Figure 9. Schematic the coordinated response of airway epithelium	37
Figure 10. Prevalence of various atopic diseases	40
Figure 11. Epicutaneous sensitization by patch method	48

Mouse lines	
TSLP$^{iep-/-}$	Mice bearing an inducible TSLP ablation in epidermal keratinocytes through i.p. Tam injection to K14-Cre-ER$^{T2(tg/0)}$/TSLP$^{L2/L2}$ mice
TSLP$^{cep-/-}$	Mice bearing TSLP constitutive ablation of in epidermal keratinocytes (K14-Cre/TSLP$^{L2/L2}$ mice)
TSLP-/-	Germline TSLP null mice
RXRαβ$^{iep-/-}$	Mice bearing RXRα and RXRβ inducible ablation in epidermal keratinocytes through Tam i.p. injection to K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/L2}$/RXRβ$^{L2/L2}$ mice
RXRαβTSLP$^{iep-/-}$	Mice bearing RXRα, RXRβ and TSLP inducible ablation in epidermal keratinocytes through Tam i.p. injection to K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/L2}$/RXRβ$^{L2/L2}$/TSLP$^{L2/L2}$ mice
CD11c-DTR	Mice bearing an inducible depletion of CD11c+ cells through an i.p. injection of DT to CD11-DTR^{Tg} mice
Balb/c	Albino in-bred strain
C57BL/6	Black in-bred strain
RAG1-/-	Germline GATA1 null mice
GATA1-/-	Germline RAG1 null mice
Langerin-DTR	Mice bearing an inducible depletion of Langerin+ cells through an i.p. injection of DT to Langerin-DTREGFP$^{+/-}$ mice
MyD88-/-	Germline MyD88 null mice
DO11.10	Mice bearing a MHC class II restricted rearranged T cell receptor transgene that respond to OVA peptide (amino acids 323-339)

Abbreviation	Definition
ACTL	Actin-like
AD	atopic dermatitis
AHR	Airway hyperresponsiveness
ALOX5	Arachidonate 5-lipoxygenase
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APOC	Apolipoprotein C
BAL	Bronchoalveolar
Bcl	B-cell CLL/lymphoma
BIR	Baculovirus inhibitor repeat
Btk	Bruton's tyrosine kinase
C-terminal	Carboxy-terminal
CARD	Caspase recruitment domain
CC	β-chemokine
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte antigen
CLR	C-type lectine receptor
CpG	Cytosine—phosphate—Guanine
CTACK	Cutaneous T-cell-attracting chemokine
CXC	α-chemokine
CXCR	CXC-chemokine receptor
DC	Dendritic cell
dDC	Dermal dendritic cell
DEAD	Aspartic acid (D)-Glutamic acid (E)-Alanine (A)-Aspartic acid (D)
DEFA	defensin alpha
DEFB	defensin beta
DEXH	Aspartic acid (D)-Glutamic acid (E)-Any aminoacid (X)-Histidine (H)
Dicer	Double-stranded RNA-specific endoribonuclease
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulfate
EDC	Epidermal differentiation complex

Abbreviation	Definition
Erk	Extracellular signal-regulated kinase
FasL	CD95-ligand
FcεRIα	IgE fragment crystallizable receptor subunit alpha
FLG	Filaggrin
FOXP3	Forkhead-box P3
GR	Glucocorticoid receptor
GSTM	Glutathione S-transferase mu
GSTP	Glutathione S-transferase pi
H&E	Hematoxylin and eosin
hBD	Human beta defensin
HDM	House dust mite
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
IDC	Inflammatory dendritic cell
IFN	interferon
IL	Interleukin
IRAK	IL-1 receptor-associated kinase
Jak	Janus kinase
JNK	C-jun N-terminal kinase
LC	Langerhans cell
LRR	Leucine-rich repeat
LTC	Leukotriene
MAIL	Molecule possessing ankyrin repeats induced by lipopolysaccharide
MBP	Major basic protein
MCP	Monocyte chemotactic protein
MDA5	melanoma differentiation-associated gene 5
mDC	myeloid DC
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mRNA	messenger RNA
N-terminal	Amino-terminal
NC	Nishiki Nezumi Cinnamon

Abbreviation	Definition
NK cells	Natural killer cells
NKRP	Killer cell lectin-like receptor subfamily B
NKT cell	Natural killer T cell
NLR	NOD-like receptor
NOA	Naruto Research Institute Otsuka Atrichia
NOD	Nucleotide oligomerization domain
OVA	Ovalbumin
OVOL	Ovo-like
OX40L	OX40-ligand
PAMP	Pathogen-associated molecular pattern
PAS	Periodic acid-Schiff
pDC	Plasmocytoid DC
PI-3	Phosphoinositide 3
PRR	Pattern recognition receptor
PYD	Pyrin domain
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RAR	Retionic acid receptor
RelB	Reticuloendotheliosis viral oncogene homolog B
RIG	Retioic acid-inducible gene
RLR	RIG-like receptor
RNA	Ribonucleic acid
RXR	Retinoid X receptor
shRNA	Small hairpin RNA
SPINK	Serine peptidase inhibitor, Kazal type
Src	Sarcoma
SSCE	Stratum corneum chymotryptic enzyme
ssRNA	single-strand RNA
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation-regulated chemokine
TBX	Thromboxane
TCR	T-cell receptor
TEWL	Transepidermal water loss

Abbreviation	Definition
TGF	Tumor growth factor
Th	Helper T cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOLLIP	Toll interacting protein
Tregs	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
TSLPR	TSLP receptor
VDR	Vitamin D receptor
VV	Vaccinia virus
WT	Wildtype

RESUME DE LA THESE DE DOCTORAT

Etude du rôle de la cytokine TSLP (Thymic Stromal LymphoPoietin) produite par les keratinocytes dans la marche atopique.

La dermatite atopique (DA) est une maladie multifactorielle inflammatoire de la peau, résultant de l'interaction entre des prédispositions génétiques et des expositions environnementales. La DA est une dermatose inflammatoire prurigineuse chronique et récurrente, caractérisée par un dysfonctionnement de la barrière cutanée, une inflammation de type T helper type 2 (Th2) accompagnée d'une éosinophilie et d'une hyper-immunoglobulinémie IgE.

La DA est associée à des antécédents personnels ou familiaux d'atopie. L'atopie est une prédisposition héréditaire à développer des réactions d'hypersensibilité médiées par les IgE. Ce terme d'atopie regroupe la DA, l'asthme, la rhinite allergique et l'allergie alimentaire.

La marche atopique désigne la progression séquentielle des maladies atopiques, en particulier l'apparition d'asthme chez les enfants précédée par celle d'une DA sévère chez les nourrissons. Dans ce cadre, des études épidémiologiques ont montré que plus de 50% de patients atteints d'une DA modérée à sévère développent ultérieurement un asthme. De plus, l'asthme est influencé par le degré de sévérité de la DA, qui pourrait ainsi être considérée comme la porte d'entrée pour le développement ultérieur d'une inflammation allergique des voies aériennes. Les mécanismes moléculaires et cellulaires de la marche atopique restent cependant mal définis.

Une sensibilisation cutanée précoce et la sévérité de la maladie sont les facteurs de risque les plus significatifs du passage à l'asthme pour les patients atteint de DA. Cela donne à penser que des facteurs qui aggravent l'inflammation de la peau et qui promeuvent la sensibilisation cutanée sont impliqués dans cette progression.

La lymphopoïétine stromale thymique (TSLP) est une cytokine dont plusieurs fonctions importantes ont été récemment identifiées. Elle est produite par les cellules épithéliales et stromales, et joue un rôle clé dans les réactions allergiques. Elle est abondamment produite par les keratinocytes de patients souffrant d'une DA, et elle

est détectée dans le poumon de patients atteint d’asthme.

Des études antérieures réalisées au sein du laboratoire ont révélé que l’invalidation sélective des gènes codant pour les récepteurs nucléaires RXRα et RXRβ dans les kératinocytes épidermiques de la souris adulte (souris $RXR\alpha\beta^{ep-/-}$), ou l’application cutanée d’un analogue de la vitamin D3, le calcipotriol (MC903), induisait l’expression de TSLP dans ces mêmes kératinocytes, et déclenchait l’apparition d’une dermatite dont les caractéristiques étaient très semblables à la DA humaine. De plus, le laboratoire a montré que des souris transgéniques exprimant sélectivement la cytokine TSLP dans les kératinocytes développent une DA semblable à celle des souris $RXR\alpha\beta^{ep-/-}$, démontrant que la production de la cytokine TSLP par les kératinocytes épidermiques est suffisante pour déclencher une DA. Récemment, le laboratoire a également montré que l’expression augmentée de TSLP dans les kératinocytes épidermiques par application cutanée de MC903 ou chez les souris $RXR\alpha\beta^{ep-/-}$, non seulement déclenche localement une inflammation de type DA, mais provoque aussi une aggravation de l’inflammation pulmonaire de type asthmatique concomitamment induite par la mise en œuvre d’un modèle expérimental d’asthme allergique. Ces résultats indiquaient que la surproduction de TSLP pendant la DA pourrait être un facteur de risque pour l’apparition ultérieure d’un asthme chez ces patients.

Il est important de noter que dans le modèle expérimental d’asthme allergique, la sensibilisation à l’allergène est réalisée par injection intra-péritonéale avec l’aide d’un adjuvant. Cependant, la peau est reconnue comme l’un des sites d’initiation de la sensibilisation aux allergènes pendant la DA, et certaines indications font supposer que la sensibilisation cutanée joue un rôle important dans la marche atopique. Les éléments qui déclenchent l’inflammation et la sensibilisation cutanée dans la DA et leur participation dans la progression de la marche atopique restent toutefois inconnus.

Mon travail de thèse a consisté à déterminer l’implication de la cytokine TSLP produite par les kératinocytes pendant la DA et sa participation dans la marche atopique. Pour atteindre cet objectif, j’ai développé un nouveau modèle murin, en essayant de reproduire au mieux le parcours observé chez les patients devant la marche atopique. Dans ce protocole, la sensibilisation à l’allergène est induite par

voie cutanée, sans adjuvant, suivie d'une phase de restimulation par instillation intra-nasale avec le même allergène, pour développer une inflammation pulmonaire de type asthmatique (figure 1).

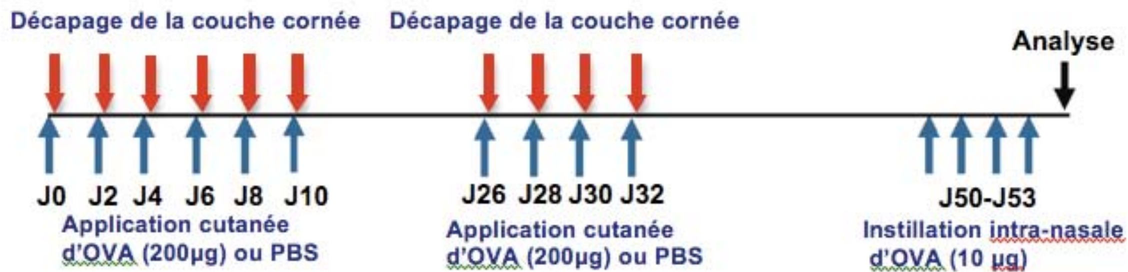

Figure 1. Représentation schématique du modèle murin de la marche atopique. La peau du dos de souris adultes a été traitée avec de l'ovalbumine (OVA) ou le véhicule (PBS) après décapage de la couche cornée à l'aide d'un ruban adhésif (tape stripping) quotidiennement tous les 2 jours, du jour zéro au jour 10 (J0-J10) et du jour 26 au jour 32 (J26-J32). Une restimulation par instillation intra-nasale avec de l'OVA a été effectuée quotidiennement pendant 4 jours (J50-J53) trois semaines après le dernier traitement cutané.

Nous avons démontré que l'application cutanée de l'ovalbumine (OVA), après décapage de la surface de la couche cornée de la peau de souris à l'aide d'un ruban adhésif (tape stripping) induit une dermatite atopique, caractérisée par une infiltration de cellules immunitaires (éosinophiles, basophiles, cellules CD4+ et les mastocytes) dans le derme, une hyperplasie de l'épiderme, une augmentation de l'expression des cytokines de type Th2 dans les ganglions lymphatiques qui drainent la peau, et une production d' immunoglobulines spécifiques à l'ovalbumine. Suite à la restimulation par instillation intra-nasale avec l'ovalbumine des souris sensibilisées par voie cutanée, celles-ci développent un asthme, caractérisé par une augmentation du nombre d'éosinophiles dans le lavage broncho-alveoaire (BAL), des infiltrats riches en cellules inflammatoires péri-vasculaires et péri-bronchiques, d'une production accrue de mucus et d'une augmentation de l'expression des cytokines de type Th2, ainsi qu'une hyperréactivité bronchique. Ainsi, le modèle développé est pertinent pour l'étude de la progression de la DA en asthme.

Pour évaluer la participation de la cytokine TSLP exprimée dans les kératinocytes dans la marche atopique, nous avons utilisé des souris présentant une invalidation sélective du gène de la cytokine TSLP dans les kératinocytes épidermiques à l'âge adulte (souris $TSLP^{iep-/-}$). En soumettant des souris $TSLP^{iep-/-}$ au protocole induisant la marche atopique que nous avons développé, nous avons montré que la production

de la cytokine TSLP dans les kératinocytes est un facteur nécessaire, non seulement pour l'inflammation cutanée, mais aussi pour générer une réponse immunitaire systémique à l'allergène, et développer un phénotype asthmatique après restimulation des voies aériennes au même allergène. De plus, la surexpression de la cytokine TSLP, par traitement topique au MC903 au cours de la sensibilisation cutanée, provoque une aggravation de la DA, une augmentation de la production d'anticorps spécifiques à l'allergène, et un phénotype asthmatique plus sévère suite à la restimulation avec l'allergène par instillation intra-nasale de façon dose-dépendante. Ainsi la quantité de cytokine TSLP produite dans les kératinocytes pendant le développement d'une inflammation allergique cutanée a un rôle très important, non seulement dans la réaction inflammatoire locale, mais aussi pour produire une réponse immunitaire systémique à l'allergène, qui se traduit par le développement d'un asthme à l'occasion d'un contact ultérieur avec le même allergène par les voies aériennes.

Des études cliniques sont nécessaires pour déterminer si l'inhibition de l'expression de la cytokine TSLP et/ou son activité pendant une DA peut réduire l'inflammation cutanée, prévenir la sensibilisation aux allergènes et arrêter la progression d'affections allergiques des voies respiratoires.

ABSTRACT

Atopic march refers to the natural history of allergic diseases, which is characterized by a typical sequence of sensitization and manifestation of symptoms in different tissues. Commonly, the clinical manifestations of atopic dermatitis (AD) appear in the early life and precede the development of airway allergic diseases. AD has been proposed as an entry point for subsequent atopic diseases.

The objectives of my thesis was: 1) to better understand the role of thymic stromal lymphopoietin (TSLP) in the atopic march and 2) to dissect TSLP-initiated immune cascade leading to AD pathogenesis. To reach my thesis objectives we used mouse models of atopic diseases in combination with various deficient-mouse lines.

In the first part of this work, using a novel atopic march mouse model, we demonstrate that keratinocytic TSLP is required not only for the development of allergic skin inflammation, but also for the generation of the allergen-specific immune response. Moreover, we demonstrate that the defective immune response against the allergen in $TSLP^{iep-/-}$ (in which keratinocytic TSLP is specific ablated in adult epidermal keratinocytes) leads to less severe asthma. In addition, using a $TSLP^{over}$ mice (in which keratinocytic TSLP overexpression is induced by topical application of MC903, a low-calcemic vitamin D analog), we demonstrate that keratinocytic TSLP overproduction during allergen skin contact, boosts allergen sensitization and triggers an aggravated asthma. These data together reveal that keratinocyte-derived TSLP plays an important role in promoting skin inflammation and allergen sensitization, which is involved in the progression to asthma (atopic march: from AD to asthma).

In the second part of this work, using a TSLP-induced AD mouse model (topical MC903 treatment), we demonstrate that skin TSLP induces an early innate recruitment of basophils in the skin, followed by a late basophil recruitment involving adaptive immunity. In addition, we demonstrate that TSLP-induced Th2 response requires an orchestrated cooperation of dendritic cells, $CD4^+$ T cells and basophils.

This work provide new knowledge in the cellular and molecular mechanisms implicated in atopic diseases involving TSLP, and provide new insights for the development of therapeutic options of these diseases.

INTRODUCTION

INTRODUCTION.

The immune system is a remarkable natural defense mechanism. It provides the means to make rapid, highly specific and protective responses against potentially pathogenic microorganism, thus creating a state of protection from infectious diseases. Immunodeficiency illustrate the central role of the immune response in protection against microbial and viral infection. However, not only a deficient, but also an excessive immune response, as seen in autoimmunity and allergic reactions, can lead to tissue damage and fatal outcome. Therefore, a balanced response, which discriminates between innocuous and harmful, is the prime challenge of the immune system.

1. Overview of the immune system.

The mammalian immune system is divided between innate and adaptive immunity, which cooperate to protect the host against microbial and viral infection. The innate immunity is the older system to control microbe invasion and represents a primary and nonspecific immune response. Conversely, the adaptive immunity represents a specific and secondary immune response.

1.1 Innate immunity.

The innate immune system include defense mechanisms that include a range from external physical and biochemical barriers (epithelial cells, mucous surfaces) to internal defense, involving cytokines, chemokines, enzymes and lipid mediators released by innate immune cells, as well as soluble mediators constitutively present in biological fluids such as plasma proteins (complement cascade, C-reactive protein). The immune innate cells present several pattern recognition receptors (PRRs) that bind pathogen-associated molecular patterns (PAMPs) expressed on the surface of invading microbes or virus.

1.1.1 Pattern-Recognition Receptors (PRRs).

PRRs are molecules on or in host cells that are able to recognize or bind to PAMPs, molecules unique to microbes that are not associated with host cells, responsible for sensing the presence of microorganism. Currently, four classes of PRR families have been identified. These families include transmembrane proteins such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as Retinoic acid-inducible gene (RIG)-like receptors (RLR) and nucleotide oligomerization domain (NOD)-like receptors (NLRs). In most of the case the sensing of PAMPs by PRRs up-regulates the transcription of genes in inflammatory response. These genes encode pro-inflammatory cytokines, type I interferons (IFNs), chemokines and antimicrobial peptides (Takeuchi and Akira, 2010).

Toll-like receptors (TLRs).

Toll, the founding member of the TLR family, was initially identified as a gene product essential for the development of embryonic dorsoventral polarity in Drosophila. Later, it was also shown to play a critical role in the anti-fungal response of flies (Lemaitre et al., 1996). TLRs are evolutionarily conserved from the worm *C. elegans* to mammals (Janeway and Medzhitov, 2002). The TLR family is one of the best-characterized PRR families, and is responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes (Akira et al., 2006). Ten TLRs have been identified in humans and 12 in mice. Different TLRs recognize various molecular patterns of microorganisms and self-components (Table 1).

C-type lectin receptors (CLRs).

C-type lectin is a type of carbohydrate-binding protein domain know as lectin. The C-type designation refers to their requirement for calcium for binding. CLRs comprise a transmembrane receptor family that recognize carbohydrates on microorganisms such as viruses, bacteria and fungi (Table 1). CLRs either stimulate the production of

pro-inflammatory cytokines or inhibit the TLR-mediated immune responses (Geijtenbeek and Gringhuis, 2009).

Retinoic acid-inducible gene (RIG)-like receptors (RLR).

RLRs are composed of two N-terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain, and a C-terminal regulatory domain. They are localized in the cytoplasm and recognize genomic RNA of dsRNA viruses and dsRNA generated as the replication intermediate of ssRNA viruses (Table 1). The RLR family is composed of retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and DEXH box polypeptide 58 (LGP2) (Takeuchi and Akira, 2010).

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5′triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

Table 1. Pathogen Recognition Receptors and their ligands [Taken from **(Takeuchi and Akira, 2010)].**

Nucleotide oligomerization domain (NOD)-like receptors (NLRs).

NLRs are a specialized group of intracellular receptors that represent a key component of the host innate immune system. This PPR family is defined by a tripartite structure consisting of a variable N-terminal protein-protein interaction domain (caspase recruitment domain [CARD], pyrin domain [PYD], acidic transactivating domain or baculovirus inhibitor repeat [BIR]), a central nucleotide-

binding oligomerization (NOD) domain and a C-terminal leucine-rich repeat (LRR). NOD1 and NOD2, which harbor CARDs, NOD and LRR domains, induce transcriptional upregulation of pro-inflammatory cytokines. NOD1 and NOD2 recognize structures of bacterial peptidoglycans (Table 1). NLRs and TLRs synergistically activate pro-inflammatory cytokine production (Chen et al., 2009).

1.1.2 Innate immune cells.

The innate immune cells include: epithelial cells from structural surface of the skin, airway, reproductive and gastrointestinal tract, and hematopoietic cells, such as natural killer (NK) cells, mast cells, eosinophils, basophils and phagocytes (including macrophages, neutrophils and dendritic cells).

Epithelial cells.

The mucosal epithelia is one of the most ancient and universal modules of the innate immunity. The mucosal epithelia from the skin, airways, reproductive and gastrointestinal tract are the main interface between the host and external factors (including microorganism and environmental factors), and therefore vulnerable to colonization and invasion of pathogens (bacteria, viruses, fungi or parasites), and to damage by environmental factors (irritants, protease, etc) (Artis, 2008).

Epithelial cells present a complete array of PRRs, and those cells have the potential to produce soluble factors secreted after their activation, like anti-microbial peptides (AMPs), cytokines, growth factors and chemokines, which are potent immunoregulators. In addition, epithelial cells can produce factors that inhibit the attachment and entry of pathogens (Medzhitov, 2007).

Natural killer (NK) cells.

NK cells represent a subgroup of white blood cells. Since their identification, NK cells have been classified as lymphocytes on the basis of their morphology, their expression of many lymphoid markers and their origin from the common lymphoid progenitor in the bone marrow. However, these cells are generally considered to be

component of the innate immune system because they lack antigen-specific cell surface receptor.

NK cells have been shown to participate in the early control against virus infection and in tumor immunosurveillance in humans and in mice (Vivier et al., 2011).

Mast cells.

Mast cells are specialized secretory cells that contain many metachromatic granules. These granules are rich in histamine and heparin, enzymes (chymase, tryptase), lipid mediators (prostaglandins and leukotrienes) and cytokines (TNFα, IL-4, IL13, etc). They are dispersed throughout most tissues, but are crucially located at the host's interfaces with the environment, such as the skin and mucosa, supporting a role in the recognition of pathogens and other environmental factors.

Mast cells contribute to controlling a wide range of pathogenic infections, including those by parasites, bacteria and probably viruses (Abraham and St John, 2010).

Eosinophils.

Eosinophils are multifunctional leukocytes implicated in the pathogenesis of numerous inflammatory processes, including parasitic helminth infections and allergic diseases. In response to diverse stimuli, eosinophils are recruited from the circulation into inflammatory foci, where they modulate immune responses through an array of mechanisms. Triggering of eosinophils by engagement of receptors for cytokines, immunoglobulins, and complement can lead to the secretion of an array of proinflammatory cytokines [IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, and TGF (transforming growth factor)-α/β], chemokines (RANTES and eotaxin-1), granule proteins (Major Basic Protein [MBP] and eosinophil cationic protein) and lipid mediators (prostaglandins, leukotrienes and thromboxane).

These molecules have proinflammatory effects, including upregulation of adhesion systems, modulation of cellular trafficking, and activation and regulation of vascular permeability, mucus secretion, and smooth muscle constriction (Rothenberg and Hogan, 2006).

Basophils.

Basophils are basophilic granulocytes circulating in the peripheral blood. They are very rare and account for less than 1% of blood leukocytes. In addition to their basophilic granules, basophils share certain features with tissue-resident mast cells. These cells have often erroneously been considered as minor and possibly redundant relatives of mast cells or as blood-circulating precursors of tissue-resident mast cells. Basophils rapidly secrete large quantities of IL-4 and IL-13, histamine and leukotriene C4 (LTC4) in response to various stimuli (Karasuyama et al., 2011).

Macrophages.

Macrophages are cells produced by differentiation of monocytes in tissues. Tissue macrophages have a broad role in maintenance of tissue homeostasis, through the clearance of senescent cells and remodeling and repair after inflammation. Macrophages have specialized functions that are adopted by the macrophages in different anatomical location. For example, alveolar macrophages which express high levels of PRRs are involved in clearing microorganism, viruses and environmental particles in the lung (Gordon and Taylor, 2005).

Neutrophils.

Neutrophils are the most abundant type of white blood cells in mammals, and form an essential component of the innate immune system. These cells are the first immune cells to arrive at the site of infection, and help to recruit and activate other cells of the immune system. In addition, neutrophils play a key role in the front-line defense against invading pathogens. Neutrophils have three strategies for directly attacking microorganisms: phagocytosis, release of soluble anti-microbials (including granule proteins) and generation of neutrophils extracellular traps (Nathan, 2006).

Dendritic cells.

Dendritic cells are “professional” antigen presenting cells (APCs), since the principal function of these cells is to present antigens. To perform this function, DCs are capable of capturing antigens, processing them, and presenting them on the cell surface of T cells along with appropriate costimulatory molecules. DCs are positioned

at the boundaries between the inner and the outside world, thus bridging innate and adaptive immunity (Banchereau and Steinman, 1998).

DCs are heterogenous in origin, morphology, phenotype and function. Two distinct DC subpopulations were originally defined in human blood, based on the expression of CD11c. They have been subsequently characterized as myeloid DCs (mDCs) and plasmocytoid DCs (pDCs) (Colonna et al., 2004).

mDCs are the most efficient APCs. They can directly prime naive T cells and can act, under different stimuli, in an immunogenic or tolerogenic manner (Steinman and Banchereau, 2007).

pDCs are less efficient than mDCs as APCs. However, they secrete high amounts of type-I interferon in response to TLRs signaling induced by single strand RNA and unmethylated CpG-containing DNA (Reizis et al., 2011).

1.2 Adaptive immunity.

The adaptive immune response is characterized by high degree of specificity to individual pathogen, because of antigen-specific receptor and the ability to form a stable memory ensuring increased protection against re-infection. T cells, together with B cells, form the major part of the adaptive immunity.

Two main pathways encompass the adaptive immune system: humoral and cell-mediated immunity. Humoral immunity, mediated by B cell secreted antibodies, protects mostly against extracellular microbes and microbial toxins, while cell-mediated immunity, orchestrated by T cells, serves as a defense mechanism against microbes that survive within phagocytes or infect nonphagocytic cells (Janeway and Medzhitov, 2002).

1.2.1 Cell-mediated immunity.

T cells fail to recognize antigens in the absence of APCs. The T cell receptor is restricted to recognizing antigen peptides only when bound to appropriate molecules of the major histocompatibility complex (MHC), also know in humans as human leukocyte antigen (HLA). This process is know as MHC restriction. There are two classes of MHC molecules: MHC class I and MHC class II.

Antigens presented by MHC class I molecules are described as “endogenous peptides” or self antigens, because they are derived from protein turnover and defective ribosomal products. During viral infection, intracellular microorganism infection, or cancerous transformation, such proteins degraded in the proteasome are as well loaded onto MHC class I molecules and displayed on the cell surface. MHC class I extracellular domains are expressed in all nucleated cells, and interact only with CD8+ cytotoxic T lymphocytes (Neefjes et al., 2011).

In contrast to MHC class I molecules, MHC class II molecules are only expressed in particular cells called professional APCs (DCs, macrophages and B cells). Antigenic peptides presented by MHC class II molecules result from lysosomal and endosomal degradation of phagosized products. Thus, MHC class II molecules present exogenous antigens and interact only with CD4+ helper T cells (Neefjes et al., 2011).

1.2.1.1 T cells.

T cells develop from a common lymphoid progenitor in the bone marrow. The progeny destined to give rise to T cells leave the bone marrow and migrate to the thymus. In the thymus T cell progenitors become mature naive T cells, which are released to peripheral tissues. There are several types of T cells such as T helper cells, cytotoxic T cells and regulatory T cells.

T helper (Th) cells

Th cells are the main regulators of the immune response, because they can activate and direct other cells, such as B cells or cytotoxic T cells. Mature naive Th cells express the surface protein CD4 and can be activated by recognition of MHC class II molecules. In addition, naive Th cells can be polarized to generate three functional subsets, depending of the microenvironment present during their activation (Figure 1). Th1 cells, induced by IL-12, express the transcription factor T-bet and secrete interferon-gamma (IFNγ). IL-4 promotes Th2 cells, which express the transcription factor GATA-3 and secrete interleukin-4 (IL-4). A combination of IL-6 and TGFβ induces Th17 cells, which express the transcription factor RORγ and secrete Interleukin-17 (IL-17) (Reiner, 2009).

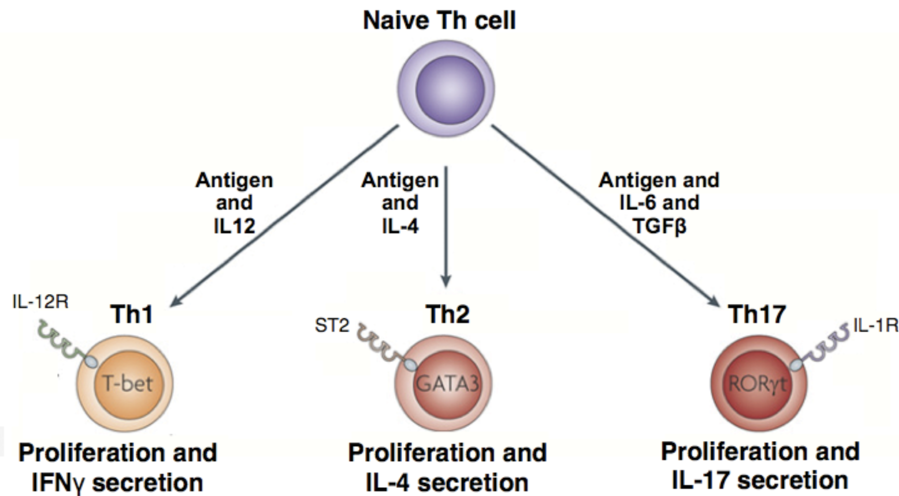

Figure 1. CD4+ T helper subsets. A CD4 T cell (Th) can differentiate into unique effector subsets determined in part by the cytokine milieu that is present when the cell encounters antigens.

Beside the differential expression of transcription factors and cytokine secretion, the Th cell subsets express different cell surface markers. Th1 are characterized by the expression of IL-12R, IL-18R WSX-1, IFNγ R2, CCR5 and CXCR3; Th2 by the expression of IL-4R, ST2, CXCR4, CCR3, CCR4 and CCR8; and Th17 by the expression of IL-1R1, IL-23R, CCR2 and CCR6 (Brusselle et al., 2011).

Cytotoxic T cells

Cytotoxic T cells are important mediators of adaptive immunity against certain viral, protozoan and bacterial pathogens. Mature cytotoxic T cells express the surface protein CD8 and can be activated by the recognition of MHC class I molecules. Activation of cytotoxic T cells promotes cytolysis (osmotic lysis) of damaged cells and the production of cytokines, chemokines and antimicrobial molecules.

Activated cytotoxic T cells are able to induce cytolysis by two distinct molecular pathway: the granule exocytosis pathway, dependent on the pore-forming molecule perforin, or by upregulation of FasL, which can initiate programmed cell death by aggregation of Fas on target cells. In addition, cytotoxic T cells secrete cytokines, including IFNγ and TNF, as well as chemokines that recruit and/or activate effector cells, such as macrophages and neutrophils (Harty et al., 2000).

Regulatory T (Treg) cells.

Treg cells (Tregs) are a subpopulation of T cells which downregulates the immune response and maintains tolerance to self-antigens. Tregs constitutively express high amounts of IL-2R α- chain (CD25) and the transcription factor FOXP3.

From a functional perspective, the various regulation mechanisms of Tregs can be grouped into four basic modes of suppression (Figure 2): by inhibitory cytokines, by cytolysis, by metabolic disruption, and by modulation of DCs maturation or function (Josefowicz et al., 2012).

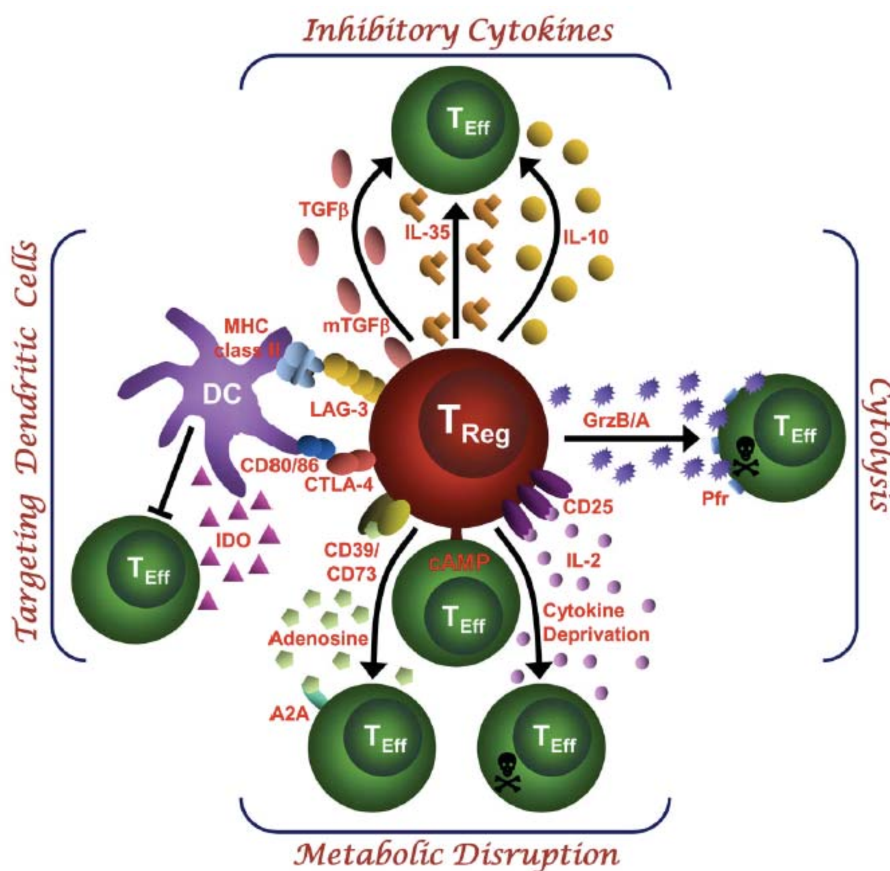

Figure 2. Basic mechanisms used by Treg cells. Treg cells suppress the immune response by i) inhibitory cytokines including IL-10, IL-35 and TGFβ. ii) Cytolysis induced by granzyme A and granzime B and perforin dependent killing mechanisms. iii) Metabolic disruption includes CD25-dependent cytokine deprivation-mediated apoptosis, cyclic AMP mediated inhibition, and CD39-and/or CD73-generated, adenosine-purinergic adenosine receptor (A2A)-mediated immnuosupression. iv) Tatgeting DCs includes mechanisms that module DC maturation and/or function such as lymphocyte astivation gene-3 (LAG-3)-MHC class II mediated suppression of DC maturation, and cytotoxic T lymphocyte antigen 4 (CTLA4)-CD80/CD86 mediated induction of DC- produced immunosuppressive molecule indoleamine 2,3 dioxygenase (IDO) [taken from **(Vignali et al., 2008)**].

Natural Killer T (NKT) cells.

NKT cells are T lymphocytes that express a TCR. This distinguishes them from NK cells, although NKT cells share some markers characteristic of NK cells (CD161 and NKR-P1). In contrast to conventional T lymphocytes and others Tregs, the NKT cell TCR does not interact with peptide antigens, but instead recognizes glycolipids presented by CD1d, a nonclassical antigen-presenting molecules (Godfrey et al., 2004).

1.2.2 Humoral immunity.

The humoral response starts when an external agent enters into the body, and is recognize by B cells. This encounter leads to the activation of naive B cells. These cells differentiate into antibody-producing plasma cells and memory cells. The antibody response to protein antigens requires the participation of both T cells and B cells. The humoral immune response has the capacity to generate different types of antibodies. The nature and magnitude of the humoral immune response are influenced by the relative amounts of different cytokines produced by Th cells at the site of B cell stimulation. Th1 cells promote the production of immunoglobulin isotype IgG2a, while Th2 cells induce the production IgE and IgG4 isotypes.

1.2.2.1 B cells.

B cells develop from a common lymphoid progenitor in the bone marrow. Immature B cells migrate to a secondary lymphoid organ (spleen or lymph nodes) where they become naive mature B cells. Mature B cells express the cell surface markers CD19, CD45RB (B220), CD21, MHC class II and B-cell receptor (IgM). The main function of mature B cells is the production of antibodies, also know as immunoglobulins (Edwards and Cambridge, 2006).

In contrast to T cells, B cells can recognize free antigens, using their BCR, and process them. After recognition of antigens, naive B cells start a clonal expansion and terminal differentiation in plasma cells, that produce a large amount of antibodies (Mauri and Bosma, 2012).

2. Biology of the skin.

The skin is the human body's largest organ, which separates the organism from its environment. The skin has two main layers: the epidermis (upper layer), and the dermis (lower layer).

2.1 Epidermis

The epidermis is a stratified squamous epithelium, which acts as the body's major barrier against environment. It also regulate the amount of water released from the body into the atmosphere through transepidermal water loss (TEWL). The epidermis, constituted at 95% of keratinocytes, and containing melanocytes, Langerhans cells and Merkell cells, is aneural and avascular, nourished by diffusion from the dermis.
The epidermis is composed of proliferating basal and differentiated suprabasal keratinocytes, divided in 3 layers: spinous, granular and cornified (Figure 3).
The basal layer is composed mainly of proliferating and non-proliferating keratinocytes, attached to the basement membrane.
In the spinous layer, keratinocytes become connected through desmosomes and start to produce lamellar bodies, glycosphingolipids, free sterols, phospholipids and catabolic enzymes. Langerhans cells, a DCs found only in the epidermis, are located in the middle of this layer.
In the granular layer, the keratinocytes loose their nuclei, and their cytoplasm appears granular. Keratinocyte-derived lipids are released into the extracellular space through exocytosis to form a lipid barrier.
The cornified layer or stratum corneum is composed of 10 to 30 layer of polyhedral, anuclated keratinocytes (know as well as corneocytes). Corneocytes are surrounded by a protein envelope, filled with water-retaining keratin proteins, attached through corneodesmosomes and surrounded in the extracellular space by stacked layers of lipids (Simpson et al., 2011). Most of the physical barrier functions of the epidermis are localized in this layer.
The epidermis serves as a barrier to protect the body against microbial pathogens, oxidant stress and chemical compounds, and provides mechanical resistance.

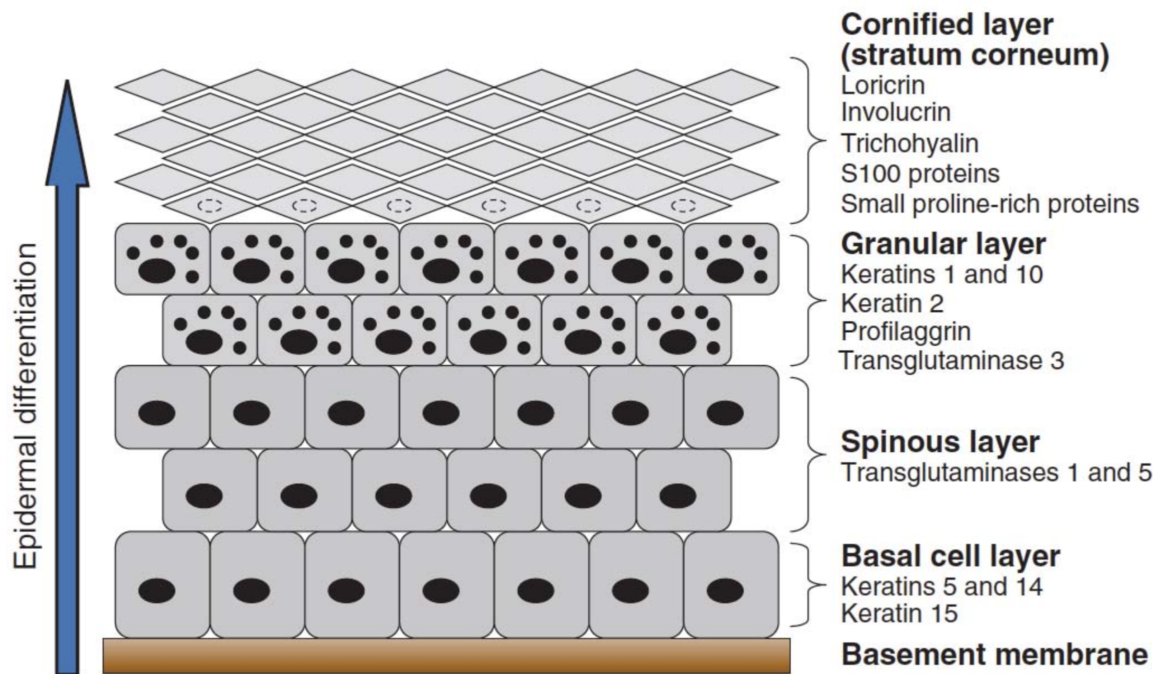

Figure 3. Structure of the epidermis. Keratinocytes, which compose the epidermis, proliferate within the basal cell layer. As differentiation proceeds, keratinocytes progress upwards through the different epidermal layers (the spinous layer, granular layer and cornified layer or stratum corneum). Each stage of epidermal differentiation is characterized by the expression of specific proteins, examples of which are listed on the figure [taken from **(Sandilands et al., 2009)**].

The physical barrier function by preventing water loss, prevents entry of microbes, allergens and irritants, and provides mechanical support. Nucleated cells with their cytoskeleton, tight and gap junctions also contribute to the physical barrier.

The chemical barrier is formed by lipids and the acid mantle (provided by free fatty acids, lactic acid from sweat secretion and urocanic acid from filaggrin protein break down), antimicrobial peptides secreted by keratinocytes from lamellar bodies and filagrin protein that aggregates keratin filaments and produces natural moisturising substances.

The immunological barrier is mediated by the physical barrier, cells (Langherhans cells and γδT cells) and secreted molecules like antimicrobial peptides, cytokines and chemokines (De Benedetto et al., 2009).

2.2 Dermis.

The dermis consists of connective tissue and protects the body from stress and strain. It is divided into two layers, the superficial area adjacent to the epidermis,

called the papillary region, and a deep thicker area known as the reticular dermis. The dermis is tightly connected to the epidermis through a basement membrane. Fibroblasts, macrophages, mast cells, dendritic cells, T cells and adipocytes are the major types of cells. Apart from these cells, the dermis is also composed of matrix components such as collagen, elastin and glycosaminoglycans (Sorrell and Caplan, 2004).

2.3 Skin immunity.

The skin serve as an immuno-protective organ that actively defends deeper body tissues. The skin exploits the immune surveillance versatility of a well-coordinated system of epithelial and immune cells. Collectively, they ensure adequate immune responses against trauma, toxins and infections, while maintaining self-tolerance, preventing allergy and inhibiting autoimmunity.

Keratinocytes can be considered the first immune sentinels encountered by xenobiotics in the skin. They need to be quick and efficient in sensing and responding to danger. Keratinocytes express a vast array of PRRs like TLRs. PRRs activated keratinocytes produce proinflammatory cytokines (IL-1, IL-18, IL-6 and TNF), antimicrobial peptides (cathelicidin, defensins and S100) and chemokines (CXCL9, CXCL10, CXCL11, CCL27, and CCL20), which lead to recruitment and activation of immune cells (Di Meglio et al., 2011).

The three populations of DCs present in normal skin are epidermal Langerhans cells pDCs and myeloid dermal DCs (dDCs). The major functions of these cells are antigen presentation of the pathogens and maintenance of the tolerance in the skin (Toebak et al., 2009).

The T cell compartment in the epidermis is exclusively composed of γδ+ T cells, while the compartment in the dermis is composed of αβ+ T cells expressing cutaneous lymphocyte antigen (CLA). The function of these cells are immune surveillance and skin homeostasis (Clark, 2010; Havran and Jameson, 2010).

Mast cells in the skin are normally located near to blood and lymphatic vessels, where they can encounter substances delivered through these two streams. Mast cells derived molecules regulate the recruitment, trafficking and function of other immune and structural skin cells (Navi et al., 2007).

3. Thymic stromal lymphopoietin (TSLP)

3.1 TSLP and its receptor.

TSLP is a member of the IL-2 cytokine family, and a distant paralog of IL-7. Murine TSLP was discovered in thymic stromal cell line supernatants that supported B cell development. Like IL-7, TSLP can stimulate thymocytes and promote B cell lymphopoiesis. A human homolog was subsequently identified, and further characterization of this cytokine revealed a four helix bundle structure containing six conserved cysteine residues and multiple potential sites for N-linked carbohydrate addition.

Several groups identified a receptor that binds TSLP with low affinity (TSLPR). Upon further characterization, the functional receptor in humans and mice was shown to be a heterodimer of TSLPR and IL-7Rα. This receptor is expressed in a variety of hematopoietic cell population, such as T cells, B cells, NK cells, monocytes, basophils, eosinophils and DCs (Roan et al., 2012).

Binding of TSLP to its receptor leads to activation of the transcription factor STAT5. It is, however, not clear how the TSLP-TSLPR-IL7Rα complex transmits the signal. Recently, a proteomic analysis indicated that Jak2, Erk1/2, JNK1/2 and p38 were inducibly phosphorylated by TSLP stimulation. Using a panel of kinase inhibitors, it was shown that inhibition of PI-3 kinase, Jak family kinase, Src family kinase or Btk suppressed TSLP-dependent cellular proliferation of the pre-B cell line Ba/F3 (Zhong et al., 2012), suggesting that these kinases are implicated in TSLP signaling.

3.2 TSLP expression.

TSLP is expressed mainly by epithelial cells including keratinocytes at barrier surfaces, and is expressed in the thymus and intestinal epithelial cells, suggesting a possible role of this cytokine in the homeostasis of these tissues. Several cell types such as mast cells, fibroblast, DCs, basophils, airway smooth muscle cells and trophoblast express TSLP. Several environmental stimuli like allergens, viruses, microbes, helminths, cigarette smoke and chemical compounds trigger TSLP

production in different cell lines, suggesting that TSLP could have a function as alarm signal against xenobiotics. Pro-inflammatory cytokines, Th2 related cytokines and IgE contribute to TSLP production, indicating an amplification cycle for the Th2 response (Takai, 2012).

In vitro studies have shown that mouse bone marrow derived DCs and splenic DCs produce TSLP in response to TLR stimulation, and that IL4 increases TLR-induced TSLP expression in these cells. In addition, human monocytes and monocyte-derived DCs produce TSLP in response to TLR stimulation. House dust mite (HDM) intratracheally-treated mice exhibit an increased production of TSLP in lung epithelial cells and DCs (Kashyap et al., 2011). In addition, TSLP is produced by a subset of gut DCs (CD103+ DCs). It acts directly on T cells by reducing their capacity to produce IL-17 and fostering the development of FOXP3+ T cells (Spadoni et al., 2012).

Earlier studies in our laboratory have shown that nuclear receptors such as RXR, RAR, VDR and GR regulate the TSLP gene expression (Li et al., 2006; Li et al., 2005; Surjit et al., 2011), demonstrating that nuclear receptors play an important role in the regulation of TSLP.

Inducible gut-specific ablation of Dicer1, a ribonuclease required in microRNA (miRNA) processing, results in lower levels of colon epithelial TSLP mRNA (Biton et al., 2011) and inducible epidermal keratinocytic specific ablation of Dicer1 in mice (Hener et al., 2011) results in increased TSLP production after MC903 (a low-calcemic vitamin D3 analog) treatment, indicating a post-transcriptional regulation of TSLP by miRNA.

3.3 TSLP effects on immune cells.

Several cellular targets of TSLP have been identified including DCs, lymphocytes and granulocytes (Figure 4).

B cells

The exact role of TSLP on B-cell development remains unclear. In vitro studies support the idea that TSLP induces the differentiation of B-cell progenitors into

mature B cells, by mechanisms distinct from IL-7 (Levin et al., 1999). B cell progenitors from fetal liver or adult bone marrow express functional TSLP receptors, but only fetal cells respond to TSLP induction, suggesting that factors differentially expressed in these cells lead to the response to this cytokine only at fetal stage (Vosshenrich et al., 2003).

The function of TSLP in the development of B cells *in vivo* remains controversial. A possible role of TSLP in B cell homeostasis was suggested based on a 10-fold reduction of B cell progenitors and mature B cells in IL7Rα (subunit used by IL-7 and TSLP) deficient mice, compared with a IL-2 receptor gamma (also know as common gamma chain) deficient mice (subunit used by IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21). These results suggest that TSLP is the main factor stimulating IL-7 independent B-lymphopoiesis (Vosshenrich et al., 2003). However, examination of TSLPR deficient mice revealed normal B-cell development (Carpino et al., 2004). There are not reports on the B-cell compartment in TSLP deficient mice.

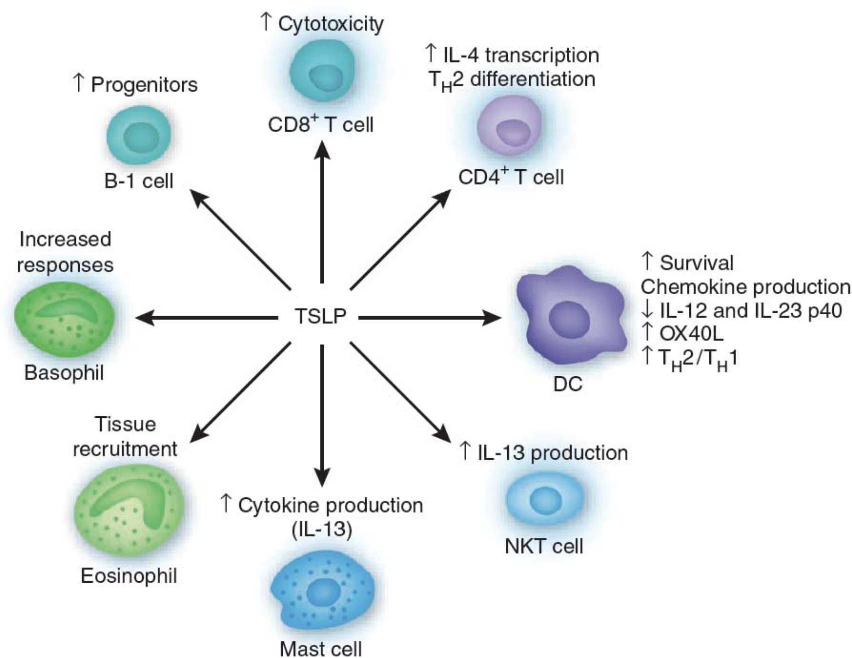

Figure 4. Immune cell responding to TSLP and its effects [taken from **(Ziegler and Artis, 2010)**].

Transgenic expression of TSLP using β-actin promoter disrupts hematopoietic homeostasis by causing decreased B lymphopoiesis and increased myelopoiesis (Osborn et al., 2004), suggesting that TSLP has a regulatory effects during hematopoiesis. Nevertheless, inducible TSLP expression in keratinocytes results in an enhanced expansion of all immature and mature B cell populations in the

periphery (Astrakhan et al., 2007). Moreover, constitutive TSLP overexpression in keratinocytes restores B cell development in IL-7 deficient mice (Chappaz et al., 2007), and exposure to high TSLP levels during neonatal hematopoiesis results in a drastic expansion of the immature B cells, causing a B-lymphoproliferative disorder (Demehri et al., 2008).

Dendritic cells

In vitro studies have shown that human TSLP induces the production of costimulatory molecules and secretion of chemokines in mDCs. TSLP treatment induces increased CD40 and CD80 expression, and the release of CCL17 and CCL22 in mDCs (Reche et al., 2001). In addition, TSLP-treated human DCs promote differentiation of naive Th cells in Th2 cells through up-regulation of OX40L expression, and decrease the production of the Th1-polarizing cytokine IL-12 (Ito et al., 2005). In mice, TSLP induces the expression of the chemokine CCL17, and increases the production of MHCII and of the costimulatory molecules CD40, CD80 and CD86 (Al-Shami et al., 2005; Zhou et al., 2005). In addition, mouse myeloid DCs express OX40L after TSLP treatment, which induces Th2 polarization of naive CD4+ T cells (Seshasayee et al., 2007). These data suggest that TSLP programs human and mouse DCs to promote a Th2 immune response. However, the cytokines and costimulatory molecules produced by DCs with physiological TSLP levels are still elusive. A recent report propose that epithelial cell-conditioned DCs with physiological amounts of TSLP drives non-inflammatory Th2 but not Th1 polarization (Rimoldi et al., 2005).

T cells

Beside the Th2 polarization induced by TSLP-treated DCs, T cells respond directly to TSLP. In cultures of double negative (CD4-CD8-) thymocytes, TSLP has a minimal proliferative activity, that is enhanced by combination with IL-1 (Sims et al., 2000), suggesting a possible role of TSLP in T cell development. In addition, T cell development is apparently normal in TSLPR-/- mice, although TSLPR-gamma chain double deficient mice show a reduction in T cell lymphocytes compared with gamma chain deficient mice, suggesting some non-redundant influence of TSLP in T cell development. Futhermore, CD4+ T cells from TSLPR-deficient mice expand less

efficiently than those from wild-type mice after sublethal irradiation (Al-Shami et al., 2004). Moreover, constitutive TSLP overexpression in keratinocytes restores T cell development in IL-7 deficient mice (Chappaz et al., 2007). These results suggest that TSLP promotes IL-7 independent T-lymphopoiesis

In vitro studies have shown that TSLP acts directly on T cells in the presence of TCR stimulation, promotes the proliferation of human and mouse naive CD4+ T cells, and the differentiation of mouse naive CD4+T cells in Th2 cells through induction of IL-4 gene transcription (Omori and Ziegler, 2007; Rochman et al., 2007). Moreover, *in vitro* differentiated Th2 cells express TSLPR at higher levels than Th1 and TH17 cells, which correlates with the ability of TSLP to promote proliferation and survival of activated Th2 cells (Kitajima et al., 2011).
Mouse CD8+ T cells also express TSLPR, although TSLPR expression is low or absent on naive CD8+T cells. However, following TCR activation, TSLPR expression is upregulated in mouse and human CD8+T cells (Akamatsu et al., 2008; Rochman and Leonard, 2008). In activated CD4+ and CD8+ T cells, TSLP stimulation up-regulates the survival protein Bcl-2 in a STAT-5-dependent manner (Kitajima et al., 2011; Rochman and Leonard, 2008), suggesting that TSLP might play a role in the production of memory T cells.

Mast cells

Human progenitor derived-mast cells express the functional receptor for TSLP, and respond to TSLP by increasing the production of various cytokines, including IL-5, IL-16, IL-10 and IL-13. However, TSLP does not induce mast cells degranulation or release of lipid mediators. In addition, TSLP mRNA is overexpressed in bronchial mucosa and TSLPR is also expressed *in vivo* in mast cells infiltrating the bronchial mucosa of asthmatic patients (Allakhverdi et al., 2007), suggesting that TSLP produced by epithelial cells might enhance Th2 responses through an increased response in mast cells. Nevertheless, mast cells deficient mice ($Kit^{w\text{-}sh}$) present a skin inflammation similar to that of wild-type mice after intradermal injection of TSLP (Jessup et al., 2008) or after MC903 treatment (our unpublished data).

Natural Killer T (NKT) cells.

Mouse NKT cells express functional TSLPR, and respond to TSLP by increasing the production of IL-13. In addition, in an allergen induced asthma model, increased airway hyperreactivity was no observed in TSLP transgenic mice lacking NKT cells, while airway eosinophilia and IgE levels were similar to NKT sufficient mice (Nagata et al., 2007).

Human NKT cells from healthy donors express both TSLPR and IL-7Ra mRNA and protein, and TSLP-treated cells secrete high amounts of IL-4 and IL-13, but not IFNγ (Wu et al., 2010). These results suggest that TSLP may directly activate human and mouse NKT cells to secrete Th2 cytokines.

Eosinophils

TSLPR and IL-7Ra are constitutively expressed in isolated human eosinophils, and TSLP enhances eosinophil survival and decreases apoptosis. In addition, TSLP-treated eosinophils release the inflammatory cytokine IL-6 and the chemokines CXCL8, CXCL1 and CCL2, without degranulation (Wong et al., 2010).

Basophils

Recombinant TSLP injection in mice results in selective accumulation of basophils expressing IL-4 in the blood (Perrigoue et al., 2009). In addition, mice receiving hydrodynamic tail vein injections of a plasmid encoding TSLP have an increased basophil number in the spleen, blood, lung and bone marrow (Siracusa et al., 2011), suggesting a possible TSLP function in basophil production. Basophil progenitors from bone marrow characterized as non-B, non-T, CD34+, c-kit- and FcERI+ express a functional TSLP receptor, and respond to TSLP producing mature basophils (Siracusa et al., 2011).

The effect of TSLP on various immune cell types demonstrates that this cytokine can affect the development of the immune response. Several studies implicate TSLP not only in allergic disorders, but also in other diseases, such as cancer, autoimmunity or infections.

4. Atopic dermatitis.

Atopic dermatitis (AD) is a common pruritic inflammatory skin disease often associated with a family and/or personal history of allergy. The prevalence of the disease is on the rise all over the world, but particularly in Western and industrialized societies (Leung and Bieber, 2003; Novak et al., 2003). The disease causes a tremendous physical, psychological, and financial burden to patients and their families, manifested by loss of school attendance in children, loss of productivity in adults and in substantial consumption of health care resources.

The hallmarks of AD are skin barrier dysfunctions, which result in dry itchy skin, and leads to scratching that inflicts mechanical injury and allergic sensitization to environmental antigens and allergic skin inflammation.

Histopathological analysis of AD skin lesions revealed an intense mononuclear cell infiltrate in the dermis with T cells, monocytes, macrophages, dendritic cells, mast cells and eosinophils and products secreted by these cells. In addition, there is fibrosis and collagen deposition in chronic skin lesions.

Two hypotheses have been proposed for the pathogenesis of AD. One hypothesis holds that the primary defect is intrinsic to skin epithelial cells and results in a defective skin barrier function with a secondary immune response to antigens that enter through the defective skin barrier (inside-outside) (Elias et al., 1999; Taieb, 1999). The other hypothesis holds that the primary abnormality is in the immune system and results in Th2/IgE-dominated immune response that causes a secondary defect in barrier skin function (outside-inside) (Leung et al., 2004).

4.1. Epidemiology.

The prevalence of AD differs between countries/regions. In industrialized countries, the prevalence of AD has at least doubled in the last three decades (Stensen et al., 2008; Tay et al., 2002; Yura and Shimizu, 2001), affecting approximately 15-30% of children (Williams and Flohr, 2006). Conversely, in developing countries it has been reported to be less than 10% (Ergin et al., 2008). The lifetime prevalence is estimated to be between 10 and 20% (Schultz Larsen et al., 1996).

The onset of AD occurs during the first 6 months of life in 45% of children, during the first year of life in 60% of patients and before the age of 5 years in at least 85% of affected individuals (Kay et al., 1994). In children with onset before the age of 2 years, 20% will have persisting manifestations of the disease, and an additional 17% will have intermittent symptoms by the age of 7 years (Illi et al., 2004).

4.2 Clinical aspects of atopic dermatitis.

The clinical manifestations of AD vary with age. In infancy, the skin lesions are located on the cheeks and scalp (Figure 5A). In childhood, lesions are located on flexures, nape of the neck and on dorsal aspects of the limbs (Figure 5B). In adolescents and adults, lichenified plaques (fibrosis and increased collagen deposition in the skin) affect the flexures, head and neck (Figure 5C).
Acute AD skin lesions show intensely pruritic, erythematous papules associated with excoriation and serous exudation (Figure 5A and B).

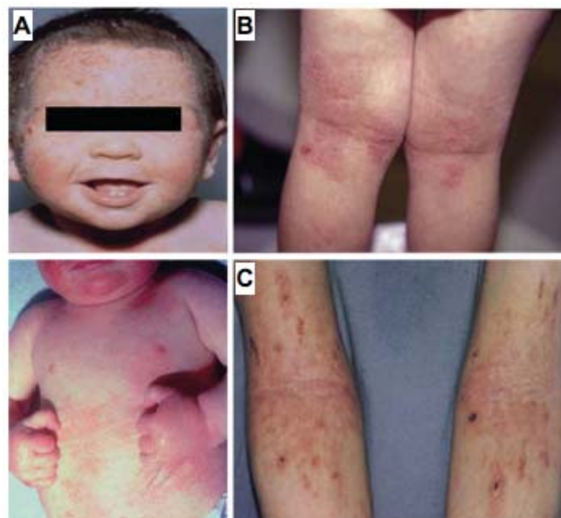

Figure 5. Clinical aspects of AD skin lesions. Lesions on cheeks and scalp in infant with AD (A), lesions on knee flexures of children with AD (B) and lichenified plaques on elbow flexures in adolescent with AD (C) [adapted from **(Oyoshi et al., 2009)**].

AD skin lesions have reduced terminal differentiation of keratinocytes, decreased cornification, and reduced lipid levels. Histological analysis of acute AD tissue samples shows acute eczematous dermatitis with highly spongiotic epidermis, whereas that of chronic AD shows a hyperplastic epidermis with much less spongiosis (Figure 6). AD is characterized by increased numbers of eosinophils and

mast cells, and absence of neutrophils. Patients with AD present orthokeratosis (absence of nuclei from stratum corneum cells), and hypogranulosis (Figure 6).

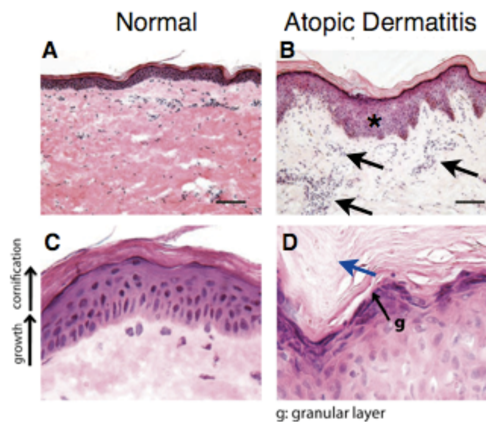

Figure 6. Histological analysis of normal skin and skin from patients with AD. H&E staining from healthy skin (A and C) and AD skin lesion (B and D). Black arrows indicate increased leukocyte infiltration, asterisk indicates epidermal hyperplasia and blue arrow indicates hyperkeratosis in AD skin lesion [Adapted from **(Guttman-Yassky et al., 2011)**].

Non-lesional skin in patients with AD is frequently dry and has a greater irritant response to chemicals or physical agents than normal skin (Oyoshi et al., 2009).

Patients with AD are highly susceptible to cutaneous bacterial, fungal and viral infections. Bacterial colonization with *Staphylococcus aureus* is the most common skin infection in AD. It occurs in more than 90% of lesional skin and in more than 70% of nonlesional skin. The number of bacteria correlates with the degree of skin inflammation. Moreover, the presence of IgE antibodies specific against staphylococcal superantigens correlate with the severity of AD, and total seum IgE levels (Mrabet-Dahbi et al., 2005). In addition to bacterial superinfections (infection following a previous infection), yeast Malassezia species is also present in superinfected AD lesions. Patients with AD run also a higher risk of developing severe skin superinfection with a number of viruses, that includes Herpes simplex virus (HSV), Molluscum contagiosum virus and Vaccinia virus (VV). These conditions exacerbate AD skin lesions.

Scratching induces skin mechanical injury that results in damage epidermal barrier and the release of a panel of proinflammatory cytokines and chemokines, which are believed to play an important role for initiating an allergic skin inflammation.

4.3 Genetics

AD is a genetically complex disease with a high familial occurrence. Twin studies of AD have shown concordance rated of 0.72-0.86 in monozygotic, and 0.21-0.23 in dizygotic, twin pairs, indicating that genetic factors play an important role in the development of this diseases (Wuthrich et al., 1981). However, the results of numerous studies on affected individuals and their families show that the heredity does not follow classical Mendel's laws, because AD is a multigenic disease influenced by the interactions of affected genes and environmental factors.

The results of genome-wide linkage studies point to various candidate regions, in different chromosomes (Bradley et al., 2002; Christensen et al., 2009; Cookson, 2001; Enomoto et al., 2007; Guilloud-Bataille et al., 2008). To date, several candidate genes are identified in AD which can be categorized into three groups: genes involved in skin barrier function, genes involved in innate immunity and genes involved in adaptive immunity.

Genes involved in skin barrier function.

AD shows a strong genetic linkage to chromosome 1q21, which contains the human epidermal differentiation complex (EDC) (Cookson, 2004). Mutations in the filaggrin (FLG) gene located in the EDC are identified in populations from Europe, Japan and USA. However, only a fraction of AD patients are carriers of these mutation, suggesting that other genes involved in skin barrier function are implicated. Moreover, mutation in genes encoding proteins involved in epidermal differentiation and proliferation, e.g. SCCE (Vasilopoulos et al., 2004), SPINK5 (Kusunoki et al., 2005), C11orf30 (Esparza-Gordillo et al., 2009), OVOL1 and ACTL9 (Paternoster et al., 2012), have been associated with AD.

Genes involved in innate immunity.

High susceptibility to infection in AD patients suggest a defective recognition of microbial products. Several polymorphism in PRRs like TLR2, TLR9, NOD1, NOD2 and CD14 (Bussmann et al., 2011), downstream molecules like TOLLIP and IRAK3,

or antimicrobial peptides like DEFA4, DEFA5, DEFA6 and DEFB1 (Barnes, 2010), are linked with this disease.

Genes involved in adaptive immunity.

The Th2 response is dominant in skin lesions of AD patients. Several polymorphism in Th2 related molecules, like cytokines [IL-4, IL-5, IL-10, IL-13, IL-18, IL31 (Schulz et al., 2007) and TSLP (Gao et al., 2010)], cytokine receptors [IL4R, IL5R, IL13Ra (Barnes, 2010), IL7Ra and TSLPr (Gao et al., 2010)], chemokines [eotaxin-1, MCP1, MIP1A, RANTES and TARC (Barnes, 2010)], or transcription factors [GATA3 and STAT6 (Barnes, 2010)], are linked with AD and atopy.

4.4 Environmental factors

The difference in prevalence of AD between urbanized and rural communities cannot be explain by a genetic predisposition, this suggest that environmental factors have a major role in the development of this disease. A major theory explaining the increase in prevalence and incidence of AD and others atopic diseases is the “hygiene hypothesis” (Schram et al., 2010). This view indicates that modern life style results in insufficient microbial stimulation of the immune system in newborns, which leads an inappropriate modulation of the immune response, leading to autoimmune or allergic diseases later in the life.

There are several factors that differ between rural and urban areas: family size, exposure to animals, maternal age, way of newborn delivery, diet, housing style, exposure to infections, exposure to pollution, water intake, vaccination status and use of antibiotics, to name a few.

4.5 Physiopathology

The cause of atopic dermatitis is unknown, but the disease seems to results from a combination of skin barrier and immunological defects.

The inside-outside theory does not explain a group of patients with a clinically indistinguishable skin phenotype, without detection of serum specific IgE (Novembre et al., 2001), suggesting that skin defects lead to a local inflammation without systemic immune response. However, the outside-inside theory does not explain that around 50% of patients with intrinsic defects in skin barrier related molecules, such as filaggrin, do not present an AD phenotype, suggesting that other factors than skin barrier dysfunction are implicated in the physiopathology of AD. These data suggest that one theory completes the other.

Role of epidermal barrier in AD

The skin barrier defect in AD patients is reflected by increased transepidermal water loss (TEWL) in both lesional or non-lesional skin, and by augmented penetration of chemical compounds (Proksch et al., 2009).

Lipid analysis of stratum corneum from skin of patients with AD shows a significant reduction in ceramide content, increase in cholesterol (Di Nardo et al., 1998) and abnormally low levels of omega-6 fatty acids (Melnik and Plewig, 1992).

Mutations in the filagrin (FLG) gene were identified initially as a cause of ichthyosis vulgaris, and subsequently as a major predisposing factor for AD.

FLG deficiency may play a role in the development of the features of AD. Indeed, a number of mutations in this gene lead to a functional barrier defect, with enhanced cutaneous allergen penetration (Scharschmidt et al., 2009), priming an allergen sensitization and increased TEWL values. The elevation in skin-surface pH observed in FLG-deficient persons (Kezic et al., 2011) could induce a protease hyperactivity and the development of a Th2 inflammation without presence of allergen (Lee et al., 2010b), and enhance *S. aureus* colonization (Miajlovic et al., 2010). In addition, AD patients with FLG mutations present increased expression of inflammatory cytokines (Kezic et al., 2012).

Decreased levels of other structural skin barrier components, such as involucrin, loricrin (Kim et al., 2008) and keratin-10 (Jensen et al., 2004) are also associated with AD.

Increased serine protease activities in acute eczematous AD are associated with impaired barrier function (Voegeli et al., 2009). Mutation in the skin-specific serine

protease inhibitor gene, SPINK5, are implicated in Netherton's syndrome, which has many features of AD, including dermatitis, eosinophilia and high IgE levels (Chavanas et al., 2000).

In addition to intrinsic factors that lead to an impairment of the skin barrier, several external factors, such as physical stress (mechanical, thermal or radiation damage), chemical stress (use of solvents and soaps) and environmental conditions (ambient temperature, humidity, UV radiation) can affect the function of the skin barrier.

Role of immunity in AD

Components of both innate and adaptive immunity contribute to the immunopathology of AD. The skin lesions of patients with AD are characterized by production of chemokines and cytokines by keratinocytes, and by the infiltration of activated T cells, eosinophils, mast cells, dendritic cells and macrophages (Figure 7).

Keratinocytes

Epidermal keratinocytes from AD patients produce a unique profile of chemokines and cytokines, accompanied by reduced AMP expression. Thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 constitute keratinocyte-derived cytokines, which collectively drive Th2 polarization through complementary and sometimes synergistic mechanisms (Carmi-Levy et al., 2011). Skin from patients with AD exhibit increased levels of IL33 (Pushparaj et al., 2009), IL25 and its cognate receptor IL25R (Wang et al., 2007) and TSLP (Soumelis et al., 2002).

Increased expression of TARC/CCL17 and CTACK/CCL27 (Tamaki, 2008) is observed in skin from patients with AD. Such elevated levels of chemokines could enhance the recruitment and homing of T cells into the skin, enhancing the Th2-type immune response observed in AD patients.

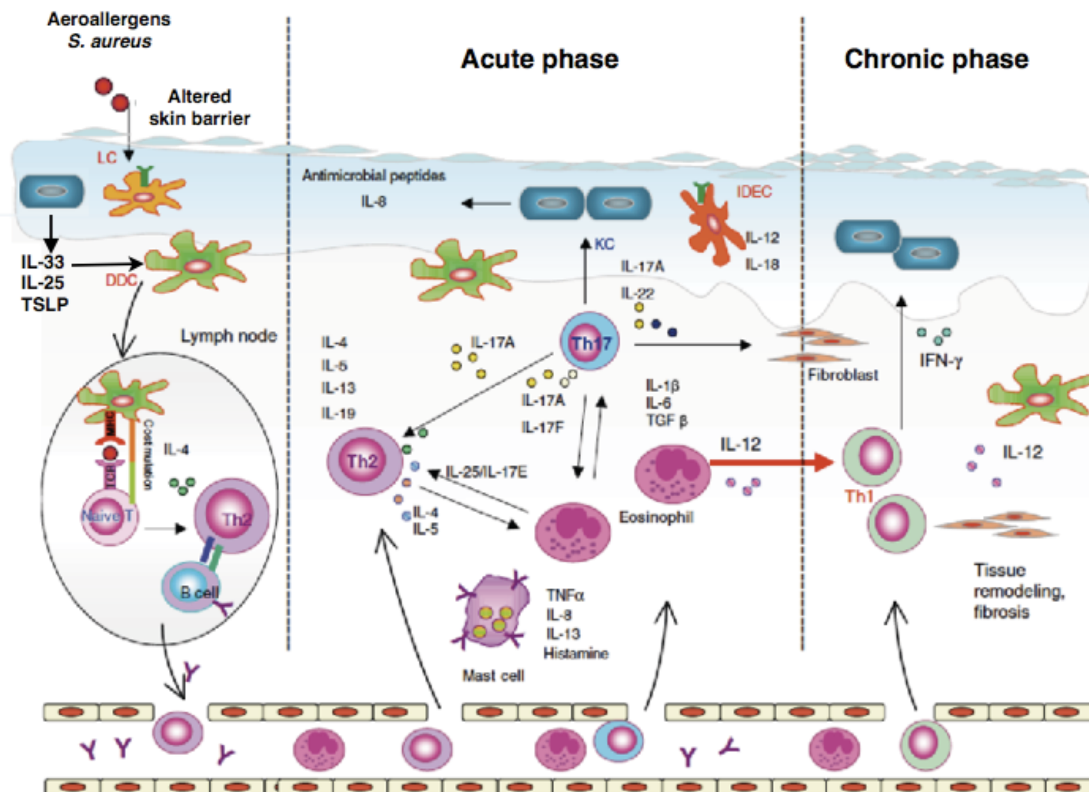

Figure 7. Schematic representation of the Immune complexity involved in AD pathogenesis. During the first contact with an allergen, keratinocytes produce Th2-promoting cytokines that activate cutaneous dendritic cells [dermal dendritic cells (DDCs) and/or Langerhans cells (LC)], and then these cells migrate to draining lymph nodes, where they induce Th2 polarization and IgE production. A second exposure to the antigen (acute phase) causes cell reactivation and release of inflammatory molecules causing an influx of eosinophils, Th2 and TH17 cells. Secretion of Th1-promoting cytokines by eosinophils, DDCs and inflammatory dendritic cells (IDECs) drives the shift toward the chronic phase of AD [adapted from **(Di Cesare et al., 2008)**].

Patients with AD often suffer from bacterial, fungal and viral infection of the skin, suggesting a defective innate response in the skin. Reduced expression of human beta defensin 2 (hBD-2) and cathelicidin antimicrobial peptide (LL-37) in the epidermis of patients with AD (Schroder, 2011) could explain the high susceptibility to cutaneous infections.

TSLP and AD

TSLP protein is highly expressed in lesions of patients with AD, but undetectable in nonlesional skin from the same patients, or other skin diseases (Soumelis et al., 2002), suggesting that increased TSLP levels are characteristic of AD skin inflammation. In addition, serum TSLP levels are augmented in patient with AD, independently of the sensitization status (Alysandratos et al., 2010; Lee et al., 2010a).

Mice studies revealed the implication of TSLP in the AD pathogenesis. Transgenic expression of TSLP in mouse keratinocytes promotes an AD-like phenotype characterized by skin inflammation with eosinophil infiltration, epidermal hyperplasia and increased levels of Th2 cytokines and serum IgE (Li et al., 2005; Yoo et al., 2005). In addition, TSLP induced expression by ablation of RXRα and RXRβ, Notch or SPINK5 in epidermal keratinocytes (Briot et al., 2009; Demehri et al., 2009; Li et al., 2005), or by MC903 topical treatment (Li et al., 2006) promotes an AD-like skin inflammation. Moreover, intradermal recombinant TSLP injection induces an AD skin inflammation (Jessup et al., 2008), demonstrating that TSLP is sufficient to induce an AD-like skin inflammation.

The role of TSLP during allergic skin inflammation is however not well know. Allergic skin inflammation is reduced in TSLPR-deficient mice after epicutaneous sensitization by the patch method (see below), as evidenced by decreased dermal infiltration with eosinophils and decreased expression of Th2 cytokines in allergen-treated skin and skin-draining lymph nodes. However, splenocytes of allergen-treated TSLPR-deficient mice proliferate and produce similar levels of Th2 cytokines as WT mice. In addition, there is not difference in T cells infiltrating the skin of OVA-treated TSLPR-deficient mice and WT controls, suggesting that TSLP plays an important role in the effector phase of the allergic inflammation rather than in the induction phase (He et al., 2008).

Dendritic cells (DCs)

During inflammation there appears to be an additional population of myeloid dermal "inflammatory" DCs (IDCs) (Zaba et al., 2009).

Skin from patients with AD exhibit increased numbers of LCs, dermal DCs and IDCs, showing high surface expression of FCεRI (Novak, 2012), which enables specific allergen uptake.

The decreased number of plasmocytoid DCs (pDCs) in the skin of patients with AD, and decreased release of type I interferons after allergen challenge might further contribute to the high susceptibility to viral infection (Novak, 2012).

In vitro studies revealed that DCs enriched from skin of patients with chronic AD (a Th2-related disease) or chronic psoriasis (a Th1, Th17-related disease) are able to

induce any kind of T-cell response (Fujita et al., 2011), suggesting that exposure of DCs to different stimuli during these skin diseases might influence the nature of the immune response.

CD4+ T cells

Recruitment of T cells into the skin and their effector responses are considered to be key features in the pathogenesis of AD. Inflammation in atopic dermatitis consists in three phases: an initial Th2 phase which precedes an acute phase, in which Th17 is predominant, and finally a shift to Th1 in the chronic phase.

Th2 cells

Acute skin lesions in patients with AD exhibit a Th2 dominant inflammation, characterized by dermal infiltration of CD4+ cells and eosinophils, and increased expression of the Th2 cytokines IL-4, IL-5 and IL-13 in skin.

The selective homing of CD4+ T cells to skin represents an important immunological event in the development of allergic skin inflammation (Santamaria-Babi, 2004). Peripheral blood and skin of patient with AD exhibit a high proportion of CLA +CCR4+CD4+ cells, and skin from these patients exhibits a high level of the chemokines TARC/CCL17, a ligand of the CCR4 receptor, suggesting that TARC may be an important signal for lymphocyte homing in the skin of AD patients (Vestergaard et al., 2000).

The increased expression of Th2-cytokines in the skin of patients with AD contribute not only to aggravate the allergic response, but also to increase skin barrier defects. Th2 cytokines impair permeability recovery after acute perturbation, and decrease the expression of genes in the epidermal differentiation complex (Kim et al., 2008).

The implication of Th2 cytokines in the development of AD has been demonstrated by several mouse model (see below).

Th17 cells

Peripheral blood from patients with AD exhibit an increase in IL-17+CD4+ T cells compared with healthy controls. Additionally, the percentage of Th17 cells in the circulation correlates with the severity of the disease, suggesting a potential role of Th17 cells in exacerbation of the disease.

Skin from patients with AD exhibit increased levels of cells producing IL-17 compared to controls. The expression of this cytokine is more evident in acute than in chronic lesions (Koga et al., 2008; Toda et al., 2003).
IL-17 and IL-22 have a synergistic effect in the induction of some cytokines and chemokines in keratinocytes in culture, suggesting that Th17 cells could promote the aggravation of cutaneous lesions in patients with AD.

Th1 cells

It is proposed that the imbalance between Th1/Th2 subsets leads to the development the atopic dermatitis and allergy.
Peripheral blood from patients with AD have a reduced percentage of IFNγ+CD4+ T cells compared to healthy controls (Lonati et al., 1999), possibly by increased apoptosis of these cells mediated by increased levels of Th2 cytokines in the circulation (Akkoc et al., 2008). Decreased levels CCR5 and CXCR3 (surface markers of Th1 cells) on CLA+ T cells from blood of patients with AD might be responsible for keeping the Th2-cytokine profile in the skin of these patients (Seneviratne et al., 2007).

Regulatory T cells

Peripheral blood from patients with AD exhibit a similar or higher number of Tregs than healthy controls, with a comparable suppressive activity (Ou et al., 2004). It was initially reported that Tregs were absent in AD skin lesions (Verhagen et al., 2006). Later on, however, the presence of Tregs in AD skin lesions was reported (Schnopp et al., 2007).

Eosinophils

The presence of eosinophils in the cutaneous inflammatory infiltration of AD has been established for a long time. Tissue and blood eosinophilia is a feature of acute and chronic AD, and correlates with disease severity (Liu et al., 2011).
Eosinophil degranulation and dermal deposits of MBP is observed in AD skin lesions. In addition, increased levels of eosinophil-specific granule proteins are present in blood of patients with AD, and correlate with disease severity.

Because Th2 cytokines induce IL-12 production by eosinophils, a possible role of eosinophils in the switch from Th2 response in acute lesions to Th1 response in chronic AD is proposed (Liu et al., 2011).

Mast cells

In acute AD lesions, mast cells are present in similar number than healthy skin, but they present degranulation. In contrast, chronic lesions exhibit increased mast cell number, especially in areas of lymphocytic infiltration.
Mast cells can function as effectors and regulatory cells during AD pathogenesis. IL-4 and IL-13 are expressed by mast cells after different stimuli. These cytokines are key factors for Th2 polarization, suggesting a possible role during Th2 polarization in the skin of patients with AD. Histamine released by mast cells affects the functions of keratinocytes and dendritic cells, by promoting the expression of various arrays of chemokines, cytokines and growth factors, thus exacerbating skin inflammation. Mast cells can bind IgE via their cell surface high-affinity IgE receptor (FcεRI). After binding, mast cells release lipids mediators, and produce a large variety of cytokines, chemokines and growth factors (Liu et al., 2011).

Basophils

The discovery of a unique basophil-specific marker, basogranulin, has enabled their identification in different skin diseases (Ito et al., 2011). In humans, however, is not clear if these cells contribute to the pathogenesis of allergic skin diseases.

5. Asthma

Asthma is a complex airway disorder manifested by a variable degree of airflow obstruction, bronchial hyperresponsiveness and airway inflammation. Depending on the underlying causes, asthma is divided in: allergic asthma, aspirin-sensitive asthma, exercise-induced asthma and irritant-induced asthma. Allergic asthma is the most common type of asthma.

5.1 Epidemiology.

The prevalence of asthma differs between countries/regions. A wide variation in prevalence rates of asthma is documented: studies of both children and adults reveal a low prevalence in developing countries and high prevalence in developed countries. It is estimated that asthma has a 7-10% prevalence worldwide (Asher et al., 2006).

5.2 Clinical aspects of asthma.

Asthma is clinically characterized by symptoms of wheeze, dyspnea and cough, and by objective evidence of variable airflow obstruction and airway hyperresponsiveness (AHR). Most patients with asthma have increased IgE serum levels and present allergen-specific IgEs (allergic asthma). Few patients have however no IgE involvement (Wenzel, 2006).

Histopathological features of lung from asthma patients are typically described as: (1) infiltration of eosinophils, lymphocytes, neutrophils, mast cells, macrophages and dendritic cells; (2) hyperplasia and hypertrophy of bronchial smooth muscle; (3) denudation of airway epithelium and deposition of collagen beneath the basement membrane in lungs; (4) increased blood vessel number and vessel endothelium proliferation; and (5) mucus hyperplasia, with increased number of mucus-secreting goblet cells in the epithelium (Figure 8) (Bai and Knight, 2005).

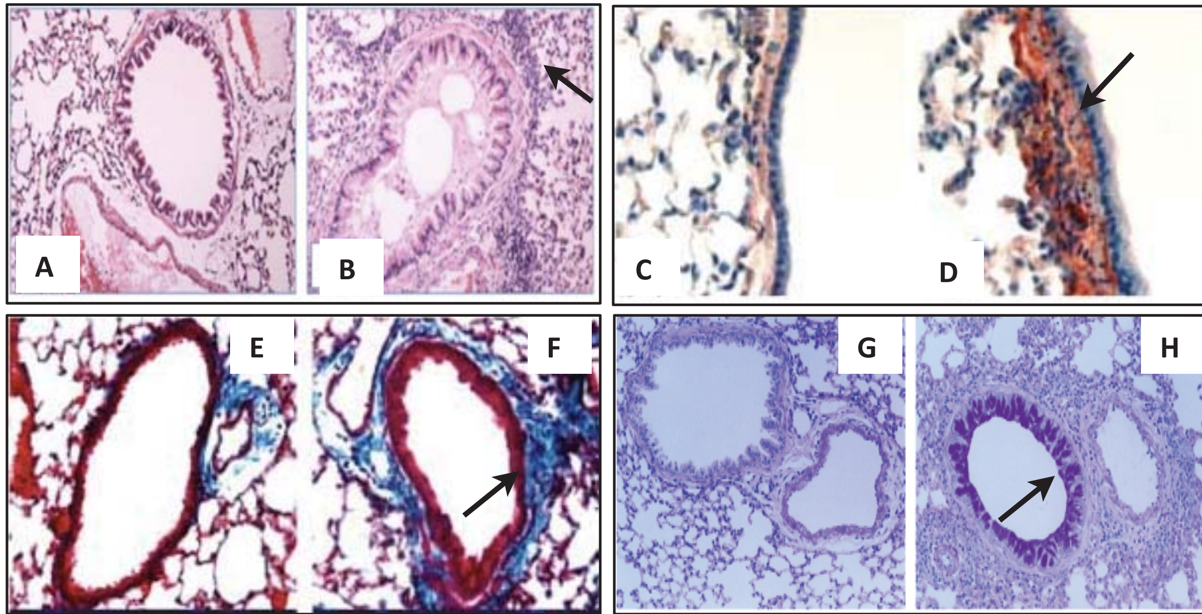

Figure 8. Histological analysis of healthy and asthmatic lungs. H&E staining from healthy (A) and asthmatic (B) lung. Black arrow indicates peribronchial cell infiltrates. α-smooth muscle actin immunostaining from healthy (C) and asthmatic (D) lung. Black arrow indicates bronchial muscle hyperplasia. Masson's trichrome staining from healthy (E) and asthmatic (F) lung. Black arrow indicates increased collagen deposition. PAS staining from healthy (G) and asthmatic (H) lung. Black arrow indicates goblet cell hyperplasia.

5.3 Genetics and environmental factors.

The pathogenesis of asthma is multifactorial, and reflects a complex interaction of genetics and environmental factors. Commonly, in genetically susceptible individuals, the exposure to ordinary environmental factors can either induce or exacerbate the disease.

Asthma susceptibility genes fall into four main groups: (1) genes associated with innate immunity and immunoregulation, such as CD14, TLR 2, TLR4,TLR6, TLR10, NOD1, NOD2, IL-6, IL-10 and TGFβ; (2) genes associated with Th2 polarization and effector functions such as GATA-3, T-bet, IL-4, IL4-RA, STAT6, IL-12p40, IL13, FcεR1 and TSLP; (3) genes associated with epithelial biology and mucosal immunity such as CCL11, CCL24, CCL26, DEFB1 and FLG and (4) genes associated with lung function, airway remodeling and disease severity, such as LTC4S, GSTP1, GSTM1, TBXA2 and ALOX5 (Vercelli, 2008).

Westernized lifestyle is correlated with asthma and allergy. However the factors implicated in the increased prevalence of atopic diseases in the urbanized areas are still unknown.

5.4 Physiopathology.

Asthma shares common immunological features with other atopic diseases, including Th2 inflammation, peripheral and lung eosinophilia, and elevated IgE. Asthma pathophysiological mechanisms involve a coordinated response of the airway epithelium, airway smooth muscle and immune cells to environmental stimuli (Figure 9).

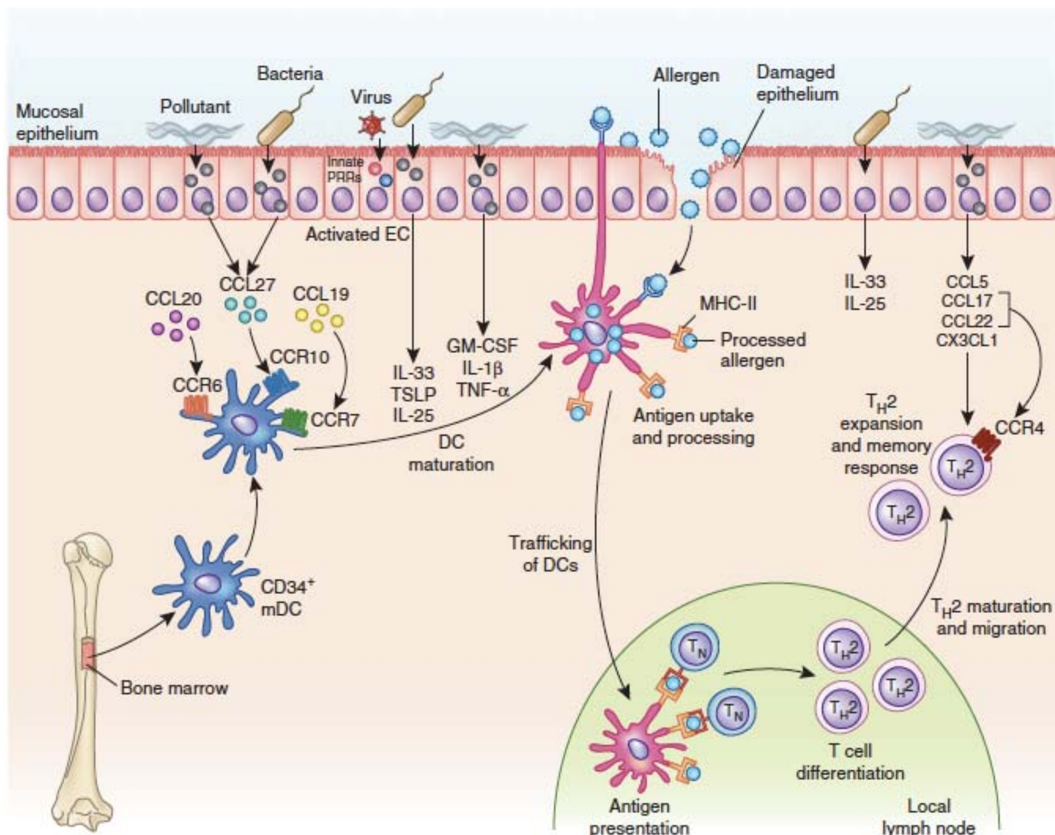

Figure 9. Schematic representation of the coordinated response of airway epithelium and immune cells in asthma pathogenesis. Airway epithelium produces cytokines and chemokines in response to environmental factors that attract and activate myeloid dendritic cells (mDCs), which then migrate to draining lymph nodes, where they induce a Th2 polarization [taken from **(Holgate, 2012)**].

Airway epithelial cells may play an active role in asthmatic inflammation through the release of many inflammatory mediators, cytokines, chemokines and growth factors (Lambrecht and Hammad, 2012). DCs attracted and activated by factors produced by airway epithelial cells induce the production of allergen-specific T-cells producing Th2 cytokines (Holgate, 2012). The production of Th2 cytokines in the lung results in the

recruitment and survival of eosinophils, and in the maintenance of mast cells in the airways (Spencer and Weller, 2010).

5.5 TSLP and allergic airway diseases.

Allergic rhinitis

Increased TSLP mRNA and protein levels in the nasal epithelium have been found in biopsies from patients with allergic rhinitis. TSLP levels correlate with Th2 cytokine levels and eosinophil number in nasal polyps of patients with allergic rhinitis (Kimura et al., 2011; Mou et al., 2009).
Blocking TSLP by antibodies in a mouse model of allergic rhinitis reduces the frequency of nasal rubs, the infiltration of leukocytes and the mucus production in the nasal epithelium and submucosa (Miyata et al., 2008), suggesting a possible implication of TSLP during the pathogenesis of the allergic rhinitis.

Asthma

Increased number of cells expressing TSLP mRNA in the bronchial epithelium and submucosa are present in asthmatic patients, which correlates with an increased expression of Th2-attracting chemokines (TARC/CCL17 and MDC/CCL22) and with decreased lung function (Ying et al., 2005). Moreover, TSLP protein is increased in both airway epithelium and lamina propria of patients with asthma, and TSLP levels correlate with the severity of the disease and the expression of the Th2 cytokine IL-13 (Shikotra et al., 2012). In addition, increased TSLP levels in bronchoalveolar (BAL) fluids of asthmatic patients is observed (Ying et al., 2008). These results suggest that TSLP produced by epithelial cells might contribute to the pathogenesis or aggravation of the asthma.
The role of TSLP in the pathophysiology of asthma has been well supported by studies in mouse models. Mice expressing increased levels of TSLP specifically in the lung exhibit a spontaneous lung inflammation similar to asthma (Zhou et al., 2005). In addition, instillation of TSLP intranasally induces a significant inflammatory infiltrate composed of lymphocytes and eosinophils in the BAL fluid with increased

levels of Th2 cytokines and elevation of serum IgE levels (Seshasayee et al., 2007). These results demonstrate that TSLP is sufficient to develop an asthma-like phenotype.

Using an asthma mouse model induced by intraperitoneal sensitization followed by an intranasal challenge with allergen, TSLP mRNA levels and TSLP protein levels were increased in lung and BAL fluid, respectively (Zhou et al., 2005), suggesting a possible involvement of this cytokine in of development or maintenance asthma. In addition, using the same asthma mouse model, TSLPR deficient mice exhibited a decreased lung allergic inflammation (Al-Shami et al., 2005; Zhou et al., 2005).

5.6 Resemblance with AD

Allergic asthma shares various similarities with AD: (1) the prevalence of the disease is rising particularly in Western and industrialized societies, (2) AD shares genetic determinants with asthma, (3) AD and asthma are complex genetic diseases that arise from gene-gene and gene-environment interactions, (4) AD and asthma can be both characterized as manifestations of an exaggerated inflammatory response to environmental triggers, including irritants and allergens and (5) increased production of IgE, tissue eosinophilia and development and activation of Th2 cells are key features of both diseases.

Differences in clinical and histopathological manifestations may lie more in differences between the skin and lungs themselves (their distinct microenvironments, resident cells, types of environmental exposures and unique, specialized immune response), than in underlying mechanisms (Eichenfield et al., 2003), suggesting that AD and allergic asthma are a single disease with different target organ, or a progression from the skin inflammation to the lung disease.

6. Atopic march: from AD to asthma.

The term atopic march refers to the natural history of atopic manifestations, which is characterized by a typical sequence of clinical symptoms and conditions appearing during a certain age period and persisting over a number of years. Commonly, the clinical features of AD precede the development of asthma and allergic rhinitis (Figure 10).

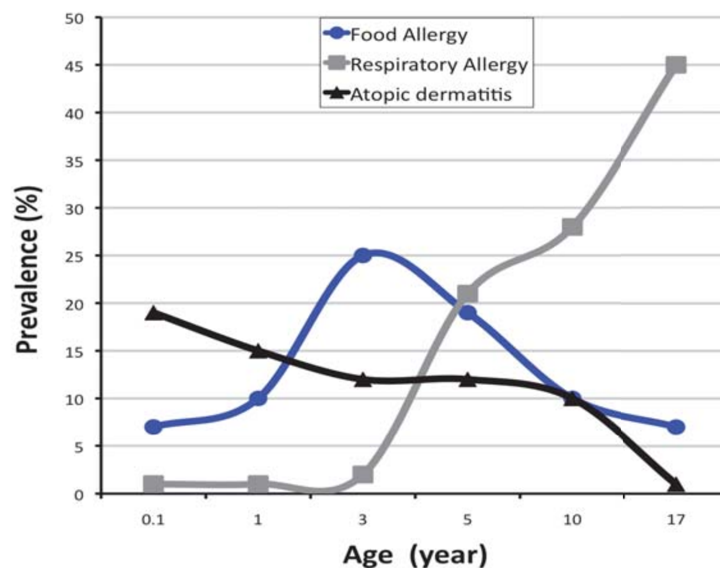

Figure 10. Prevalence of various atopic diseases. Prevalence of AD is highest during infancy and then decreases gradually. Food allergy prevalence peaks at 3 years of age and decreases at adolescence. In contrast, respiratory allergy is more common at school age and adolescence [adapted from **(Spergel and Paller, 2003)**].

Several longitudinal studies indicate that the severity of AD can influence the course of asthma and allergic rhinitis. Seventy percent of patients with severe AD develop asthma, compared with 30% of the patients with mild AD and approximately 8% in the general population (Gustafsson et al., 2000), suggesting that AD is the “entry point” for subsequent allergic diseases.

AD patients with specific IgE antibodies to common environmental allergens have a higher risk for progressing in the atopic march to allergic rhinitis and asthma that those with AD without IgE sensitization (Wuthrich and Schmid-Grendelmeier, 2002), suggesting that sensitization during AD is an important factor for progression into an allergic airway disease later in life.

Disease severity and sensitization are the two major determinants of increased risk of subsequent allergic airway disease in patients with AD (Illi et al., 2004), suggesting

that factors exacerbating skin inflammation and promoting sensitization through the skin are implicated in the atopic march.

The progression of AD to allergic airway diseases is supported by experimental evidence of mouse models. Epicutaneous sensitization with OVA (patch method, see below) induces localized AD and increases the number of eosinophils in the bronchoalveolar lavage (BAL) fluid, and airway hyperresponsivenes after a single challenge with aerosolized OVA (Spergel et al., 1998). In addition, epicutaneous sensitization with *A. fumigatus* induces AD, and a single intranasal challenge with the same allergen induces experimental allergic rhinitis (Akei et al., 2006).

6.1 TSLP and atopic march

Even though the implication of TSLP in the atopic march in humans is not confirmed, studies in mice have demonstrated that this cytokine is important for the progression of AD to asthma.

Indeed, increased expression of TSLP in skin, either by ablation of RXRs in keratinocytes or upon MC903 skin topical treatment, not only triggers an AD-like syndrome, but also leads to an aggravation of an allergic lung inflammation of mice in which asthma is induced by intraperitoneal sensitization followed to intranasal challenge (Zhang et al., 2009). In addition, an aggravated asthma phenotype has been reported in mice with increased TSLP expression in skin, either by ablation of RPB-j in keratinocytes or by transgenic expression (Demehri et al., 2009), suggesting that increased systemic TSLP levels could be a risk factor for the development or aggravation of the allergic airway inflammation. However, the implication of TSLP at physiological levels during the atopic march still unknown.

7. Mouse models of atopic dermatitis and asthma.

Our understanding of human atopic dermatitis and asthma have enormously expanded with the use of animal models, because they allow in-depth investigation of their pathogenesis, and provide invaluable tools for diagnostic and pharmaceutical purposes.

7.1 Mouse models of AD.

Since the description of Nc/Nga mouse as the first mouse model of AD, several additional mouse models have been developed (Table 2). These models can be categorized into four groups: (1) mice that spontaneously develop AD-like skin lesions, (2) genetically engineered models, (3) models induced by topical application of chemical compounds and (4) models induced by epicutaneous sensitization with allergens.

7.1.1 Spontaneous mouse models of AD.

Nc/Nga mice spontaneously develop severe dermatitis in the presence of nonspecific allergens. Morbid Nc/Nga mice exhibit AD symptoms, including itching, erythema, drying, hyperplasia of the epidermis, increased TEWL and impaired ceramide metabolism. Furthermore, Nc/Nga mice display some of the characteristic histopathological features of AD, such as skin eosinophilia, as well as increased number and activation of mast cells and lymphocytes. Along with these skin changes, Nc/Nga mice exhibit an increased Th2 response in the spleen and an increased serum level of total IgE (Matsuda et al., 1997). Mice kept under specific pathogen free conditions fail to develop skin lesions, thus strongly suggesting that environmental factors play an important role in their skin inflammation. Genetic analysis of Nc/Nga mice has demonstrated that the mutation responsible for the skin phenotype is on chromosome 9 at the derm1 locus (Kohara et al., 2001), but no specific gene has yet been identified.

The flaky tail mice exhibit sporadic superficial dermal cellular infiltration that includes lymphocytes, eosinophils, mononuclear cells and increased mast cell number. In addition, increased TEWL and penetration of fluorescent molecules in these mice confirm a defective skin barrier function (Moniaga et al., 2010). A recent study reveals that the gene responsible for the phenotype of flaky tail mice is a single nucleotide deletion of the profilaggrin gene, resulting in a frameshift mutation and premature truncation of the protein (Fallon et al., 2009).
Other strains of mice that spontaneously develop dermatitis are NOA mice, DS-Nh mice and KOR-adjm (Haraguchi et al., 1997; Matsushima et al., 2010; Watanabe et al., 1999).

7.1.2 Genetically engineered mouse models.

Multiple mouse models establish a definite role of Th2 cytokines in the pathogenesis of AD. Transgenic mice overexpressing IL-4 or IL-13 specifically in skin keratinocytes develop hallmarks of AD, including pruritus, increased T cells, mast cells, mononuclear cells and eosinophil skin infiltration, bacterial infection of the skin and elevated IgE and IgG1 (Chan et al., 2001; Zheng et al., 2009).
Transgenic mice overexpressing IL-31 exhibit signs of dermatitis, including pruritus, hair loss and skin thickening. Histological examination of the skin reveals acanthosis, inflammatory cell infiltration and skin mastocytosis, which resemble skin lesions of human AD. However, these mice show normal IgE serum levels, suggesting that IL-31 is not implicated in the systemic immune response (Dillon et al., 2004).
Skin lesions from skin-specific TSLP transgenic mice show a phenotype similar to patients with AD, including hyperkeratosis, spongiosis and dermal infiltration with lymphocytes, macrophages as well as increased mast cell and eosinophil number. Skin lesions of these mice exhibit increased levels of Th2 cytokines. These mice also show elevated serum levels of IgE and IgG1 (Li et al., 2005; Yoo et al., 2005). Moreover, deficiency of Notch or both nuclear receptors RXRα and β in epidermal keratinocytes lead to increased TSLP levels in epidermal keratinocytes, and such mutant mice develop of an AD-like phenotype (Demehri et al., 2009; Li et al., 2005).

Several other mouse models of AD has been published, such as caspase-1, SCCE, APOC1 and IL-18 transgenic mice and Cathepsin E, RelB, FOXP3 and MAIL/IkBz knockout mice (Barton et al., 2000; Godfrey et al., 1991; Konishi et al., 2002; Nagelkerken et al., 2008; Ny and Egelrud, 2004; Shiina et al., 2004; Tsukuba et al., 2003; Yamanaka et al., 2000).

Spontaneous mouse models of AD.				
Strain	**Skin histology**	**Skin infiltration**	**Skin cytokines/ chemokines**	**Systemic response**
Nc/Nga	Moderate epidermal hyperplasia, prominent hyperkeratosis and inflammatory cells infiltration	Mast cells, eosinophils, macrophages, CD4+ cells and few CD8+ cells	IL-4 and IL-5 TARC and MDC	Increased serum IgE and IgG levels
Flaky tail	Epidermal hyperplasia, acanthosis, increased inflammatory cells and fibrous bundles in the dermis	Mast cells, eosinophils, neutrophils and CD4+ cells	IL-17, IL-6, and IL-23 CXCL2	Increased serum IgE and IgG1 levels
NOA	Epidermis desquamation, hyperkeratosis, thickening of the dermis	Mast cells and eosinophils	Eotaxin-1	Increased serum IgE levels
DS/Nh	Keratosis, thickening and necrosis of the epidermis, leukocytic infiltration into the dermis	Mast cells, macrophages and CD4+ cells	IL-4	Increased serum IgE levels

<table>
<tr><td>KOR-adjm</td><td>Thickening of the epidermis with massive cell infiltration in the dermis</td><td>Eosinophils</td><td colspan="2"></td><td>Increased serum IgE levels</td></tr>
<tr><th colspan="6">Genetically engineered mouse models</th></tr>
<tr><th>Strain</th><th>Target cell</th><th>Skin histology</th><th>Skin infiltration</th><th>Skin cytokines/ chemokine s</th><th>Systemic response</th></tr>
<tr><td>Transgenic IL-4</td><td>Keratinocytes</td><td>Mild spongiosis and acanthosis, hyperkeratosis and a dermal and epidermal infiltration of mononuclear cells</td><td>Mast cells, eosinophils, macrophages and T cells</td><td>IL-4
CCL27</td><td>Increased serum IgE and IgG1 levels</td></tr>
<tr><td>Transgenic IL-13</td><td>Keratinocytes</td><td>Thickening of the epidermal and dermal layers, spongiosis, hyperkeratosis, fibrosis and marked cellular infiltration</td><td>Mast cells, eosinophils, LCs and CD4+ cells</td><td>TSLP and TGFβ
Eotaxin, TARC, CCL27, MCP-1, MDC and RANTES</td><td>Increased serum IgE and IgG1 levels</td></tr>
<tr><td>Transgenic IL-31</td><td>Ubiquitous</td><td>Hyperkeratosis, acanthosis and inflammatory cell infiltration</td><td>Mast cells</td><td></td><td>Normal serum IgE and IgG1 levels</td></tr>
<tr><td>Transgenic TSLP</td><td>Keratinocytes</td><td>Hyperplasia of the epidermis with a heavy dermal cell infiltrate</td><td>Mast cells, eosinophils, DCs and CD4+ cells</td><td>IL-4, IL-5, IL13, IL10 and IL-31
TARC, MDC, MCP2, CXCL10 and CCL20</td><td>Increased serum IgE and IgG levels</td></tr>
<tr><td>Notch deficient</td><td>Keratinocytes</td><td>Acanthosis, hyperkeratosis and spongiosis with massive dermal cell infiltration</td><td>Mast cells, eosinophils, T cells, DCs, LCs and neutrophils</td><td>TSLP, IL-1β, IL-6 and TNFα.
MCP1, MIP3a, ICAM-1 and G-CSF</td><td>Increased serum IgE and IgG levels</td></tr>
</table>

RXRα and RXRβ deficient	Keratinocytes	Hyperplasia of the epidermis with a heavy dermal cell infiltrate	Mast cells, eosinophils, DCs and CD4+ cells	IL-4, IL-5, IL13, IL10 and IL-31 TARC, MDC, MCP2, CXCL10 and CCL20	Increased serum IgE and IgG levels
FOXP3 knockout	Ubiquitous	Epidermal hyperplasia with prominent dermal cell infiltrate	Eosinophils		Increased serum IgE and IgG levels
Models induced by topical application of chemical compounds					
Compound	**Skin histology**	**Skin infiltration**	**Skin cytokines/ chemokines**		**Systemic response**
Oxazolone	Epidermal hyperplasia with prominent dermal cell infiltrate	Mast cells, eosinophils and CRTH2+ T cells	IL-4		Increased serum IgE levels
MC903	Hyperplasia of the epidermis with a heavy dermal cell infiltrate	Mast cells, eosinophils, DCs and CD4+ cells	IL-4, IL-5, IL13, IL10 and IL-31 TARC, MDC, MCP2, CXCL10 and CCL20		Increased serum IgE and IgG levels
Models induced by epicutaneous sensitization with allergens					
Method	**Skin histology**	**Skin infiltration**	**Skin cytokines/ chemokines**		**Systemic response**
Patch method	Epidermal hyperplasia, focal acanthosis, mild spongiosis and dermal cell infiltration	Neutrophils, lymphocytes, mast cells, and eosinophils	IL-4, IL-5, IFNγ and IL-17		Increased serum IgE and IgG levels and presence of allergen-specific Immunoglo bulins

Table 2. Mouse models of atopic dermatitis.

7.1.3 Models induced by topical application of chemical compounds

Oxazolone is commonly used to induce allergic contact dermatitis and evokes a Th1-dominated response. However, multiple challenges with oxazolone to the skin of hairless mice cause a shift from typical delayed-type hypersensitivity, into a more chronic dermatosis, with multiples features of AD, including impairment of the skin barrier, decreased expression of three structural protein markers of differentiation (loricrin, involucrin and filaggrin), prominent inflammatory infiltrate, including T cells, mast cells and eosinophils, and increased serum IgE levels (Man et al., 2008).
Topical application of MC903 (a low-calcemic vitamin D3 analog) induces TSLP expression in epidermal keratinocytes, which results in an atopic dermatitis-like syndrome, characterized by a red, scaly and lesioned skin, accompanied by an epidermal hyperplasia and a dermal infiltration of CD4+ cells, eosinophils, dendritic cells and mast cells, as well as by an increase of Th2 cytokines in the skin, and elevated serum IgE and blood eosinophilia (Li et al., 2006).

7.1.4 Models induced by epicutaneous sensitization with allergens

Epicutaneous sensitization with allergens plays an important role in the pathogenesis of AD. Epicutaneous exposure of protein allergens (e.g. ovalbumin [OVA]), in absence of adjuvant, by the patch method (Figure 11), can prime and sensitize mice to mount a Th2 immune response with high levels of IgE, and production of allergen specific immunoglobulins. The treated skin of these mice show lymphocyte infiltration in both dermis and epidermis (Wang et al., 1996). Histopathological and immunopathological examination of the skin lesions of these mice reveal increased infiltrate of neutrophils, eosinophils, mononuclear cells, mast cells, CD3+ cells and CD4+ cells. In addition, skin lesions of these mice exhibit an increased IL-4, IL-5 and IFNγ expression (Spergel et al., 1998).
Serum OVA-specific IgG1, IgE and IgG2a, and increased level of IL-4, IL-5, IL-13 and IFNγ, in response to OVA stimulation demonstrate the systemic response to the allergen after epicutaneous treatment.

The main advantage of this model is that it can be generated in any mouse strain. The use of this protocol with gene-deficient mice could help to investigate the implication the various cytokines, chemokines or cells in the physiophatology of AD. In these respect, alfa-beta T cells, IL-4, IL-5, IFNγ, IL-10, IL-21R and TSLPR are implicated in some aspects of this model. However, gamma-delta T cells, B cells, mast cells, iNKT cells or CD40L-CD40 interaction are not critical for skin inflammation and Th2 response (Alenius et al., 2002; Elkhal et al., 2006; He et al., 2008; Jin et al., 2009; Laouini et al., 2003; Spergel et al., 1999; Woodward et al., 2001).

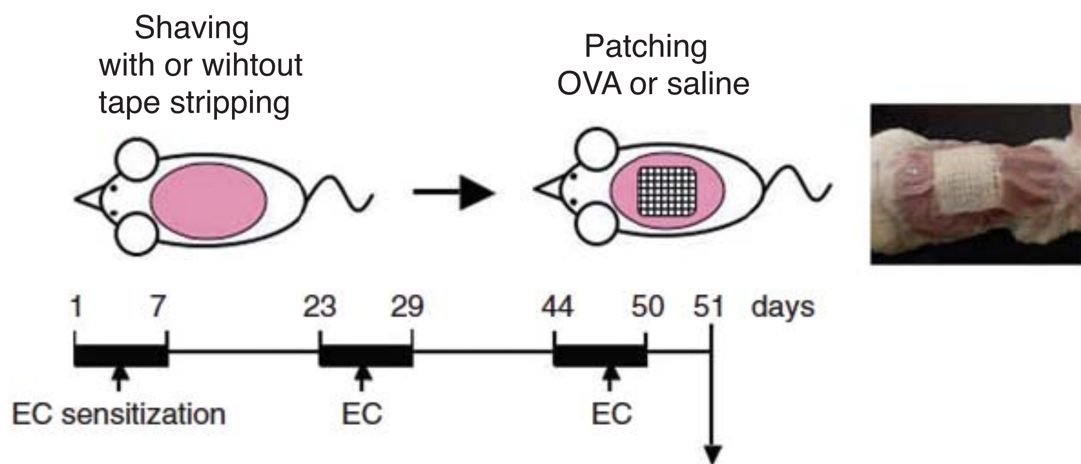

Figure 11. Epicutaneous sensitization by patch method. Mice are sensitized with OVA or saline applied in a sterile patch. The patch is placed for 1 week period and then removed. Two weeks later, similar patch is reapplied to the same skin site. Each mouse has a total of three 1-week exposure separated from each other by 2-week intervals.

7.2 Mouse models of allergic asthma.

Since the first report of an experimental mouse allergic asthma (Curtis and Kaltreider, 1989), mice are the most extensively studied model system. Experimental asthma mouse models can be divided in acute challenge models and chronic allergen exposure models.

7.2.1 Acute challenge asthma models.

These protocols usually require multiple systemic administration of the allergen in the presence of an adjuvant (generally Alum [$AlOH_3$]), followed of a challenge with the same allergen through the airways. Allergen may be inhaled as a nebulized formulation, or administered by intratracheal or intranasal instillation of an aqueous formulation (Nials and Uddin, 2008).
The acute challenge mouse models reproduce many key features of clinical asthma, induces elevated IgE levels, airway inflammation, goblet cell hyperplasia, epithelial hypertrophy and AHR. However, these models do not reproduce the lung remodeling observed in asthmatic patients. Furthermore, many of the key features appear to be short-lived and, in some models, airways inflammation and AHR is resolved within a few days after the final allergen challenge.

7.2.2 Chronic allergen exposure asthma models.

Chronic allergen exposure asthma models involve repeated exposure of the airways to low levels of allergen for long periods of times (with or without the use of systemic sensitization in presence of an adjuvant).
Chronic allergen exposure in mice reproduce some of the hallmarks of human asthma, including allergen sensitization, a Th2 dependent allergic lung inflammation, airway eosinophilia and AHR. In addition in some models, there is evidence of airway remodeling (goblet cell hyperplasia, epithelial hypertrophy, and fibrosis). Some of the characteristic features of this model persist after the final challenge.

7.3 Inconvenience of mouse models of AD and allergic asthma.

Mouse models for atopic diseases, such as AD and asthma, provide an invaluable tool to better understand the pathogenesis of these diseases. However, the allergen-induced mouse models used until now do not mimic faithfully the physiological

situation observed in AD and asthmatic patients. First, atopic patients have sporadic contact with the allergen (not constant, like AD patch method model). Second, sensitization observed in these patients is through the epithelium without help of adjuvants (not systemic, like in acute challenge asthma model). Third, some patients develop an atopic march (not independent disease [AD or allergic asthma]). Thus, development of new mouse models that faithfully mimic the pathophysiology observed in atopic patients are required.

OBJECTIVES

At the time when I joined the laboratory, studies had shown that increased expression of TSLP in skin keratinocytes, induced either by ablation of RXRα and RXRβ in adult mouse keratinocytes, or upon MC903 skin topical treatment, not only triggered an AD, but also led to aggravation of an asthma-like lung inflammation. The aggravation of asthma was mediated by TSLP produced in keratinocytes, as $TSLP^{ep-/-}$ mice in which TSLP was selectively ablated in epidermal keratinocytes, failed to exhibit this aggravation. These studies indicated that TSLP may play a critical role in the atopic march. However, in these studies, allergen sensitization was artificially achieved by systemic sensitization with the help of the an adjuvant, which does not mimic the sensitization occurring through the skin in AD patients. In addition, the cascade of events induced by increased TSLP production was unknown.

My thesis research was aimed to further exploring the role of keratinocytic TSLP in triggering atopic dermatitis and atopic march.
My thesis was focussed on three objectives:
1.- To generate a new mouse model that faithfully mimics the onset of atopic march
2.- To investigate the implication of TSLP in a mouse model of atopic march
3.- To unravel the mechanism by which TSLP induces Th2-mediated skin inflammation.

The results and discussion of the above studies are described in the following section:
i) TSLP produced by keratinocytes promotes allergen sensitization through skin and thereby triggers atopic march in mice. (Part 1, manuscript in press)
ii) TSLP promotes skin basophilia in both T and B cell-independent and -dependent manner (Part 2A, manuscript in preparation).
iii) TSLP-induced Th2 polarization requires reciprocal action of Basophil, Dendritic cells and CD4+T cells (Part 2B, manuscript in preparation).

RESULTS

Part 1

TSLP produced by keratinocytes promotes allergen sensitization through skin and thereby triggers atopic march in mice.

Juan Manuel Leyva-Castillo, Pierre Hener, Hua Jiang and Mei Li.

Journal of Investigative Dermatology (in press).

In this manuscript we report that mechanical disruption of the skin barrier by tape stripping induces TSLP protein production by keratinocytes. We established a new mouse model of atopic march which faithfully mimics the onset of this progression (AD to asthma), induced by topical allergen application after mechanic disruption of the skin barrier to induce an allergic skin inflammation and systemic sensitization followed of the intranasal challenge with the same allergen to induce an airway allergic inflammation. Our results demonstrate that keratinocytic TSLP induced by skin barrier impairment is essentially required to generate a Th2 allergic response in this model, as ablation of TSLP in keratinocytes leads a decreased allergic skin inflammation, and reduced production of allergen-specific immunoglobulins. In addition, upon intranasal challenge with the same allergen, epicutaneous treated keratinocytic TSLP-deficient mice developed a less severe asthma-like phenotype. In contrast, overproduction of keratinocytic TSLP enhances skin inflammation, boosts the systemic sensitization through the skin and triggers an aggravate allergic airway inflammation in a dose-dependent manner.

Our study uncovers a crucial role of keratinocytic TSLP in the “atopic march” by promoting allergen sensitization occurring in barrier-impaired skin, which ultimately leads to allergic asthma.

TSLP Produced by Keratinocytes Promotes Allergen Sensitization through Skin and Thereby Triggers Atopic March in Mice

Juan Manuel Leyva-Castillo[1], Pierre Hener[1], Hua Jiang[1] and Mei Li[1]

Atopic dermatitis often precedes the development of asthma, a phenomenon known as "atopic march". An important role of allergen sensitization developed through barrier-defective skin has been recognized in the onset of atopic march; however, the underlying mechanism remains poorly understood. In this study, we use an experimental atopic march mouse model, in which the sensitization to allergen is achieved through barrier-impaired skin, followed by allergen challenge in the airway. By using thymic stromal lymphopoietin $(TSLP)^{iep-/-}$ mice in which the cytokine TSLP is selectively and inducibly ablated in epidermal keratinocytes, we demonstrate that keratinocytic TSLP, the expression of which is induced by skin barrier impairment, is essential for generating skin allergic inflammation and allergen-induced T helper type 2 response, for developing sensitization to allergen, and for triggering a subsequent allergic asthma. Furthermore, using $TSLP^{over}$ mice in which overexpression of keratinocytic TSLP is induced by skin topical application of MC903 (a vitamin D3 analog) in a dose-dependent manner, we show that keratinocytic TSLP levels are correlated with skin sensitization strength and asthma severity. Taken together, our study uncovers a crucial role of keratinocytic TSLP in the "atopic march" by promoting allergen sensitization occurring in barrier-impaired skin, which ultimately leads to allergic asthma.

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INTRODUCTION

Atopic dermatitis (AD, eczema) frequently starts in early infancy, and is characterized by skin inflammation and impaired skin barrier function (Bieber, 2008; Boguniewicz and Leung, 2011). More than 50% of AD patients with moderate to severe AD develop asthma and/or allergic rhinitis at a later stage, and the severity of AD appears to influence the course of respiratory allergy. Although this phenomenon is well known as the ''atopic march'' (Spergel and Paller, 2003; Hahn and Bacharier, 2005; Boguniewicz and Leung, 2011), how asthma progresses from AD still remains obscure, and an effective prevention of the atopic march is still lacking.

It has been recognized that, often during the course of AD, a majority of patients develop sensitization to common food and/or environmental allergens (Bieber, 2008; Cork *et al*., 2009; Boguniewicz and Leung, 2011), and moreover that the severity of AD correlates with the degree of sensitization and with the risk of developing asthma (Schafer *et al*., 1999; Oettgen and Geha, 2001). Indeed, allergen sensitization developed through barrier-defective skin during AD could be a critical event leading to subsequent allergic asthma (Bieber, 2008; Cork *et al*., 2009; Benedetto *et al*., 2012); however, the mechanism underlying skin sensitization is still poorly understood.

Thymic stromal lymphopoietin (TSLP) has emerged as a key epithelium-derived cytokine in the AD pathogenesis (Liu, 2006; Ziegler and Artis, 2010). Increased expression of TSLP has been revealed in skin keratinocytes from AD patients (Soumelis *et al*., 2002), and elevated serum TSLP level was reported in AD children (Lee *et al*., 2010a). Our previous study (Li *et al*., 2005) and that of others (Yoo *et al*., 2005) have demonstrated that transgenic mice overexpressing TSLP in keratinocytes developed a spontaneous AD-like dermatitis. We have further established an experimental $TSLP^{over}$ protocol through which a skin topical application of MC903 (calcipotriol; a low-calcemic analog of vitamin D3) induces keratinocytic TSLP expression and triggers an AD-like syndrome (Li *et al*., 2006, 2009). More recently, we reported that MC903 skin treatment aggravated experimental allergic asthma induced by intraperitoneal (i.p.) sensitization to ovalbumin

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Abbreviations: AD, atopic dermatitis; CT, wild-type control; DC, dendritic cell; e.c., epicutaneous; i.n., intranasal; i.p., intraperitoneal; LN, lymph node; MT, mutant; NT, nontreated; OVA, ovalbumin; PBS, phosphate-buffered saline; Tam, tamoxifen; Th2, T helper type 2; TSLP, thymic stromal lymphopoietin

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(OVA) and airway OVA challenge, which was mediated by TSLP produced in keratinocytes (Zhang *et al.*, 2009). Interestingly, we also found that overexpressed keratinocytic TSLP during the OVA sensitization phase was able to aggravate OVA-induced asthma (Zhang *et al.*, 2009). This study led us to posit that TSLP overproduced in the skin of AD patients could be a risk factor for the development of allergic airway inflammation. A similar suggestion was made by Demehri *et al.* (2009). However, in these previous experiments, allergen sensitization was ''artificially'' achieved by i.p. injection of OVA complexed with exogenous adjuvant (Takeda and Gelfand, 2009; Holgate, 2011), which does not mimic the sensitization occurring in epithelium sites such as skin (Bieber, 2008). In this study, by using an experimental ''atopic march'' mouse model, in which allergen sensitization is achieved through barrier-impaired skin, followed by airway challenge, our study demonstrates that keratinocyte-produced TSLP has a crucial role in promoting allergen sensitization occurring in skin, which ultimately triggers the ''atopic march'' leading to allergic asthma.

RESULTS

An experimental ''atopic march'' model induced by OVA epicutaneous (e.c.) sensitization through barrier-impaired skin and intranasal (i.n.) challenge

We established a mouse model representing the atopic march features (outlined in Figure 1a), which is initiated by an e.c. sensitization phase by OVA treatment on barrier-impaired skin through tape stripping (mimicking scratching in AD patients) (Strid *et al.*, 2004; Cork *et al.*, 2009; Jin *et al.*, 2009), and followed by an OVA i.n. challenge phase. In this model, e.c. OVA–treated wild-type Balb/c mice exhibited an AD-like skin allergic inflammation (see Figure 2), and developed an allergen sensitization evidenced by systemic immune responses, including the production of OVA-specific Igs (indicating an allergen-specific B-cell response), and cytokine production of splenocyte cells upon *in vitro* OVA stimulation (indicating an allergen-specific T-cell response; see Figure 3). Upon i.n. challenge, these e.c. OVA–sensitized mice exhibited an asthmatic phenotype (see Figure 4).

Barrier impairment induces TSLP production in keratinocytes

In skin extracts of nontreated (NT) wild-type Balb/c mice, TSLP protein levels were undetectable by ELISA. In contrast, upon phosphate-buffered saline (PBS) or OVA treatment on barrier-impaired skin (e.c. PBS or e.c. OVA), TSLP protein levels were upregulated after 3 hours, and further increased 9, 24, and 48 hours later (Figure 1b). These levels were not significantly different between e.c. OVA and e.c. PBS treatment, suggesting that TSLP induction was mainly due to skin barrier impairment but not due to OVA application.

To examine whether epidermal keratinocytes were the source of stripping-induced TSLP, we used $TSLP^{iep-/-}$ mutant (MT) mice, in which selective ablation of TSLP was induced

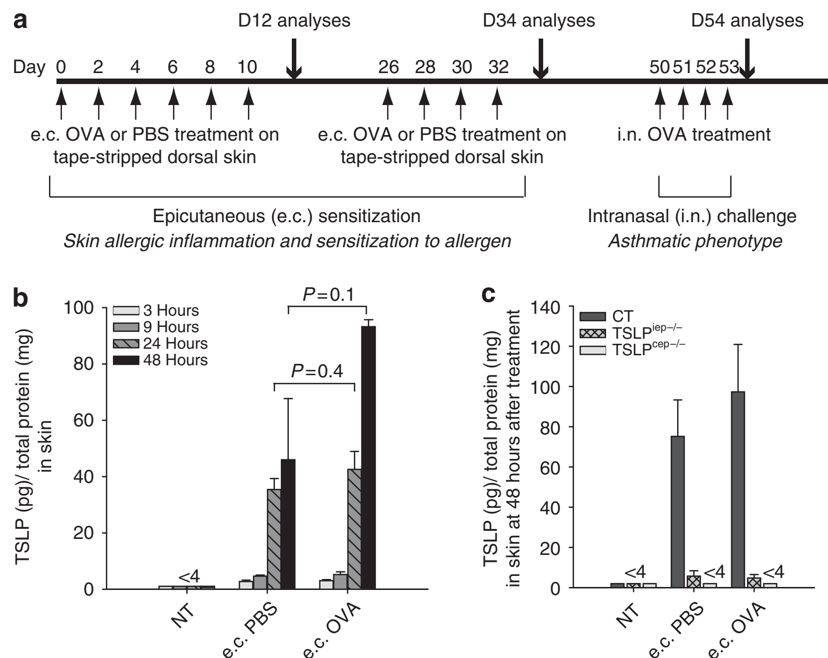

Figure 1. Thymic stromal lymphopoietin (TSLP) production is induced in keratinocytes by tape stripping during epicutaneous (e.c.) sensitization in an experimental mouse asthma model. (**a**) Experimental protocol. Dorsal skin of 10–12-week-old female mice was shaved and tape stripped (until TEWL (transepidermal water loss) value reached 15–20 $g\,m^{-2}$ per hour) and treated with ovalbumin (OVA) every other day (D) from D0 to D10. Similar e.c. treatment was repeated from D26 to D32. Nontreated (NT) mice or mice treated with phosphate-buffered saline (PBS) on tape-stripped skin (e.c. PBS) were used as controls. Three weeks later, mice were intranasally (i.n.) challenged with OVA for 4 consecutive days (D50–D53). (**b**) TSLP protein levels in the skin of wild-type Balb/c mice measured by ELISA at 3, 9, 24, and 48 hours after one e.c. PBS or e.c. OVA treatment. (**c**) TSLP protein levels measured by ELISA in the skin of wild-type control (CT), $TSLP^{iep-/-}$ (inducible ablation of TSLP in epidermal keratinocytes), and $TSLP^{cep-/-}$ (constitutive ablation of TSLP in epidermal keratinocytes) mice 48 hours after one e.c. treatment. Values are mean ± SEM ($n \geqslant 4$ mice per group).

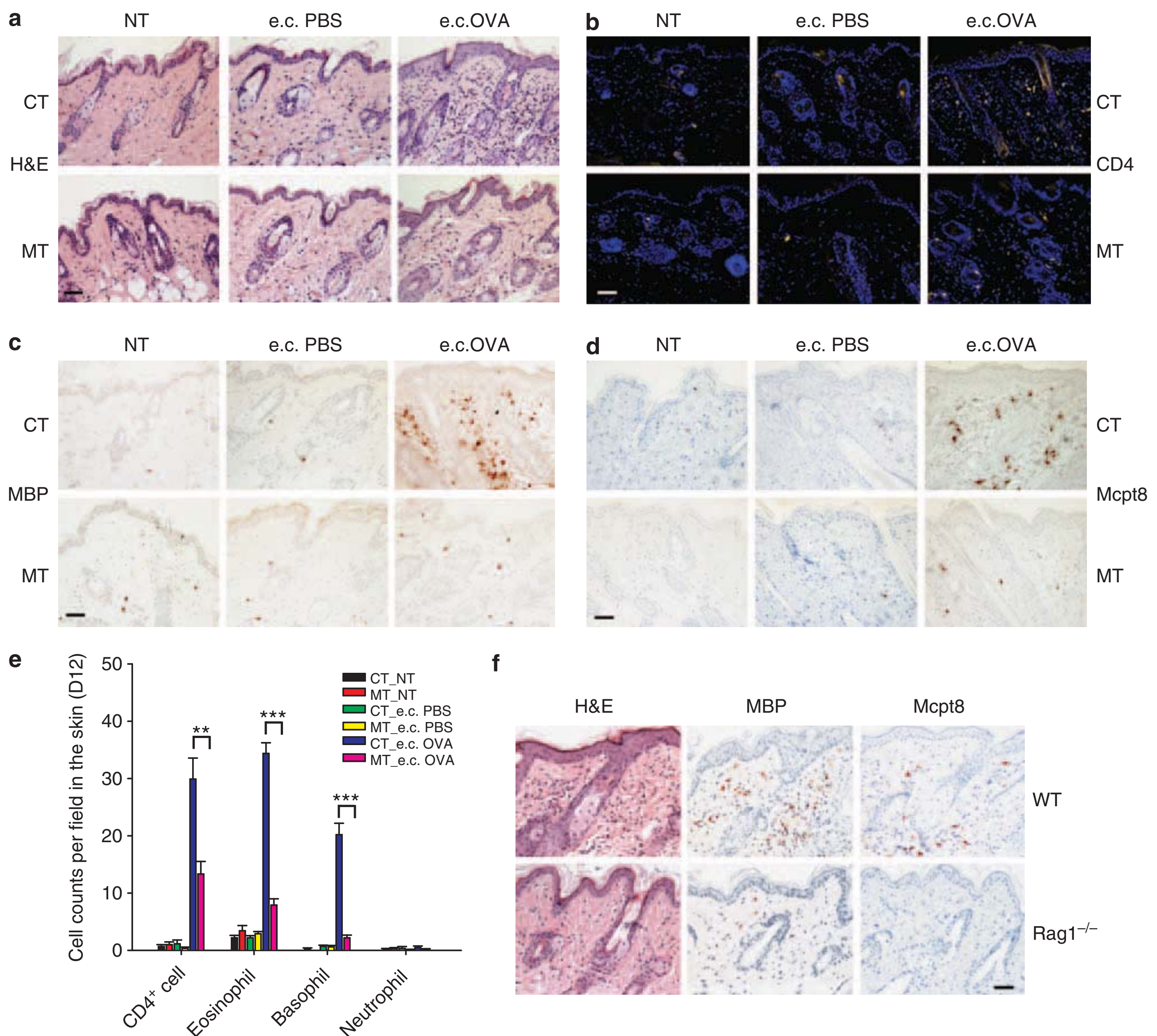

Figure 2. Reduced allergic inflammatory infiltrate in skin of epicutaneous (e.c.) ovalbumin (OVA)–treated thymic stromal lymphopoietin (TSLP)$^{iep-/-}$ mutant (MT) mice. (**a**) Hematoxylin and eosin (H&E) staining of skin paraffin sections from day (D)12, showing reduced epidermal thickening and dermal infiltration in MT mice compared with wild-type control (CT) mice, upon e.c. OVA treatment. (**b–d**) Immunohistochemical (IHC) staining performed on skin sections at D12, (**b**) with antibodies against CD4 (yellow corresponds to CD4$^+$ staining and blue for DAPI staining of nuclei), (**c**) MBP (specific for eosinophils; in dark red), or (**d**) Mcpt8 (specific for basophils; in dark red). (**e**) A summary of cell counts for skin-infiltrating immune cells at D12. Results were obtained by calculating the average number of positively stained cells per microscopic field (at $\times 200$ magnification; $n \geqslant 12$) $^{**}P<0.01$; $^{***}P<0.001$. Values are mean ± SEM. (**f**) Comparison of H&E and IHC staining with antibodies against MBP or Mcpt8 in e.c.OVA–treated wild-type (WT) and Rag1$^{-/-}$ skin at D12. NT, nontreated; e.c. phosphate-buffered saline (PBS), treatment of PBS on tape-stripped skin; e.c. OVA, treatment of OVA on tape-stripped skin. Bar = 50 μm.

at the adult stage in epidermal keratinocytes by tamoxifen (Tam) treatment of K14-CreER^{T2}/TSLP$^{L2/L2}$ mice (Li *et al.*, 2009). The TSLP increase was nearly abolished in both e.c. PBS– and e.c. OVA–treated TSLP$^{iep-/-}$ mice (Figure 1c). The residual induction of TSLP seen in e.c.-treated TSLP$^{iep-/-}$ mice was most likely due to a <100% efficiency in Tam-induced *TSLP* gene ablation in keratinocytes, as the induction of TSLP was fully abolished in TSLP$^{cep-/-}$ MT mice (K14-Cre/TSLP$^{L2/L2}$) (Li *et al.*, 2009) in which the *TSLP* gene was "constitutively" ablated in keratinocytes at the embryonic stage (Figure 1c). Therefore, barrier impairment by tape stripping induces a production of TSLP in keratinocytes.

Ablation of TSLP in mouse skin keratinocytes results in a reduced allergic inflammation upon e.c. OVA treatment

Wild-type control (CT; Tam-injected TSLP$^{L2/L2}$ mice) and TSLP$^{iep-/-}$ MT mice were treated as described in Figure 1a. At D12, e.c. OVA–treated CT mice exhibited epidermal

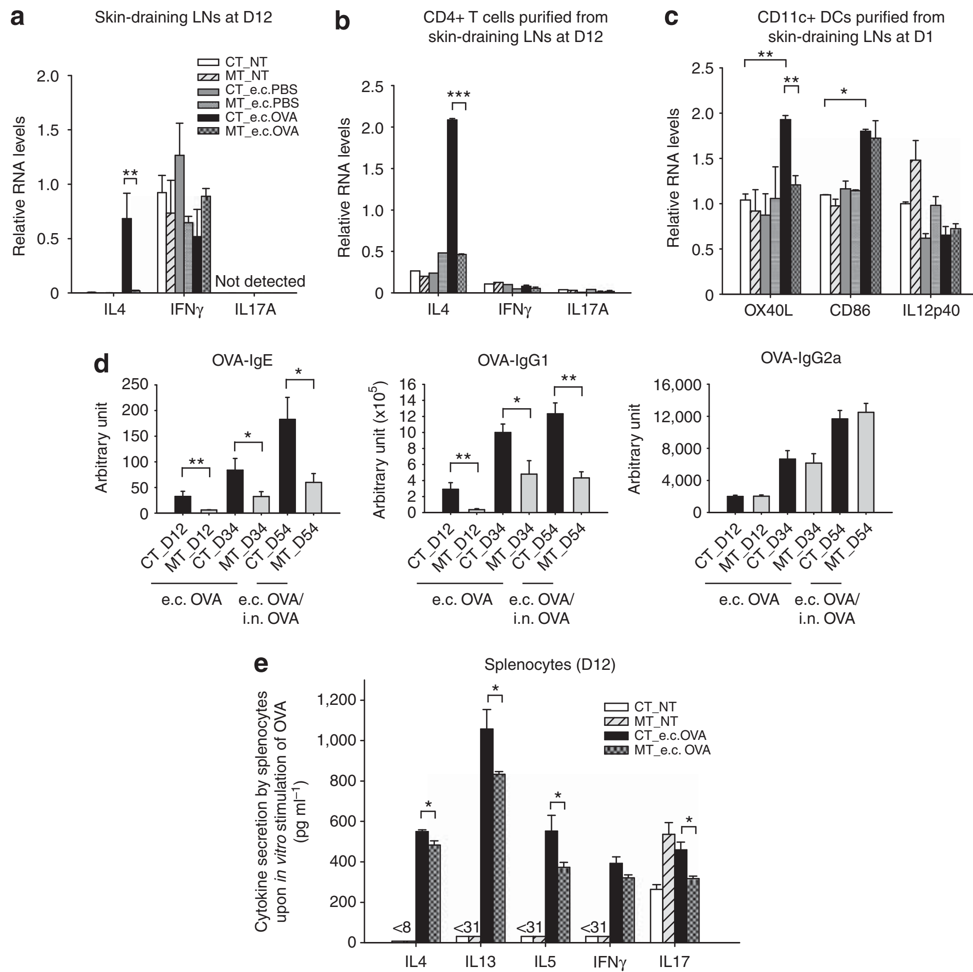

Figure 3. Impaired T helper type 2 (Th2) cell response and defective allergen sensitization in epicutaneous (e.c.) ovalbumin (OVA)–treated thymic stromal lymphopoietin (TSLP)$^{iep-/-}$ mutant (MT) mice. (**a**, **b**) Quantitative reverse-transcriptase–PCR (RT-PCR) analyses of (**a**) skin-draining lymph nodes (LN) and (**b**) purified CD4$^+$ T cells from skin-draining LNs at day (D)12. (**c**) Quantitative RT-PCR analyses of purified CD11c$^+$ dentritic cells (DCs) from skin-draining LNs at D1. (**d**) Serum OVA–specific IgE, IgG1, and IgG2a levels at various time points (D12, D34, and D54). (**e**) Cytokine secretion by splenocytes at D12, in response to *in vitro* OVA stimulation. *$P<0.05$; **$P<0.01$; ***$P<0.001$ ($n \geqslant 5$ mice per group). Values are mean ± SEM. CT, wild-type control mice; NT, nontreated.

hyperplasia and dermal cell infiltration (Figure 2a). The infiltrated cells comprised CD4$^+$ cells (Figure 2b), eosinophils (Figure 2c), and basophils (Figure 2d), all of which are characteristic cells in allergic skin inflammation (Leung *et al.*, 2004; Ito *et al.*, 2011). This local skin infiltrate was not seen in NT or e.c. PBS–treated CT skin (Figure 2a–e), indicating that it was an immune response upon allergen treatment on barrier-impaired skin, but was not induced by barrier disruption alone. Notably, the infiltrate of eosinophils and basophils was abolished in e.c. OVA–treated Rag1$^{-/-}$ mice (Figure 2f), indicating that it was a T- and B-cell-dependent, but not an innate inflammatory, response. Very few CD8$^+$ T cells or neutrophils were detected in e.c.-treated CT skin (Figure 2e and data not shown). At D34, e.c. OVA–treated CT mice showed an immune infiltrate similar to that seen on D12 (data not shown).

In contrast, e.c. OVA–treated MT mice displayed a drastically reduced skin allergic inflammation, exhibiting lesser epidermal hyperplasia and dermal infiltrate of CD4$^+$ cells, eosinophils, and basophils at D12 (Figure 2a–e), as well as at D34 (data not shown). Altogether, these results indicate that keratinocytic TSLP is essential for generating skin allergic inflammation upon OVA treatment on barrier-impaired skin.

The T helper type 2 (Th2) response induced by e.c. OVA treatment is impaired in mice lacking TSLP in keratinocytes

When examined at D12, skin-draining lymph nodes (LN) from the e.c. OVA–treated CT mice showed a significant induction of IL-4, compared with NT or e.c. PBS–treated CT mice (Figure 3a). This increase was abrogated in e.c. OVA–treated TSLP$^{iep-/-}$ MT mice (Figure 3a). In contrast, the expression of Th1 cytokine (IFN-γ) was not significantly affected, whereas that of Th17 cytokine (IL-17A) was below detectable levels (Figure 3a). Furthermore, we found that IL-4 expression was induced in purified CD4$^+$ T cells from skin-draining LNs of e.c. OVA–treated CT (Figure 3b), but not in

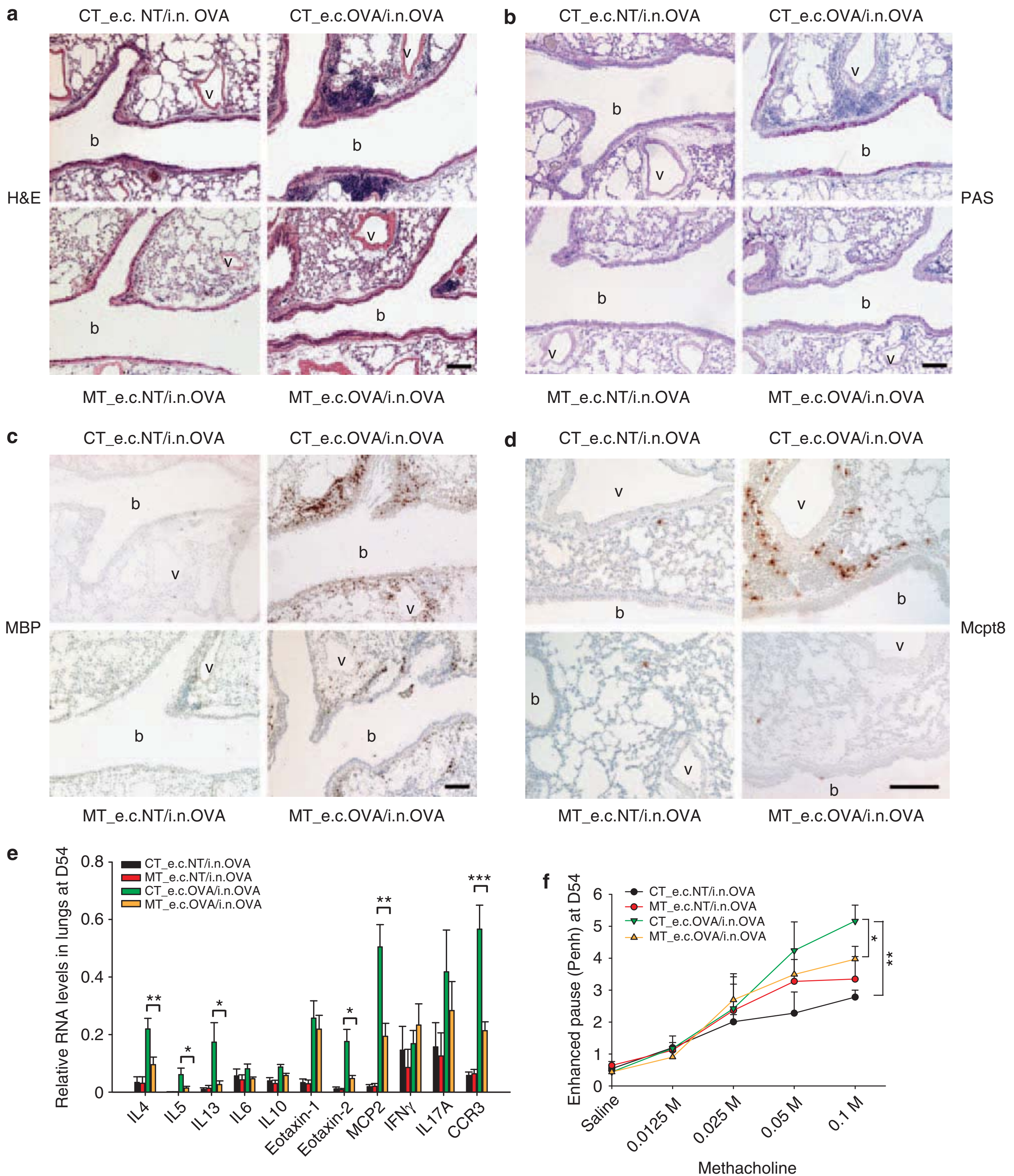

Figure 4. An attenuated allergic asthma phenotype in epicutaneous (e.c.)-sensitized thymic stromal lymphopoietin (TSLP)$^{iep-/-}$ mutant (MT) mice upon intranasal (i.n.) challenge. (**a**) Hematoxylin and eosin (H&E) staining of lung section at day (D)54. ''e.c. ovalbumin (OVA)/i.n.OVA'', mice with e.c. OVA sensitization and i.n. OVA challenge; ''e.c. nontreated (NT)/i.n.OVA'', mice without any e.c. treatment but with i.n. challenge of OVA. (**b**) Periodic acid Schiff (PAS) staining of lung sections at D54. Mucus-secreting goblet cells are stained purple in bronchioles. (**c**, **d**) Immunohistochemical (IHC) with antibodies against (**c**) MBP (for eosinophils) and (**d**) Mcpt8 (for basophils) of lung sections at D54. Dark red color stands for stained cells. (**e**) Quantitative reverse-transcriptase–PCR (RT-PCR) analyses of cytokines and chemokines in lungs at D54. (**f**) Airway hyperresponsiveness (AHR) evaluated by plethysmography after exposure to increasing doses of methacholine. $*P<0.05$; $**P<0.01$; $***P<0.001$ ($n \geqslant 6$ mice per group). Values are mean ± SEM. b, bronchiole; CT, wild-type control mice; v, vessel. Bar = 200 μm.

non-CD4$^+$ T cells (data not shown), indicating that CD4$^+$ T cells were the major source of IL-4. This IL-4 induction was clearly abolished in CD4$^+$ T cells from e.c. OVA–treated MT mice (Figure 3b). Notably, even though tape stripping on its own induced TSLP expression in keratinocytes of CT mice, it did not lead to IL-4 induction in CD4$^+$ T cells in the absence of OVA (compare CT_e.c.PBS with CT_ e.c.OVA, Figure 3b).

It has been reported that dendritic cells (DCs) targeted *in vitro* with TSLP express OX40L, which has been implicated in the initiation of the Th2 cell response (Ito *et al.*, 2005). The expression of OX40L was therefore examined in purified DCs from skin-draining LNs of CT and MT mice at D1 (i.e., 24 hours after the first e.c. treatment), a time at which skin DCs migrate to draining LNs after encountering an antigen (Inoue *et al.*, 2005). OX40L was induced in DCs from CT mice upon e.c. OVA (but not upon e.c. PBS); however, this induction was largely reduced in DCs from e.c. OVA–treated MT mice (Figure 3c), indicating that keratinocytic TSLP was required for OVA-induced OX40L expression in DCs. In contrast, other costimulatory factors including CD40, CD86, and CD80 were similarly increased in DCs from CT and MT mice upon OVA treatment (Figure 3c and data not shown). The expression of Th1-polarizing cytokine IL-12 was not different between OVA-treated CT and MT (Figure 3c). Taken together, these results indicate that keratinocytic TSLP has an essential role in promoting e.c. OVA–induced Th2 response.

Allergen sensitization through skin is defective in mice lacking TSLP in keratinocytes

To examine the sensitization to allergen, we first analyzed the production of OVA-specific IgE, IgG1, and IgG2a. TSLP$^{iep-/-}$ MT mice exhibited lower levels of OVA-specific IgE and IgG1, whereas OVA-IgG2a levels were comparable in CT and MT mice, after either e.c. sensitization (at D12 and D34) or i.n. challenge (at D54; Figure 3d). We then analyzed cytokine production by splenocytes upon *in vitro* OVA stimulation. Th2 cytokine (IL-4, IL-13, and IL-5) levels were all significantly reduced in MT mice at D12 (Figure 3e). A decrease of IL-17 was also observed, whereas IFN-γ levels were comparable (Figure 3e). Similar results were obtained at D34 (data not shown). Taken together, these data indicate that keratinocytic TSLP is required for an optimal Th2 allergen sensitization through barrier-defective skin.

Allergic asthma induced by e.c. sensitization and airway challenge is attenuated in mice lacking TSLP in keratinocytes

Upon i.n. OVA challenge, e.c. OVA–sensitized CT mice (CT_e.c.OVA/i.n.OVA) exhibited an allergic airway inflammation with peribronchiolar and perivascular infiltrates (compared with CT_e.c.NT/i.n.OVA, Figure 4a), comprising eosinophils (Figure 4c), basophils (Figure 4d), and CD4$^+$ T cells (data not shown). Very few CD8$^+$ T cells or neutrophils were observed (data not shown). These mice also exhibited hyperplasia of mucus-secreting goblet cells (Figure 4b). Reverse-transcriptase–PCR analyses of lungs showed an upregulated expression of Th2 cytokines (IL-4, IL-5, and IL-13), Th2 chemokines (eotaxin-1, eotaxin-2, and MCP-2), and CCR3 (mainly expressed by eosinophils and basophils), whereas expression levels of IFN-γ and IL-17A were not significantly affected (Figure 4e). Whole-body plethysmography analyses showed that these mice exhibited an enhanced airway hyperresponsiveness (Figure 4f).

In contrast to CT mice, TSLP$^{iep-/-}$ MT mice developed an attenuated asthmatic phenotype upon e.c.OVA/i.n.OVA treatment, as shown by much lesser infiltrates of eosinophils, basophils, and CD4$^+$ cells (Figure 4a, c and d, and data not shown), reduced goblet cell hyperplasia (Figure 4b), decreased expression of Th2 cytokines and chemokines (Figure 4e), and diminished airway hyperresponsiveness (Figure 4f).

This attenuated asthmatic phenotype in MT mice was due to defects in skin sensitization, but not due to airway challenge, because TSLP was selectively ablated in skin keratinocytes and not in airways (Li *et al.*, 2009; data not shown). Importantly, when sensitization was achieved through i.p. route (by injection of OVA/alum), and followed by i.n. OVA challenge, TSLP$^{iep-/-}$ MT and CT mice developed a similar sensitization and asthmatic phenotype (Supplementary Figure S1 online). Altogether, these data demonstrate that keratinocytic TSLP is crucial for promoting sensitization to allergen through skin, which triggers the ''atopic march'' and leads to allergic asthma.

Topical MC903 treatment induces keratinocytic TSLP expression, enhances e.c. sensitization, and promotes allergic asthma in a dose-dependent manner

It has been shown that TSLP is overexpressed in keratinocytes in lesioned skin of AD patients (Soumelis *et al.*, 2002). To examine whether overproduction of TSLP could boost skin sensitization, thus triggering the atopic march, keratinocytic TSLP was induced by MC903 application during the first phase of e.c. sensitization (Figure 5a). Note that upon MC903 treatment of wild-type Balb/c mice, TSLP expression was rapidly induced in a dose-dependent manner (Figure 5b), whereas ending the MC903 treatment normalized TSLP levels within 3 days (Zhang *et al.*, 2009; data not shown).

In e.c. OVA–treated TSLP^{over} Balb/c mice, Th2 and Th17 (but not Th1) cytokines secreted by splenocytes at D34 were upregulated (Figure 5c), and serum OVA–specific IgE and IgG1 were concomitantly increased (Figure 5d). It is interesting to note that these enhancing effects of MC903 were clearly dose dependent.

Upon i.n. OVA challenge, MC903-treated e.c. OVA–sensitized wild-type mice developed an aggravated asthmatic phenotype. They exhibited enhanced mucus secretion and increased airway inflammatory infiltrate comprising eosinophils, basophils, and CD3$^+$ T lymphocytes (consisting mainly of CD4$^+$ but not CD8$^+$ T cells; Figure 5e and f). The aggravation of these asthmatic phenotypes was again correlated with TSLP expression levels (Figure 5f). Accordingly, mRNA levels of Th2 cytokines (e.g., IL-4, IL-5, and IL-13), eosinophil-attractant chemokine (eotaxin-2) and receptor (CCR3) in lungs, as well as in bronchoalveolar lavage cells, were all dose-dependently increased (Figure 5g). Importantly, in the absence of e.c. OVA sensitization, MC903-induced

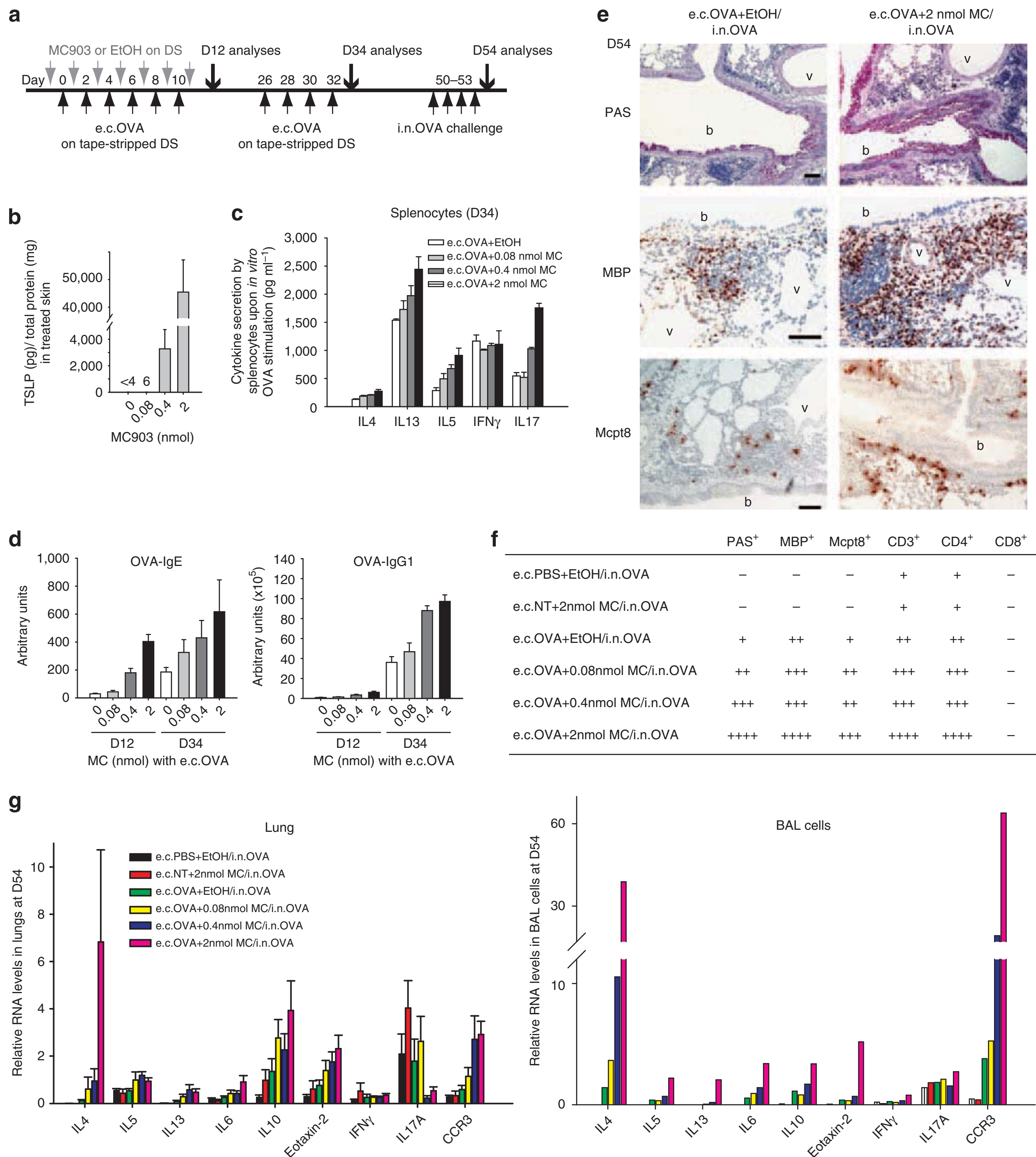

Figure 5. Skin topical application of MC903 induces keratinocytic thymic stromal lymphopoietin (TSLP) expression, enhances epicutaneous (e.c.) sensitization, and promotes allergic asthma in a dose-dependent manner. (**a**) Experimental protocol. Eight to twelve-week-old female wild-type Balb/c mice were subjected to the mouse model as described in Figure 1a, and concomitantly treated with MC903 or ethanol (EtOH) (its vehicle control) on the same area of dorsal skin (DS), during the first phase of e.c. sensitization. (**b**) TSLP protein levels measured by ELISA at day (D)2 in dorsal skin upon topical application of 0.08, 0.4, or 2 nmol MC903 at D −1 and D1. (**c**) Cytokine secretion by *in vitro* ovalbumin (OVA)–stimulated splenocytes from D34. (**d**) Serum OVA–specific IgE, IgG1, and IgG2a levels at D12 and D34. Values are mean ± SEM ($n \geq 6$ per group). (**e**) Mucus production (periodic acid Schiff (PAS) staining; in purple) and infiltrate of eosinophils (immunohistochemical (IHC) with MBP antibody) and basophils (IHC with Mcpt8 antibody) in lungs at D54. (**f**) A summary of comparison of mucus production (PAS staining) and inflammatory infiltrates of various cells (by IHC) in lungs of e.c.-sensitized and intranasal (i.n.)-challenged mice combined with different doses (0.08, 0.4, or 2 nmol) of MC903. Abundance of positive cells detected in lung sections is presented with − (not detected) to + + + + (the most abundant). (**g**) Quantitative reverse-transcriptase–PCR (RT-PCR) analyses in mouse lungs (left panel; values are mean ± SEM; $n = 6$ mice per group) and bronchoaveolar lavage (BAL) cells (right panel; pool of six mice per group) at D54. Results are representative of three independent experiments. b, bronchiole; NT, nontreated; v, vessel. Bar = 100 μm.

production of skin TSLP did not induce any asthmatic phenotype upon OVA airway challenge (see e.c.NT + 2 nmol MC/i.n.OVA; Figure 5f and g), indicating that the over-produced skin TSLP on its own (without allergen) does not promote asthma. Altogether, these data indicate that keratinocytic TSLP expression level correlates with the strength of skin sensitization and the severity of asthma.

DISCUSSION

An understanding of the mechanism of sensitization to allergen through skin is crucial for elucidating the mechanism underlying the ''atopic march'', and for possibly preventing its development. In the present study, we demonstrate that TSLP produced by keratinocytes is crucial for developing allergen sensitization through barrier-impaired skin, and for subsequent generation of allergic asthma.

The epidermis of AD patients is characterized by barrier impairment, and the majority of the patients develop sensitization to common allergens (Bieber, 2008; Cork *et al.*, 2009; Boguniewicz and Leung, 2011). We show that impairing the mouse epidermal barrier through tape stripping induces TSLP production in keratinocytes, in agreement with a recent report (Oyoshi *et al.*, 2010). Interestingly, impairment of human skin barrier by tape stripping or by application of sodium lauryl sulfate was also found to induce epidermal TSLP expression (Angelova-Fischer *et al.*, 2010). Actually, various extrinsic or intrinsic factors have been shown to impair the epidermal barrier, accompanied by TSLP production in keratinocytes (Demehri *et al.*, 2008; Briot *et al.*, 2009; Angelova-Fischer *et al.*, 2010; Takai and Ikeda, 2011). How these factors lead to TSLP expression remains to be elucidated. The induction of TSLP by tape stripping does not appear to involve the majority of TLR pathways, as TSLP expression was similarly observed in tape-stripped MyD88$^{-/-}$ mouse skin (Supplementary Figure S2 online). Whether it could be mediated via the proteinase-activated receptor 2 (Briot *et al.*, 2009; Lee *et al.*, 2010b) needs to be determined.

Our results demonstrate that keratinocytic TSLP induced by barrier impairment is essential for generating a Th2 allergic immune response, as ablation of TSLP in keratinocytes leads to the abrogation of allergic skin inflammation and Th2 response upon allergen treatment on barrier-impaired skin (Figures 2 and 3). However, TSLP induced by tape stripping does not on its own, in the absence of allergen, lead to the skin recruitment of CD4^{+} cell, eosinophils, and basophils (see Figure 2a–e). Similarly, it does not induce IL-4 (indicative of Th2 differentiation) in CD4^{+} T cells (Figure 3b), nor does it induce OX40L (a key Th2 costimulatory factor) in DCs (Figure 3c). These observations clearly suggest that TSLP acts as a crucial Th2 ''adjuvant'' contributing to the allergic immune response upon allergen treatment on barrier-impaired skin. This conclusion differs from that drawn from our own previous reports and that of others, showing that transgenic TSLP (Li *et al.*, 2005; Yoo *et al.*, 2005) or MC903-induced TSLP (Li *et al.*, 2006, 2009) overexpression in mice leads to a ''spontaneous'' AD, in the absence of allergen treatment on skin. Indeed, in those previous mouse models, TSLP expression was much higher: e.g., TSLP was detected in a high level in serum (Li *et al.*, 2005, 2006), whereas serum TSLP was below detectable level ($<7.8\,pg\,ml^{-1}$) in the present case. One possible explanation would be that above a certain threshold level TSLP could trigger an immune cascade even in the absence of penetration of any exogenous allergens into the skin. Whether such a high TSLP level exists in human AD patients is actually uncertain, but in any event our present data demonstrate that a low level of TSLP production is essential and sufficient for exerting its Th2 ''adjuvant'' effect in promoting allergen sensitization through barrier-impaired skin.

How mouse keratinocytic TSLP promotes the allergic Th2 response remains to be elucidated. Human TSLP has been shown to target DCs *in vitro* to induce a Th2 response (Soumelis *et al.*, 2002; Ito *et al.*, 2005; Liu *et al.*, 2007), but whether mouse TSLP exerts similar function was unclear (Soumelis *et al.*, 2002; Al-Shami *et al.*, 2005; He *et al.*, 2008). We found that keratinocytic TSLP was essential for OX40L expression in DCs in response to OVA, as early as 24 hours after OVA encountering in barrier-defective mouse skin (Figure 3c), suggesting that a TSLP (in keratinocytes)-OX40L (in DCs) pathway could be critically involved in the initiation of allergen-dependent Th2 response. In contrast to *in vitro* studies (Al-Shami *et al.*, 2005; Ito *et al.*, 2005), we found that e.c. OVA–induced expression of CD40, CD80, and CD86 in DCs did not require TSLP (Figure 3c and data not shown). Interestingly, a recent study has shown that the complete Th2 induction upon i.p. immunization of OVA plus adjuvant (Nod1 or Nod2 agonists) was dependent on both TSLP production in stromal cells and upregulation of OX40L on DCs (Magalhaes *et al.*, 2011). Therefore, a TSLP-OX40L axis could be one common mechanism underlying Th2 priming either through e.c. or i.p. route. In addition, we observed that antigen presentation was impaired in TSLP$^{iep-/-}$ MT mice (Supplementary Figure S3 online). Which subset(s) of DCs in the skin (Henri *et al.*, 2010) is (are) possibly responsible for the TSLP-promoted Th2 response to allergens and whether TSLP may act on other antigen-presenting cells (e.g., basophils) (Maddur *et al.*, 2010) to initiate the skin allergic response remain to be investigated.

The impaired Th2 response in TSLP$^{iep-/-}$ MT mice correlates with defective skin sensitization and subsequent asthma generation. Whereas OVA-induced IL-4 expression is abolished in skin-draining LNs in these MT mice (Figure 3a), OVA-specific IgE and IgG1, as well as OVA-stimulated Th2 cytokine production in splenocytes, were not fully abrogated (Figure 3d and e), suggesting that factors (Cork *et al.*, 2009) other than TSLP also contribute to the complete Th2 sensitization to allergen in barrier-impaired skin. In contrast to its effect on Th2 response, TSLP appears to have no or a much less effect on the Th1 or Th17 response in skin (Figure 3a). Accordingly, the expression of IFN-γ or IL-17A was not different in the lung upon OVA i.n. challenge (Figure 4e). Moreover, neutrophils were barely detected in the skin upon e.c. sensitization (Figure 2e), or in the lung upon i.n. challenge (data not shown), in either CT or TSLP$^{iep-/-}$ MT mice.

In contrast to TSLP$^{iep-/-}$ MT mice in which skin sensitization is defective, TSLP^{over} mice in which TSLP expression in keratinocytes is induced by MC903 topical application develop an enhanced e.c. sensitization and subsequently an aggravated allergic asthma (Figure 5). It is interesting to note that our data establish a significant correlation of skin TSLP levels with sensitization strength, and with asthma severity, further supporting the fact that keratinocytic TSLP represents a risk factor for sensitization developed in AD patients and for triggering the onset of the ''atopic march''.

In conclusion, our study has revealed that keratinocyte-derived cytokine TSLP is crucial for promoting allergen sensitization that occurs in barrier-impaired skin, which accounts for the ''atopic march'' leading ultimately to allergic asthma. It suggests that blocking TSLP production in skin could be therapeutically useful in preventing or limiting allergen sensitization that is commonly developed in AD patients, and halting the progress of the ''atopic march''.

MATERIALS AND METHODS

Mice

Wild-type Balb/c and Rag1$^{-/-}$ mice (in Balb/c background) were purchased from Charles River Laboratories (Lyon, France). Eight to twelve-week-old K14-Cre-ER$^{T2(tg/0)}$/TSLP$^{L2/L2}$ female mice (in a Balb/c background) were injected i.p. with Tam (0.1 mg in 100 μl sunflower oil, for 5 consecutive days) (Li *et al.*, 2000) to generate TSLP$^{iep-/-}$ mice. Tam-injected age- and sex-matched littermates (K14-Cre-ER$^{T2(0/0)}$/TSLP$^{L2/L2}$) were used as CT. TSLP$^{iep-/-}$ and CT mice were subjected to experiments 4 weeks after the first Tam injection. Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the Animal Care and Use Committee of the Institut de Génétique et de Biologie Moléculaire et Cellulaire.

OVA e.c. sensitization and i.n. challenge

An area of approximately 2 cm^2 of the dorsal skin of mice was shaved and tape stripped until TEWL (transepidermal water loss) measurement reached between 15 and 20 g m^{-2} per hour (DermaLab, Cortex Technology, Hadsund, Denmark), and then treated with 200 μg of OVA (Sigma, St Louis, MO) in 50 μl PBS with a cotton swab, every other day from D0 to D10. Similar e.c. treatment was repeated for 4 times 2 weeks later from D26 to D32. NT mice or mice treated with PBS on tape-stripped skin (e.c. PBS) were used as controls. To induce an experimental asthma, mice were i.n. challenged with OVA (50 μg in 25 μl saline) 3 weeks later, on 4 consecutive days (D50 to D53). Airway reactivity was measured with whole-body plethysmography (Emka Technologie, Paris, France) 1 day after the last i.n. treatment before mice were killed for other analyses.

MC903 topical application

MC903 (calcipotriol; Leo Pharma, Ballerup, Denmark) was dissolved in ethanol (Zhang *et al.*, 2009) and topically applied on mouse dorsal skin with the quantity indicated. Ethanol was used as vehicle control.

Skin TSLP protein levels

Mouse skin was chopped and homogenized using Mixer Mill MM301 (Retsch, Haan, Germany) in lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 1 mM DTT, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche). Protein concentration of skin extract was quantified using the Bio-Rad Protein Assay (Bio-Rad, Marnes-la-Coquette, France). TSLP levels in skin extracts were determined with the Duoset TSLP ELISA development kit (R&D, Minneapolis, MN).

Antibodies

The following antibodies were used in the immunohistochemical staining: rat anti-mouse MBP (provided by Dr James J Lee, Mayo Clinic, Scottsdale, AZ), rat anti-mouse MCP-8 (clone TUG-8, Biolegend, San Diego, CA), rat anti-mouse NIMP-R14 (Abcam, Cambridge, MA), rat anti-mouse CD3 (clone 17A2, BD, Heidelberg, Germany), rat anti-mouse CD4 (clone RM4-5, or clone GK1.5, BD) and rat anti-mouse CD8 (clone 53-6.7, BD).

Splenocyte culture

Single-cell suspensions of spleen were cultured in medium (RPMI 1640 without HEPES supplemented by 10% FCS, 2 mM glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin) at a density of 2.5×10^6 ml^{-1} in the presence of 500 μg ml^{-1} OVA in anti-CD3 (10 μg ml^{-1}; clone 145-2C11 (e-Bioscience, Paris, France))- coated 96-well plates for 3 days. Cytokine levels of IL-4, IL-5, IL-13, IL-17, and IFN-γ in culture supernatants were measured using the Duoset ELISA Development system (R&D).

Isolation of CD4$^+$ T cells and DCs from mouse LNs

LN CD4$^+$ T cells were purified using the mouse CD4$^+$ T-cell Isolation Kit II (Miltenyi Biotec, Paris, France; purity was confirmed to >94%). LN CD11c$^+$ DCs were first enriched using CD11c Microbeads (Miltenyi Biotec) and then sorted with FACS Aria II (BD; purity was confirmed to >95%).

Statistical analysis

Data were analyzed using SigmaStat (Systat Software, Richmond, CA) by the Student's *t*-test or the Mann–Whitney rank-sum nonparametric test depending on results from the Kolmogorov–Smirnov test (with Lilliefors' correction) for normality and Levene Median test for equal variance. Two-way repeated-measures ANOVA was used to compare airway hyperresponsiveness between groups responding to various doses of methacholine.

Other methods

Histopathology, immunohistochemical staining, serum Ig determination, quantitative reverse-transcriptase–PCR, and airway reactivity were performed as previously described (Zhang *et al.*, 2009; Hener *et al.*, 2011). Details of these methods, as well as *in vivo* proliferation of the adoptively transferred T cell, can be found in the Supplementary Materials and Methods online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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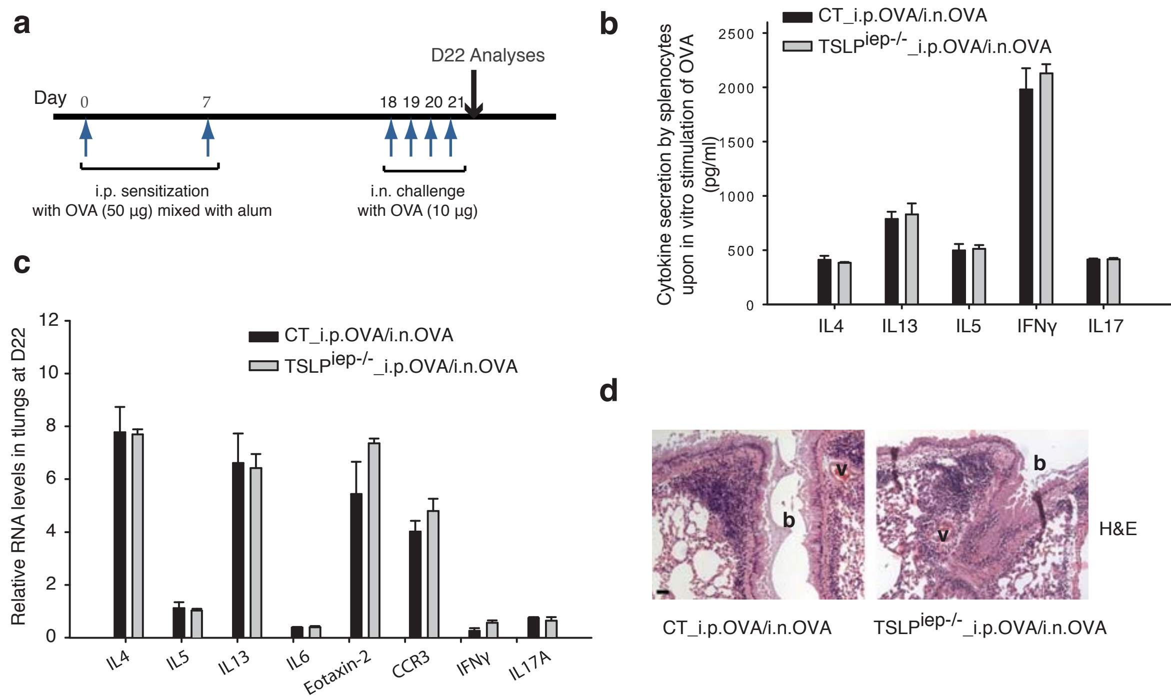
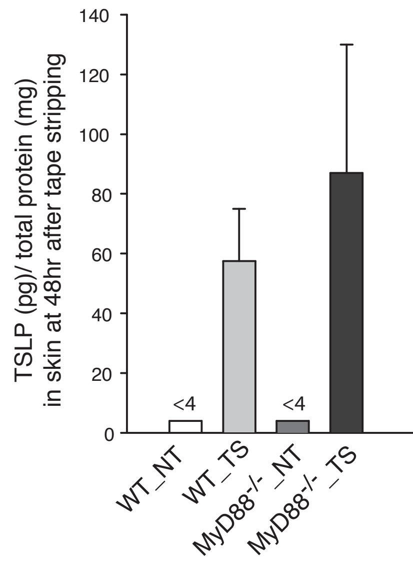
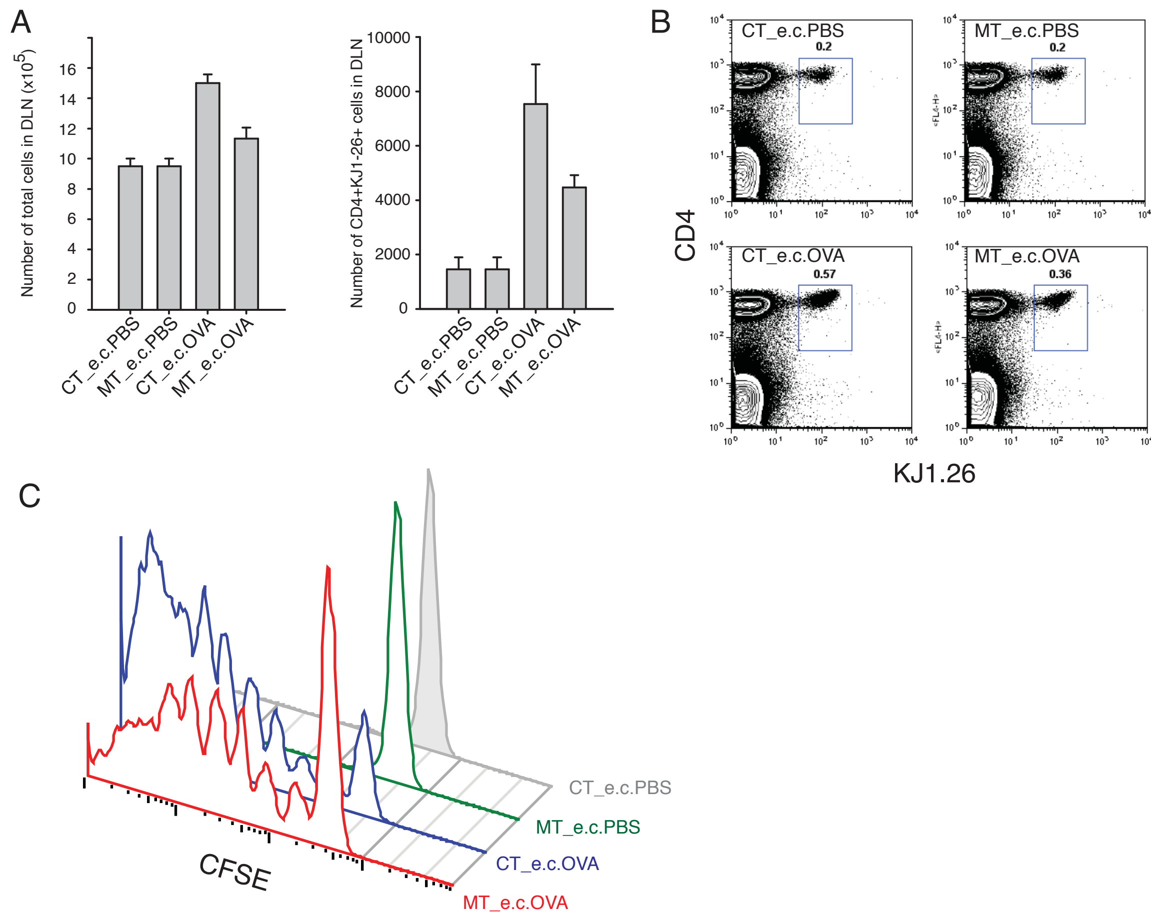


Supplementary Figure S1. Keratinocytic TSLP is not required for intraperitoneal (i.p.) sensitization with OVA/Alum, and for the generation of asthmatic phenotype upon intranasal (i.n.) OVA challenge. (**a**) An experimental asthma protocol employing i.p. sensitization of OVA mixed with alum (aluminum hydroxide) (at D0 and D7) and i.n. challenge of OVA (at D18, 19, 20 and 21). (**b**) Cytokine secretion by splenocytes at D22 upon in vitro stimulation of OVA, showing comparable levels between TSLP$^{iep-/-}$ and wildtype control (CT) mice. (**c**) Quantitative RT-PCR analyses of lungs at D22 from TSLP$^{iep-/-}$ and CT mice. Values are mean ± SEM (n≥5 mice per group). (**d**) H&E staining of lung paraffin sections from D22, showing comparable inflammatory infiltrates between TSLP$^{iep-/-}$ and CT mice. b, bronchiole; v, vessel. Scale bar, 50 µm.



Supplementary Figure S2 MyD88 is not required for tape-stripping-induced TSLP expression in keratinocytes. Dorsal skin of twelve weeks old MyD88$^{-/-}$ (in a mixed background of 75% C57BL/6 and 25% Balb/c) mice and their wildtype (WT) littermates was shaved and tape-stripped, and was analyzed 48 hrs later. TSLP protein levels in skin were measured by ELISA. NT, non-treated. TS, tape-stripped. Values are mean ± SEM (n=3 mice per group).



Supplementary Figure S3. Defective expansion of adoptively transferred TCR-OVA transgenic T cells in skin-draining lymph nodes (LNs) of e.c. OVA-treated TSLP$^{iep-/-}$ mutant (MT) mice. Naïve CD4$^+$ DO11.10 cells were adoptively transferred into wildtype control (CT) or MT recipients, and followed by e.c. treatment with OVA (200 µg) or saline on the tape-stripped dorsal skin at 48 hours later. Skin-draining LNs were analyzed 4 days later for the accumulation of CD4$^+$KJ1.26$^+$ cells. (**a**) Numbers of total cells and CD4$^+$KJ1.26$^+$ cells in skin-draining LNs. (**b**) Representative FACS plots of CD4$^+$KJ1.26$^+$ cells of skin-draining LNs of recipients of CD4$^+$ DO11.10 cells. (**c**) Less cell division of CFSE-labeled CD4$^+$ DO11.10 cells in skin-draining LNs of e.c. OVA-treated MT, compared to those of CT mice. No cell division was seen in e.c. PBS-treated CT or MT mice.

SUPPLEMENTARY MATERIALS AND METHODS

Histopathology. Skin or lung tissues were fixed overnight at 4 °C in 4% paraformaldehyde (PFA), and embedded in paraffin. Sections (3µm) were stained with hematoxylin/eosin (H&E), or Periodic acid-Schiff (for goblet cells in lung).

Immunohistochemical (IHC) staining. For IHC staining of MBP (for eosinophils) or NIMP-R14 (for neutrophils), 3 µm paraffin sections were treated with 0.6% H_2O_2 to block the endogeneous peroxidase activity, followed by digestion with Pepsin solution (Invitrogen) in order to retrieve antigen. For IHC staining of MCP-8 (for basophils), paraffin sections were treated with citric buffer (10mM citric acid, pH6) to retrieve antigen, followed by blocking the endogenous peroxidase activity with 0.6% H_2O_2. Slides were then blocked with rabbit serum (Vector Laboratories), and incubated with rat-anti-mouse MBP (provided by Dr James J Lee, Mayo Clinic, Rochester, USA), NIMP-R14 (Abcam) or rat-anti-mouse MCP-8 (clone TUG-8, Biolegend) antibodies respectively. After incubation with biotinylated rabbit anti-rat IgG, followed by AB complex (Vector Laboratories), staining was finally visualized with AEC+ high sensitivity substrate chromogen solution (Dako) and counterstained with hematoxylin.

For CD3, CD4 and CD8 labeling, 10 µm frozen sections were fixed in 4% paraformaldehyde, permeabilized with cold acetone, and blocked with normal goat serum (Vector laboratories). Slides were then incubated with primary antibody [rat monoclonal anti-CD3 (clone 17A2); rat monoclonal anti-CD4 (clone RM4-5, or clone GK1.5); or rat monoclonal anti-CD8 (clone 53-6.7)]. After washing, sections were incubated with CY3-conjugated goat anti-rat IgG antibody (Jackson ImmunoResearch) and mounted with vectashield medium (Vector laboratories) containing DAPI (invitrogen).

Serum immunoglobulin determination. For OVA-specific Igs, microtiter plates (R&D Systems) were first coated with OVA (20 μg/ml in 0.1M sodium bicarbonate buffer). After blocking with 1% BSA (Sigma), the coated plates were then incubated with serum samples, followed by incubation with a biotinylated rat anti-mouse IgE (clone R35–118, BD Biosciences PharMingen), rat anti-mouse IgG1 (clone A85-1, BD Biosciences PharMingen), or rat anti-mouse IgG2a (clone R19-15, BD Biosciences PharMingen). ExtrAvidin peroxidase (Sigma) and TMB (tetramethylbenzidine) Substrate Reagent Set (BD Biosciences PharMingen) were used for detection. Serum levels of OVA-specific Igs were related to a pooled serum from OVA-sensitized and challenged BALB/c mice (internal standard) and expressed as arbitrary units.

Quantitative RT-PCR. RNA was reverse-transcribed using random oligonucleotide hexamers and amplified by quantitative PCR with a Ligthcycler 480 (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Relative RNA levels were calculated using hypoxanthine phosphoribosyltransferase (HPRT) an internal control. Sequences of PCR primers: HPRT, TSLP, IL4, IL5, IL13, IFNγ, IL10, IL6, Eotaxin-1, Eotaxin-2, MCP2 and CCR3 were as previously reported (Hener *et al.*, 2011); IL17A (239bp) (5'-CCAGGGAGAGCTTCATCTGT-3'; 5'-ACGTGGAACGGTTGAGGTAG-3'); OX40L (204 bp) (5'-GCTAAGGCTGGTGGTCTCTG-3'; 5'-ACCGAATTGTTCTGCACCTC-3';); CCR7 (165 bp) (5'-AAAGCACAGCCTTCCTGTGT-3'; 5'-AGTCCACCGTG GTATTCTCG-3') ; CD86 (246 bp) (5'-CACGAGCTTTGACAGGAACA-3'; 5'-TTAGGTTTCGGGTGACCTTG-3').

Airway Reactivity. Airway reactivity was measured by noninvasive whole-body plethysmography (Emka Technologies, France) in response to inhaled methacholine (Sigma), using enhanced pause (Penh) as an index of airway responsiveness.

In vivo proliferation of adoptively transferred T cell. OVA-specific $CD4^+$ T cells were purified from DO11.10 mice (The Jackson Laboratory) using mouse $CD4^+$ T cell Isolation Kit II (Miltenyi), stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma), and were *i.v.* injected ($1x10^6$ cells) to $TSLP^{+/+}$ or $TSLP^{iep-/-}$ recipient mice. At 48h after, mice were e.c. treated with 200 µg OVA or PBS on tape-stripped dorsal skin. Four days after, skin-draining LNs were harvested and in vivo proliferation of the adoptively transferred DO11.10 cells was analyzed by CFSE dilution in $CD4^+KJ1\text{-}26^+$ cells.

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PART 2.A

TSLP promotes innate and adaptive recruitment of basophils in skin.

Juan Manuel Leyva-Castillo et al. (manuscript in preparation).

Basophils have been recognized as important players for Th2 immune responses. Using a cytokine thymic stromal lymphopoietin (TSLP)-dependent atopic dermatitis mouse model (induced by topical MC903 treatment), we report here that increased skin TSLP expression promotes an early innate recruitment of basophils to the skin, followed by a later recruitment of basophils, which involves adaptive immune response.

INTRODUCTION.

Basophils are the least abundant granulocyte population accounting for less than 1% of white blood cells in circulation. Basophils are recruited to the site of inflammation after exposure to stimuli such as allergens or helminth parasites (1-6), suggesting that local expression of cytokines, chemokines and/or growth factors attract basophils from the circulation.

Several studies in various mouse models have indicated that basophil migration and function are dependent on cells of the adaptive immune system. Experimental infection with the gastrointestinal nematode, *Nippostrongylus brasiliensis* (*N. brasiliensis*) leads to recruitment of IL-4 expressing basophils in the lung and liver of infected mice. Basophil accumulation and its IL-4 production in the effector sites were completely abolished in the absence of adaptive immune cells. Moreover, CD4+ T cell transfer in adaptive immune cell deficient mice restores IL-4 expressing basophil recruitment in effector sites (2, 4, 5), suggesting that $CD4^+$ T cells, but not the production of immunoglobulins, are required for basophil migration induced by *N. brasilensis*. However, antibiotic-treated and germ-free mice presented an elevated number of blood basophil that correlate with the increased IgE serum levels (7), suggesting that in some context, the humoral response might also be implicated in basophil migration.

The innate inflammatory signals inducing basophil mobilization remain unclear. It has been shown that thymic stromal lymphopoietin (TSLP), a key epithelium-derived cytokine in allergic inflammation (8-10), promotes peripheral basophilia in absence of IL-3 (11), an important basophil differentiation and activation factor (12-15). However, whether adaptive immune cells are required in TSLP-dependent basophilia was not investigated.

Our previous studies (16-18) and that of others (19, 20) have demonstrated that elevated TSLP levels in mouse skin induces a spontaneous AD-like dermatitis. Employing our

previously established mouse AD model, generated either by topical MC903 treatment (16) or by selective ablation of RXRα and RXRβ in adult epidermal keratinocytes (18), we demonstrate that TSLP overexpression not only triggers an inflammatory infiltration of $CD4^+$ T cells, mast cells and eosinophils, but also leads to skin basophilia.

Taking advantage of inducible TSLP expression by topical MC903 treatment, which provides a tool to analyze the kinetics of immune cell infiltrate in the skin, we reveal that TSLP-promoted basophil recruitment to skin exhibits an early “innate phase”, followed by a later phase in which adaptive immunity plays a role.

MATERIALS AND METHODS.

Mice. Wild-type BALB/c mice and Rag1-/- mice (in a Balb/c background) were purchased from Charles River Laboratories. CD11c-DTR (21) (in C56BL/6 background) and TSLP-/- (16) (in Balb/c background) mice have been previously described. K14-Cre$^{ERT2(tg/0)}$/RXR$\alpha^{L2/L2}$/RXR$\beta^{L2/L2}$ were crossed with TSLP$^{L2/L2}$ mice to generate K14-Cre$^{ERT2(tg/0)}$/RXR$\alpha^{L2/L2}$/RXR$\beta^{L2/L2}$/TSLP$^{L2/L2}$ mice (mice are in mixed background of 50% C56BL/6 and 50% 129SV/J). Mice were injected intraperitoneally with 0.1 mg/mouse/day of tamoxifen (Tam) for 5 days to generate RXR$\alpha\beta^{ep-/-}$ and RXR$\alpha\beta^{ep-/-}$/TSLP$^{ep-/-}$mice. Tam-injected age- and sex-matched littermates (Cre$^{ERT2(0/0)}$/RXR$\alpha^{L2/L2}$/RXR$\beta^{L2/L2}$) were used as wildttype controls (CT). Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the Animal Care and Use Committee of the Institut de Génétique et de Biologie Moléculaire et Cellulaire.

MC903 Topical Application. MC903 (calcipotriol; Leo Pharma, Denmark) was dissolved in ethanol (ETOH) and topically applied on mouse ears (1 nmol in 25 μl per ear). As vehicle control, the same volume of ETOH was applied on mouse ears.

Single cell preparations. For preparation of dermal cells, mouse ears were splited and floated (epidermis facing up) on 2.5 mg/ml Dispase (Roche) in PBS overnight at 4°C. The dermis was separated from the epidermis and digested in 1 mg/ml collagenase D (Roche), 2.5% FCS and 100 U/ml DNase I (Roche) in PBS at 37°C for 1 h, and then filtered through 70μm cell strainer (BD).

White blood cells were separated using 2% dextran (Sigma). Briefly, 300 μl of heparinized mouse blood was mixed 1:1 with a solution of 2% dextran, and incubated for 30 min at 37°C. The upper phase was taken and centrifuged (3000 rpm) for 5 min, to recover with blood cells.

Bone marrow cells from femurs of mice were flushed 8 times with 0.5% BSA and 2mM EDTA in PBS. Spleen cells were obtained by mechanical dissociation. Cells were then passed through 70 μm cell strainer. Red blood cells were removed by incubation in cold ACK lysis buffer at RT for 5 min.

Antibodies and flow cytometry. The following antibodies were used in flow cytometry analyses: purified anti-FcγRIII/II (CD32/16; Fc Block; clone 2.4G2), FITC-conjugated anti-Gr-1 (clone RB6-8C5), PE- conjugated anti-siglec-F (clone E50-2440) and V500- conjugated anti-CD3 (clone 500-A2) from BD Biosciences; biotin- conjugated anti-CD49b (clone DX5) and APC- conjugated anti-CD45 (clone 30-F11) from eBioscience; APC-Cy7- conjugated anti-CD117 (c-kit; clone 2B8), PECy7- conjugated anti-FCεRIα (clone MAR-1), FITC-conjugated anti-CD200R (clone OX-110), PE- conjugated anti-CD123 (IL-3Rα; clone 5B111), Pacific Blue- conjugated anti CD11b (clone M1/70), Alexa Fluor 647- conjugated anti-CD218a (IL-18Rα; clone BG/IL18RA) and PerCP-Cy5.5- conjugated anti-CD45R (B220; cloneRA3-6B2) from Biolegend; FITC-conjugated anti T1/ST2 (IL-33R, clone DJ8) from mdbioproducts; and Qdot-585- conjugated Streptavidin from Invitrogen.

Cells were incubated with 2.4G2 antibody for 10 min, washed and stained with a mix of antibodies for 10 min. Finally cells were washed and analyzed on a LSRII flow cytometer (BD Biosciences). Results were analyzed using FlowJo (Treestar).

Histopathology. Skin tissues were fixed overnight at 4 °C in 4% paraformaldehyde (PFA), and embedded in paraffin. Sections (3 μm) were stained with hematoxylin/eosin (H&E).

Immunohistochemical (IHC) staining. Paraffin sections were treated with citric buffer (10 mM citric acid, pH6) to retrieve antigen, followed by blocking the endogenous peroxidase activity with 0.6% H_2O_2. Slides were then blocked with rabbit serum (Vector laboratories) and incubated with rat-anti-mouse MCP-8 (clone TUG-8, Biolegend). After incubation with

biotinylated rabbit anti-rat IgG, followed by AB complex (Vector Laboratories), staining was finally visualized with AEC+ high sensitivity substrate chromogen solution (Dako) and counterstained with hematoxylin.

RNA extraction and Quantitative RT-PCR

RNA was extracted using TRI Reagent (Sigma-Aldrich). RNA was reverse-transcribed using random oligonucleotide hexamers and amplified by quantitative PCR with a Ligthcycler 480 (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Relative RNA levels were calculated using hypoxanthine phosphoribosyltransferase (HPRT) an internal control. Sequence of PCR primers:

HPRT (164bp) (5'- TGGATACAGGCCAGACTTTG -3'; 5'-GATTCAACTTGCGCTCATCTTA -3')

TSLP (194bp) (5'-AGCTTGTCTCCTGAAAATCGAG-3'; 5'-AGGTTTGATTCAGGCAGATGTT-3');

MCPT8 (159bp) (5'- GTGGGAAATCCCAGTGAGAA-3'; 5'-TCCGAATCCAAGGCATAAAG-3');

IL-3 (161bp) (5'- CTGCCTACATCTGCGAATGA-3'; 5'-TTAGGAGAGACGGAGCCAGA-3').

Depletion of CD11c$^+$ dendritic cells (DCs) in vivo

For depletion of CD11c$^+$ DCs in vivo, CD11c-DTR$^{Tg/0}$ mice received an intraperitoneal injection of diphtheria toxin (DT) (100 ng per mouse) one day before MC903 treatment. Depletion efficiency was eveluated by analysis of DCs in lymph nodes, skin and splenocytes. DT-injected littermate (CD11c-DTR$^{0/0}$) mice were used as controls.

Statistic analysis

Data were analyzed using SigmaStat (Systat Software Inc. Point Richmond, CA, USA) by the

Student t test or the Mann-Whitney rank sum nonparametric test depending on results from the Kolmogorov-Smirnov test (with Lilliefors' correction) for normality and Levene Median test for equal variance.

RESULTS

Topical application of MC903 induces a TSLP-dependent skin basophilia

To induce TSLP expression in skin keratinocytes and an AD-like inflammation, mouse ears were topically treated with MC903 (1 nmol per ear) every other day from day (D) 0 to D10 (Fig. 1A). When examined at D11, TSLP mRNA level was induced in MC903-treated wildtype (TSLP+/+) skin, but not in MC903-treated TSLP-/- skin (Fig. 1B). Immunohistochemical (IHC) staining with an antibody against MCPT8 [a basophil specific differentiation marker (22)] showed a massive infiltrate of basophils in the dermis of MC903-treated wildtype ears, but not of MC903-treated TSLP-/- ears (Fig. 1C). Correspondingly, MCPT8 mRNA levels were increased in MC903-treated wildtype, but not TSLP-/- ears, at D11 (Fig 1D).

Skin dermal cells were further analyzed by flow cytometry. A massive dermal infiltration of $CD45^{+}$ hematopoietic cells was seen in MC903-treated wildtype ears, which was largely abrogated in MC903-treated TSLP-/- ears (Fig 1E, black square gates). The inflammatory infiltrate included T-lymphocytes ($CD45^{+}CD3^{+}$), mast cells ($CD45^{+}CD3^{-}B220^{-}CD117+$ $Fc\varepsilon RI\alpha^{+}$) and eosinophils ($CD45^{+}CD3^{-}B220^{-}Gr1^{+}SiglecF^{+}$) (data not shown), as reported in our previous studies (16-18). In addition, a $CD45^{low}$ population was clearly observed in MC903-treated wildtype ears (Fig. 1E, blue circle gates). This $CD45^{low}$ population was further characterized as basophils ($CD3^{-}B220^{-}CD49b^{+}Fc\varepsilon RI\alpha^{+}CD117^{-}Gr1^{-}SiglecF^{-}$) (Fig. 1F) (2). Both frequency and total cell number of basophils were increased in MC903-treated wildtype ears (compared with ETOH-treated wildtype mice) (Fig 1G). However, these

increases were largely abolished in MC903-treated $TSLP^{-/-}$ ears (Fig. 1G). Taken together, these results indicate that TSLP expression induced by topical application of MC903 triggers skin basophilia.

In addition, MC903-treated wildtype mice also exhibited increased number of basophils in peripheral blood, spleen and bone marrow at D11 (Fig. 1H), which were all abolished in MC903-treated $TSLP^{-/-}$ mice. This is in agreement with recent reports showing that injection of recombinant TSLP leads to peripheral basophilia (11, 23).

Mice with selective ablation of RXRα and β in adult epidermal keratinocytes develop a TSLP-mediated skin basophilia

We previously reported that tamoxifen (Tam)-induced ablation of both RXRs α and β selectively in adult mouse keratinocytes ($RXR\alpha\beta^{ep-/-}$) induced the expression of TSLP in keratinocytes and generated a spontaneous AD-like skin inflammation (18). 8 weeks after Tam injection (wka), $RXR\alpha\beta^{ep-/-}$ mice showed increased TSLP mRNA levels in the skin (Fig. 2A), accompanied by the elevated frequency and total cell number of basophils in skin dermis (Fig. 2B and C). Skin basophilia in $RXR\alpha\beta^{ep-/-}$ mice was also confirmed by IHC of MCPT8 (Fig. 2D) and quantitative RT-PCR analyses of MCPT8 mRNA levels (Fig. 2E). In contrast, basophil recruitment was abolished in $RXR\alpha\beta^{ep-/-}$/$TSLP^{ep-/-}$ mice (Fig. 2B-E), demonstrating that skin basophilia in mice lacking RXRαβ selectively in epidermal keratinocytes is mediated by keratinocytic TSLP.

TSLP-induced skin basophil recruitment exhibits an earlier "innate" phase followed by a later "adaptive" phase

We further investigated the kinetics of basophil recruitment into skin dermis upon MC903 treatment. Flow cytometry analyses showed that basophil number in ear dermis was increased as early as D3, and further increased from D5 to D11 (Fig. 3A). Consistently, the increase of MCPT8 mRNA levels in MC903-treated skin exhibited similar kinetics (Fig. 3B). To investigate whether T cells could be implicated in basophil recruitment in the skin (2, 4, 5), we examined the kinetics of CD3+ T cell recruitment in skin dermis upon MC903 treatment. A significant increase of CD3+ T cell number was observed from D5, but not at D3 (Fig. 3C), suggesting that MC903-induced skin recruitment of basophils occurred earlier than that of T-lymphocytes. This early recruitment of basophils was mediated by TSLP, as it was completely abolished in MC903-treated TSLP-/- mice (Fig. 3D).

To examine whether the TSLP-induced early recruitment of basophils at D3 is dependent of adaptive immunity, RAG1-/- mice lacking mature T- and B-lymphocytes were subjected to MC903 treatment. TSLP-induced early recruitment of basophils at D3 was comparable between RAG1+/+ and RAG1-/- mice (Fig. 3E), suggesting that it is independent of T-and B-cells. As dendritic cells (DCs) are the bridge between innate and adaptive immune responses through cytokine production and T cell activation (24), we further examined the requirement of DCs in basophil recruitment. We found that the early recruitment of basophils was unaffected in CD11c^{DEP} mice, in which CD11c+ DCs were depleted upon dipheteria toxin (DT) injection to CD11c-DTR$^{Tg/0}$ mice (21) (Fig. 3F). These results thus indicate that TSLP induces an early "innate" recruitment of basophil into the skin, which is independent of adaptive immunity.

We next examined whether adaptive immunity might be involved in the recruitment of basophils at a later stage. At D5, both basophil and T-lymphocyte numbers were increased in the dermis of MC903-treated wildtype ears (Fig. 4A, see also Fig. 3A and C), but not in MC903-treated TSLP-/- ears (Fig. 4A), confirming that these are TSLP-triggered effects. Interestingly, basophil and T cell numbers at D5 were both decreased in MC903-treated CD11c^{DEP} mice, when compared to control CD11c^{CT} (DT-injected CD11c-DTR0/0 mice) (Fig 4A and B), suggesting that DCs play a role in the accumulation of T-lymphocytes and basophils in the skin. Moreover, we observed that basophil number was clearly lower in RAG1-/- ears than in RAG1+/+ ears at D5 upon MC903 treatment (Fig. 4C), with similar results obtained at D11 (Fig. 4D). Decreased basophil infiltrate in the skin was also confirmed by IHC staining of basophils using MCPT8 antibody (Fig. 4E), and by the analyses of MCPT8 mRNA levels (Fig. 4F). Note that MC903-induced skin TSLP expression was similar in CD11c^{DEP} and CD11c^{CT}, and in RAG1+/+ and RAG1-/- mice (data not shown). Altogether, these results indicate that adaptive immunity contributes to the later stage of basophil skin recruitment triggered by TSLP.

TSLP induces IL-3 expression in the skin

It has been shown that IL-3 produced by activated CD4$^+$ T cells plays a key role in the recruitment of the basophils into draining lymph nodes, lung and liver, following helminth infection (2, 5, 25). To examine whether IL-3 could be implicated in basophil recruitment in the skin, we first analyzed the kinetics of IL-3 expression in MC903-treated wildtype Balb/c ears. Whereas IL-3 mRNA levels were undetectable from D0 to D3, it was increased at D5 and D11 (Fig. 5A). In TSLP-/- mice, the induction of IL-3 expression by MC903 was

completely abolished at D5, and largely abrogated at D11 (Fig. 5B), indicating that MC903-induced IL-3 expression is triggered by TSLP.

Next, we examined IL-3 expression in MC903-treated RAG-1 ears. Results showed that IL-3 mRNAs were not detected RAG1-/- ears at D5 or D11 (Fig. 5C), suggesting that cells from adaptive immunity, most probably CD4+ T cells, could be the source of IL-3. Taken together, these above data suggest a possible role of IL-3 in later stage of TSLP-induced skin basophilia.

Skin basophils exhibit phenotypical differences in wildtype and RAG1-/- mice upon MC903 treatment

We further tested whether basophils in MC903-treated wildtype and RAG1-/- skin may have any phenotypical differences. Several basophil-associated surface markers were analyzed by flow cytometry. No difference of expression was observed for IL-18R, CD123, IL-33R and FcεRIα (Figure 6A, B, C and D). However, basophils in MC903-treated RAG1-/- skin exhibited lower expression of CD200R (Figure 6E), but higher expression of CD11b (Figure 6F). These data indicate that in the RAG1-/- mice, TSLP-induced skin basophils not only are less in quantity (see Fig. 6C), but also exhibit phenotypical differences.

DISCUSSION.

Here, using a TSLP-dependent AD-like skin inflammation mouse model triggered by topical MC903 treatment (16), we confirmed that increased TSLP expression in the skin not only induces an inflammatory infiltrate including T cells, eosinophils and mast cells, as previously reported, but also triggers skin basophilia. In addition, this was confirmed by another TSLP-induced mouse model in which RXRα and RXRβ were ablated in adult epidermal keratinocytes. We further showed that topical MC903 treatment promotes TSLP-dependent early innate skin basophil recruitment, which is followed by at later stage in which the adaptive immune cells contribute to the skin basophilia. In addition, TSLP-induced skin basophils present phenotypic differences in absence of adaptive immune cells.

Our results demonstrated that mature T and B cells, and DCs were dispensable for TSLP-induced innate basophil skin recruitment at D3 (Figure 3E and F). How elevated TSLP expression induces the innate recruitment of basophil in skin remains to be elucidated. It does not involve basophil chemoattractant factors such as IL-3, IL-5, eotaxin-1 and eotaxin-2 (26) as their mRNA levels were all unchanged at D3 (data not shown). We observed a small increase of MCP-2 (CCL8) and MCP-3 (CCL7), basophil chemoattractant factors (27), at D3 (data not shown). Whether these chemokines could be implicated in basophil recruitment or whether TSLP may attract basophils directly to skin, remains unclear.

Besides basophil recruitment in skin, we also observed that TSLP induces basophil recruitment in peripheral blood, spleen and bone marrow (Figure 1H), in agreement with recent report showing that TSLP induces basophil hematopoiesis (11, 23). These could be consequence from keratinocyte-derived TSLP entered into circulation. Interestingly, TSLP-induced peripheral basophilia did not seem to require adaptive immunity, as RAG1-/- mice

exhibit similar basophil number in blood, spleen and bone marrow as control mice at D11 (data not shown).

Our results also demonstrated that adaptive immunity was implicated in TSLP-promoted basophil skin recruitment at D5 and after (D11). Which type of adaptive immunity (cellular or humoral) was responsible for the increased TSLP-dependent basophil recruitment at this later stage remains to be investigated. However, our results showed that the adoptive transfer of $CD4^+$ T cells into RAG1-/- mice leads to increased basophil number in MC903-treated skin, suggesting that $CD4^+$ T cells contribute to basophil skin recruitment at this later stage. In this respect, various reports have shown that activated $CD4^+$ T cells are required to promote basophil migration to the liver, lungs and draining lymph node of *N. brasiliensis* infected mice (2, 4, 5, 25). Moreover, we also observed an increase of IL-3 at the later stage of TSLP-promoted skin basophil recruitment, which was completely abolished in MC903 treated RAG1-/- mice. Considering that CD4+ T cell-derived IL-3 is a key factor for basophil activation, expansion, migration and survival (2, 12, 14, 15, 25), we are under way to determine if IL-3 is required for TSLP-induced basophil recruitment at this later stage.

Our results demonstrate that skin basophils from MC903-treated wildtype (RAG+/+) mice presented higher CD200R (a basophil activation marker (28)) and lower CD11b expression compared with those from RAG1-/- mice, suggesting that although TSLP can promote basophil recruitment without the need of adaptive immune system, the full activation of basophils may be influenced by the adaptive immunity. Our preliminary results showed an higher CD200R expression in skin basophils from MC903-treated CD4+ T cell-reconstituted RAG1-/- mice compared with MC903-treated RAG1-/- mice at D5 (data not shown). Interestingly, a recent report suggest that both $CD4^+$ T cell-derived cytokines and direct cell

contact contributed to full activation of basophils (4). Whether these mechanism are involved in the full activation of skin basophils induced by TSLP remains to be determined.

In conclusion, our results demonstrate that the role of keratinocyte-derived TSLP in promoting skin basophilia involves different mechanisms in different stages. The early TSLP-driven recruitment of basophils to skin is an innate phase, whereas at a later stage adaptive immunity participates not only in the recruitment of the basophils to the skin but also in the activation of these basophils. Whether TSLP derived from other epithelia (e.g. lung and intestine) may have similar effects needs to be investigated. It remains also interesting to investigate during allergy and parasites infection, whether TSLP could mediated the innate initial basophil recruitment of basophils to the affected organs, followed by the development of adaptive immune response that further enhances the migration and activation of basophils.

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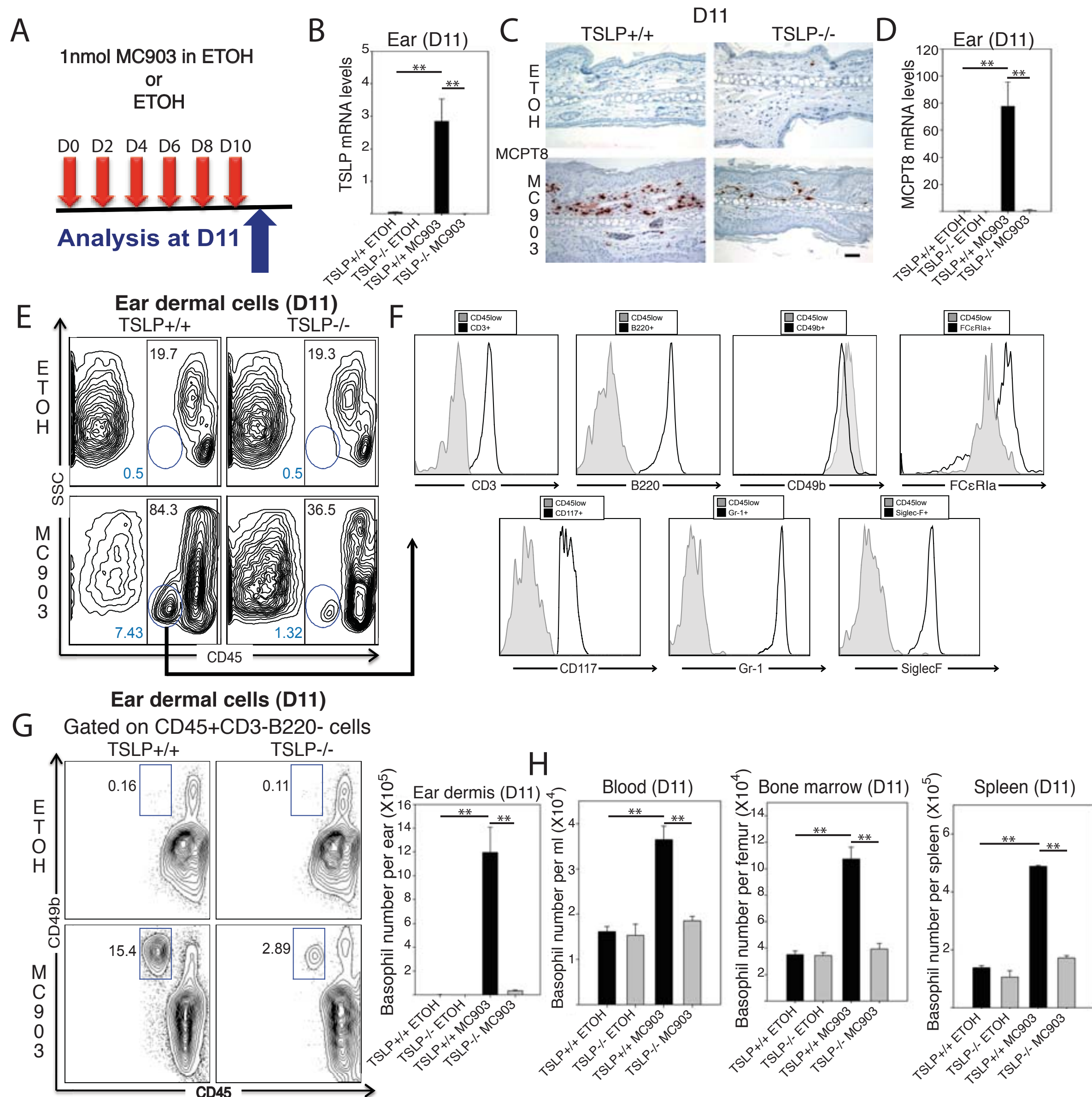

Figure 1. Topical MC903 treatment induces TSLP-dependent skin basophilia.

A) Experimental protocol. Ears of female mice were topically treated with MC903 or vehicule (ethanol; ETOH), every other day, form day (D) 0 to D10, and analyzed at D11. B) Quantitative RT-PCR analysis of TSLP mRNA levels of ears from MC903- or ETOH-treated TSLP+/+ and TSLP-/- mice at D11. C) Immunohistochemical (IHC) staining with antibody against MCPT-8 (for basophils) of ear section at D11. Scale bar, 50 µm. D) Quantitative RT-PCR analysis of MCPT-8 mRNA levels at D11. E) FACS analysis of CD45+ cells in the ear dermis of MC903- or ETOH-treated mice at D11. The black square gates show CD45+ cells and the numbers in black represent the percentage of CD45+ population in total dermal cells. The blue circle gates show CD45^{low} cells and the number in blue represent the percentage of CD45^{low} population in total dermal cells. F) The CD45^{low} cells (shaded)were further characterized as CD3-B220-CD49b+FcERIa+CD117-GR1-SiglecF-. G) Frequency (left panel) and total cell number (right panel) of basophils in dermis of MC903- or ETOH-treated ears at D11. H) Basophil numbers in blood, bone marrow and spleen of MC903- or ETOH-treated mice at D11. **$p<0.005$. Values are mean ± SEM (n≥ 3 mice per group).Data are representative of two or three independent experiments.

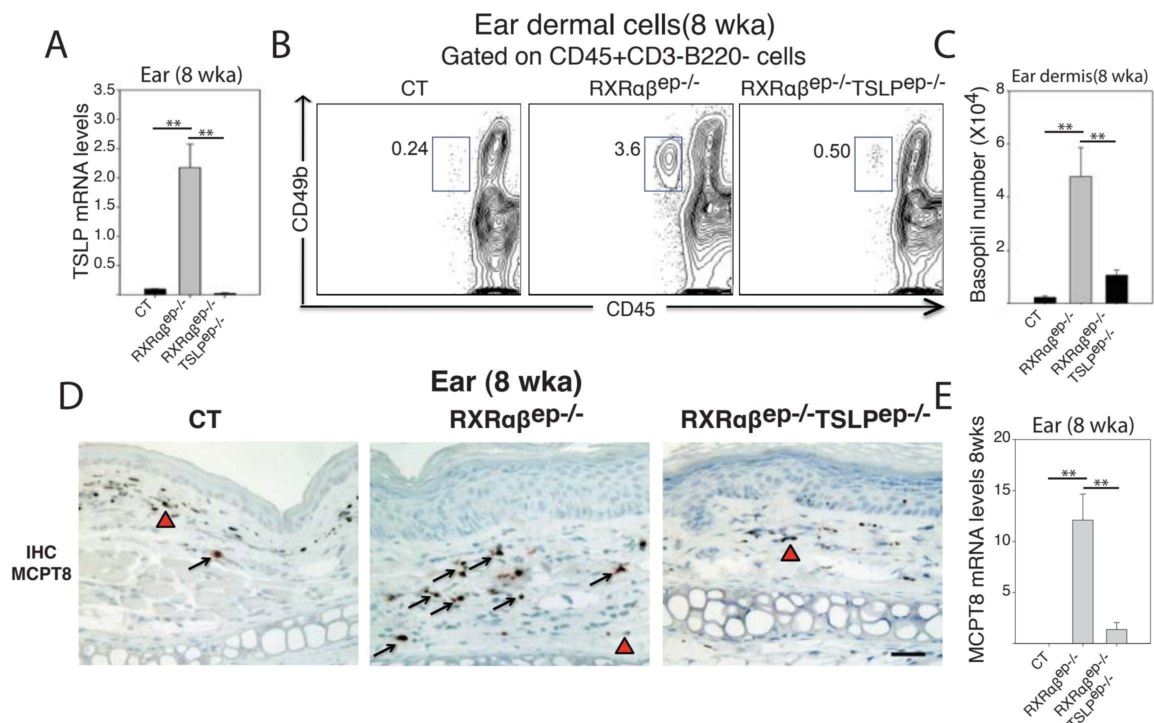

Figure 2. TSLP-dependent skin basophilia in mice with selective ablation of RXRα and RXRβ in adult keratinocytes.

A) Quantitative RT-PCR analysis of TSLP mRNA levels of ears from control [Tamoxifen (Tam)-injected K14-Cre-$^{ERT2(0/0)}$RXRα$^{L2/L2}$RXRβ$^{L2/L2}$TSLP$^{L2/L2}$; CT], RXRαβ$^{ep-/-}$ (Tam-injected K14-Cre-$^{ERT2(tg/0)}$RXRα$^{L2/L2}$RXRβ$^{L2/L2}$) or RXRαβ$^{ep-/-}$-TSLP$^{ep-/-}$(Tam-injected K14-Cre-$^{ERT2(tg/0)}$RXRα$^{L2/L2}$RXRβ$^{L2/L2}$TSLP$^{L2/L2}$) mice, 8 weeks after tamoxifen injection (8 wka). B) The frequency of basophils in ears from CT, RXRαβ$^{ep-/-}$ and RXRαβ$^{ep-/-}$-TSLP$^{ep-/-}$ mice at 8 wka. C) Basophil number per ear of CT, RXRαβ$^{ep-/-}$ and RXRαβ$^{ep-/-}$-TSLP$^{ep-/-}$ mice at 8 wka. D) IHC staining with antibody against MCPT-8 of ear sections at 8 wka. Black arrows point to MCPT8+ cells (in dark red). Note that the pigmented melanocytes (pointed by red triangles) are present in the dermis, and are not MCPT-8+ cells (mice are in mixed backgroud of 50% C56BJ/6 and 50% 129SV/J). Scale bar, 50 µm. E) Quantitative RT-PCR analysis of MCPT-8 mRNA levels of ears at 8 wka. **$p<0.005$. Values are mean ± SEM (n≥3 mice per group). Data representative of two independent experiments.

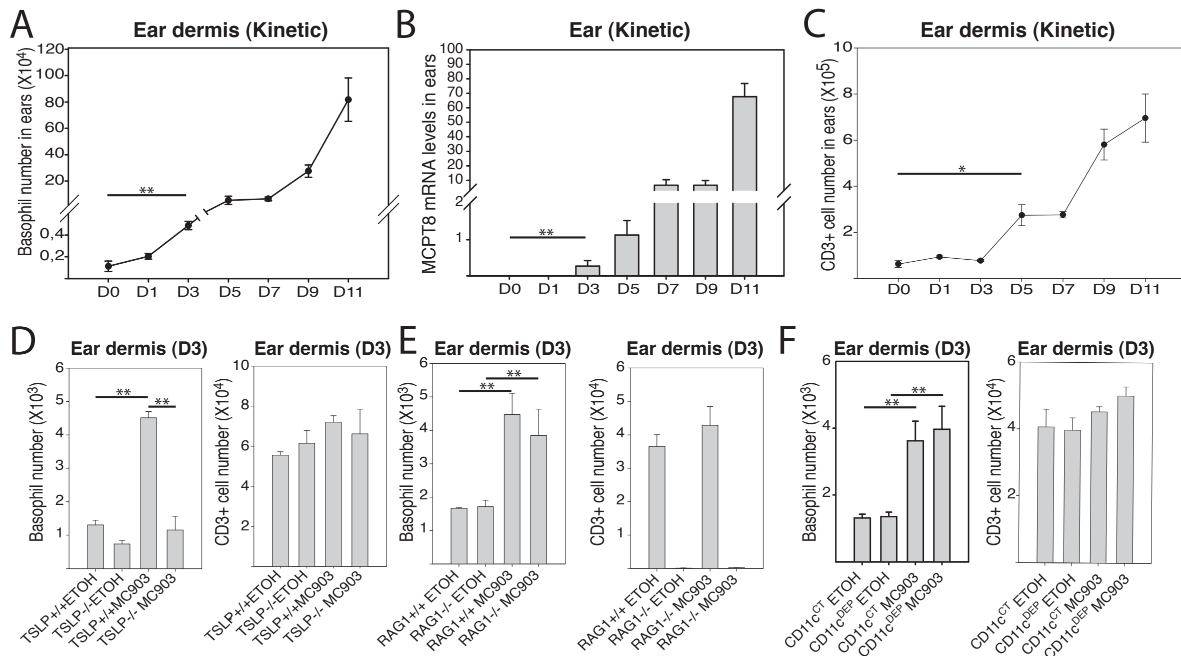

Figure 3.TSLP-induced basophil skin recruitment at the earlier phase is independent of adaptive immunity. A) Basophil number per ear of MC903-treated Balb/c mice at various time points. B) Quantitative RT-PCR analysis of MCPT-8 mRNA levels in ears of MC903-treated Balb/c mice. C) CD3+ cell number per ear of MC903-treated Balb/c mice. D) Total basophil number (left panel) and total CD3+ cell number (rigth panel) per ear of MC903- or ETOH-treated ears from TSLP+/+ and TSLP-/- mice at D3. E) Total basophil number (left panel) and total CD3+ cell number (rigth panel) per ear of MC903- or ETOH-treated ears from RAG+/+ and RAG-/- mice at D3. F) Total basophil number (left panel) and total CD3+ cell number (rigth panel) per ear of MC903- or ETOH-treated ears from CD11c^{Dep} (diphtheria toxin (DT)-injected CD11c-DTR$^{Tg/0}$) and CD11c^{CT} (DT-injected CD11c-DTR$^{0/0}$, as wildtype controls) mice at D3. *$p<0.05$, **$p<0.005$. Values are mean ± SEM (n≥3 mice per group). Data are representative of two or three independent experiments.

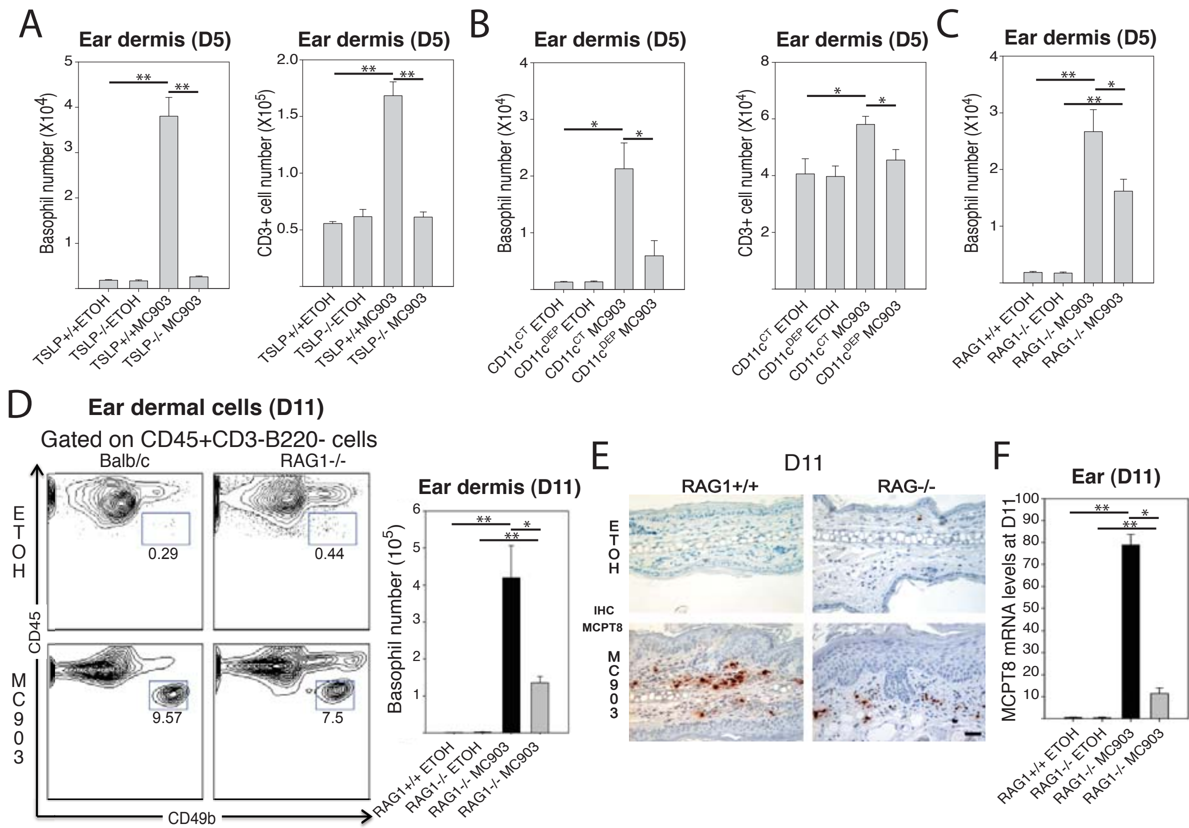

Figure 4.TSLP-induced basophil skin recruitment at the later phase requires adaptive immunity.

A) Total basophil number (left panel) and total CD3+ cell number (rigth panel) per ear of MC903- or ETOH-treated ears from TSLP+/+ and TSLP-/- mice at D5. B) Total basophil number (left panel) and total CD3+ cell number (rigth panel) per ear of MC903- or ETOH-treated ears from $CD11c^{Dep}$ and $CD11c^{CT}$ mice at D5. C) Total basophil number per ear of MC903- or ETOH-treated ears from RAG+/+ and RAG-/- mice at D5. D-F) Frequency and total number of basophils per ear (D) Immunohistochemical (IHC) staining with antibody against MCPT-8 (for basophils). Scale bar, 50 µm. (E) Quantitative RT-PCR analysis of MCPT-8 mRNA levels (F) of MC903- or ETOH-treated ears from RAG1+/+ and RAG1-/- mice at D11. *$p<0.05$, **$p<0.005$. Values are mean ± SEM (n≥3 mice per group). Data are representative of two independent experiments.

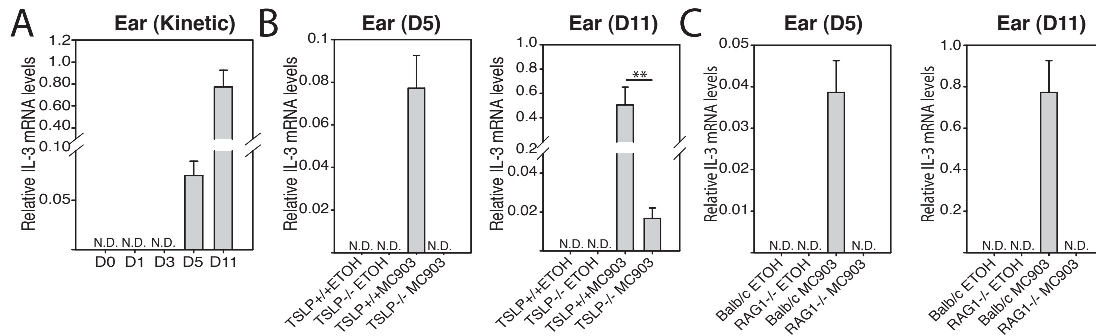

Figure 5. TSLP induces the expression of IL-3 in the skin.

A) Kinetic analysis of IL-3 mRNA levels by quantitative RT-PCR in ears from MC903-treated Balb/c mice at various time points. B and C) Quantitative RT-PCR analysis of IL-3 mRNA levels in ears from MC903- or ETOH-treated TSLP+/+ and TSLP-/- (B) and RAG1+/+ and RAG1-/- (C) mice at D5 and D11 (as indicated). N.D. not detected. **$p<0.005$. Values are mean ± SEM (n≥3 mice per group). Data are representative of two independent experiments.

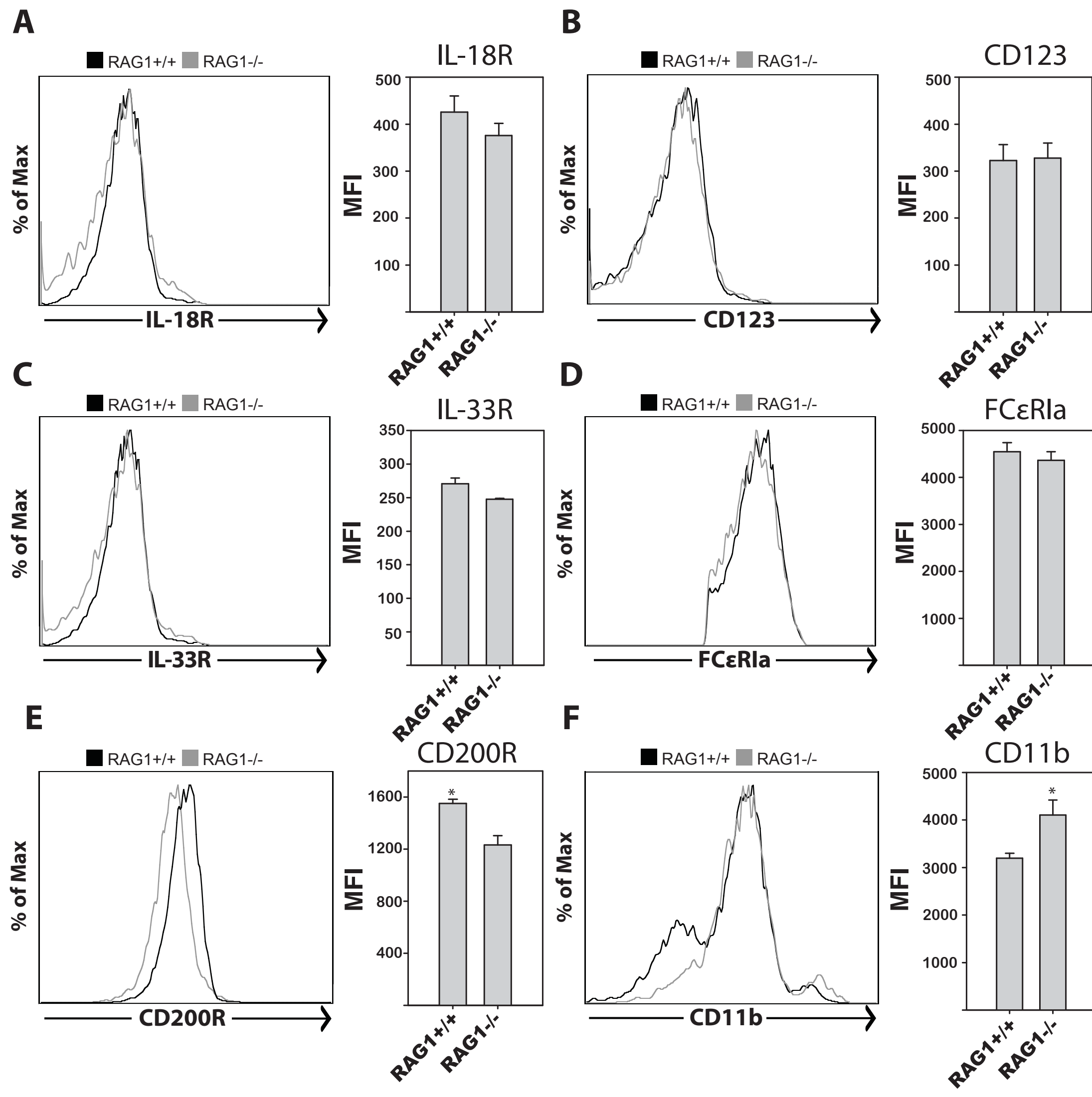

Figure 6. TSLP-induced skin basophils exhibit a heterogenous phenotype in presence or absence of adaptive immune cells.

Basophils (CD3-B220-CD45lowCD49+) in MC903-treated RAG+/+ and RAG1-/- ears at D11 were stained and compared for their expression of IL18R (A), CD123 (B), IL-33R (C), FcεRIα (D), CD200R (E) and CD11b (F). Representative FACS plot dots (left panels) and average of mean fluorescence intensity (MFI) levels (right panels) were shown. *$p<0.05$. Values are mean ± SEM (n≥3 mice per group). Data are representative of two independent experiments.

PART 2.B

Skin TSLP-induced Th2 priming requires an orchestrated cooperation of dendritic cells, $CD4^+$ T cells and basophils

Juan Manuel Leyva-Castillo et al. (manuscript in preparation).

In this study, we employed our previously established experimental protocol in which TSLP expression in skin keratinocytes is induced by topical treatment of MC903, to elucidate the cellular and molecular mechanism underlying how TSLP drives the Th2 priming. We provided novel evidences that TSLP-promoted IL-4 initiation in CD4+ T cells in skin-draining lymph nodes (LNs) requires an orchestrated cooperation of DCs, CD4+ T-lymphocytes and basophils.

INTRODUCTION.

Cell-mediated immunity is characterized by high degree of specific response to individual pathogen. T helper cells respond producing IFN γ in response to intracellular pathogens (Th1 response), whereas fungal infection induces the production of IL17 in these cells (Th17 response). In contrast parasite infection promote the production of IL-4 in T helper cells (Th2 response) (1). Although dendritic cells (DCs) provide the signals required driving Th1 and Th17 responses, the mechanisms leading to Th2 cell differentiation *in vivo* are still controversial. Recently, several reports suggested that basophils are the cells that induce a Th2 response *in vivo* to protease allergens or helminth infection, because they can act as antigen-presenting cells (APCs) and produce Th2-promoting cytokines (TSLP and IL-4) (2-5). In contrast, in a model of sensitization to inhaled allergen (House Dust Mites; HDM) in the lungs (6), in a model of helmnith infection (7), and in a model of protease allergen (8), dendritic cells, not basophils, were necessary and sufficient for inducing a Th2 response. In addition, a recent study supports that cooperation between DCs and basophils was required to promote a Th2 response *in vivo* induced by protease allergen (9). The differences in these studies suggest that there are multiple mechanisms to promote Th2 response.

Thymic stromal lymphopoietin (TSLP) is a master regulator in Th2-related diseases (AD and asthma) (10-12). Our previous studies (13-15) and that of others (16, 17) have demonstrated that elevated TSLP levels in the mouse skin trigger an AD-like dermatitis, characterized by increased levels of Th2 cytokines in the skin and skin-draining lymph nodes, accompanied by $CD4^+$ T cell and eosinophil infiltration in the skin, and elevated serum IgE levels. In addition, increased TSLP levels in mouse lung generated an asthma-like lung inflammation (11, 18, 19). However, the immune cascade triggered by TSLP-induced Th2 response is still unknown.

Here, using a TSLP-dependent AD mouse model induced by MC903 topical treatment (13), we demonstrate that TSLP-triggered Th2 priming requires the cooperative contribution of DCs, CD4+ T cells and basophils.

MATERIALS AND METHODS.

Mice

Wild-type Balb/c mice and Rag1-/- mice (in Balb/c background) were purchased from Charles River Laboratories. TSLP-/- (13), CD11c-DTR (20) and Langerin-DTREGFP (21) mice have been previously described. Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the Animal Care and Use Committee of the Institut de Génétique et de Biologie Moléculaire et Cellulaire.

Antibodies and flow cytometry

The following antibodies were used: purified anti-FcγRIII/II (CD32/16; Fc Block; clone 2.4G2), FITC-conjugated anti-Gr-1 (clone RB6-8C5), PE-1 conjugated anti-siglec-F (clone E50-2440), V500-conjugated anti-CD3 (clone 500-A2), PerCP-Cy5.5-conjugated anti-CD19 (clone1D3), biotin-conjugated anti-CD4 (clone GK1.5), PerCP-Cy5.5-conjugated anti-CD8a (clone 53-6.7), APC-conjugated anti-IL-4 (clone 11B11) and biotin-conjugated anti-CD11c (clone HL3) were from BD Biosciences; biotin-conjugated anti-CD49b (clone DX5), Alexa700-conjugated anti-CD8a (clone 53-6.7) and FITC-conjugated MHCII (clone M5/114.15.2) were from eBioscience; APC-Cy7-conjugated anti-CD117 (c-kit; clone 2B8), PECy7-conjugated anti-FCεRIα (MAR-1) and PerCP-Cy5.5- or PECy7-conjugated anti-CD45R (B220; clone RA3-6B2) were from Biolegend; Qdot-585-conjugated streptavidin was from Invitrogen; and PE-conjugated streptavidin was from Jackson ImmunoResearch.

For surface marker staining, ear-draining lymph node (EDLN) cells were incubated with 2.4G2 antibody on ice for 10 minutes to block Fc receptors, washed and incubated with a mix of antibodies on ice for 10 minutes. Finally cells were washed and analyzed.

For intracellular IL-4 staining, EDLN cells were stimulated for 5 hours at $5X10^6$ cells/ml in

the presence of PMA (50 ng/ml), ionomycin (500 ng/ml) and BD golgi stop (BD Biosciences). After washing, cells were incubated with 2.4G2 antibody, followed by surface staining for CD3, CD8 and CD4. Cells were then fixed and permeabilized using BD Cytofix/Cytoperm™ Kit (BD Biosciences) and stained with anti-IL-4 antibody.

Cells were analyzed on a LSRII flow cytometer (BD Biosciences). Results were analyzed using FlowJo (Treestar).

MC903 Topical Application

MC903 (calcipotriol; Leo Pharma, Denmark) was dissolved in EtOH and topically applied on mouse ears (1 nmol in 25 ul per ear). As vehicle control, the same volume of EtOH was applied on mouse ears .

RNA extraction and Quantitative RT-PCR

RNA was extracted using TRI Reagent (Sigma-Aldrich) or RNeasy micro kit (QIAGEN). RNA was reverse-transcribed using random oligonucleotide hexamers and amplified by quantitative PCR with a Ligthcycler 480 (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Relative RNA levels were calculated using hypoxanthine phosphoribosyltransferase (HPRT) as internal control. Sequences of PCR primers: HPRT, TSLP, IL4, IL-3, IFNγ, IL17a, OX40L, CD40, CD86, CD80, IL12p35 and IL12p40 were as previously reported (22).

Isolation of dendritic cells and $CD4^+$ cells from mouse lymph nodes (LN)

For DCs isolation, EDLN were chopped and digested in 2 mg/ml collagenase D (Roche), 2.5% FCS and 100 U/ml DNAse I (Roche) in PBS at 37°C for 30 min, and then filtered through 70μm cell strainer. Single-cell suspensions were incubated with Fc-block (Fcγ RII/III m Ab 2.4G2) for 10 min, and DCs were enriched using CD11c Microbeads (Miltenyi Biotec). CD11c+MHCII+ cells were then sorted with FACS Aria II (BD) (purity was > 95%).

For CD4+ cells, EDLN were chopped and mechanically disrupted, and then filtered through 70 μm cell strainer. $CD4^+$ cells were isolated using $CD4^+$ T cell isolation kit II (Miltenyi Biotec) (purity was > 90%).

Depletion of dendritic cells or Langerhans cells in vivo

For depletion of CD11c+ DCs, CD11c-$DTR^{Tg/0}$ mice were intraperitoneal (i.p.) injected with diphtheria toxin (DT) (100ng per mouse) one day before MC903 treatment. DT-injected CD11c-$DTR^{0/0}$ mice were used as control. Ablation efficiency was eveluated by analysis of DCs in lymph nodes, skin and splenocytes. For depletion of Langerhans cells, Langerin-$DTREGFP^{+/-}$ knockin mice received an i.p. injection of DT (1 μg per mouse) one day before MC903 treatment. DT-injected wildtype littermatte mice were used as control.

Basophil depletion in vivo

For depletion of basophils, wildtype Balb/c mice were injected twice daily for 3 days with 5 μg of anti-FcεRIα (clone MAR-1) or an isotype control antibody (Armenian Hamster IgG; clone eBio299Arm) (eBioscience) (23) 5 days before of MC903 treatment. The efficiency of basophil depletion was evaluated in spleen, ear-draining lymph node and ears.

Adoptive transfer of $CD4^+$ T cells.

$CD4^+$ cells were purified from spleens of Balb/c mice using the mouse $CD4^+$ T cell Isolation Kit II (Miltenyi) and were intravenously (*i.v).* injected ($5x10^6$ cells) to RAG1-/- recipient mice 24 h prior to MC903 treatment.

Naive T cell culture.

Naive $CD4^+$ cells from spleen were isolated using the $CD4^+CD62L^+$ T cell isolation kit II (Miltenyi Biotec), following the manufacturer's instructions. Purified naive T cells ($1X10^6$/ml) were cultured in anti-CD3 (2μg/ml) coated wells, with a combination of anti-OX40 (5μg/ml) and anti-CD28 (2μg/ml) antibodies in RPMI 1640 supplemented with 10%

of FCS and 2mM of L-glutamine. Cells and supernatants were harvested 72 h after.

Statistic analysis

Data were analyzed using SigmaStat (Systat Software Inc. Point Richmond, CA, USA) by the Student t test or the Mann-Whitney rank sum nonparametric test depending on results from the Kolmogorov-Smirnov test (with Lilliefors' correction) for normality and Levene Median test for equal variance.

RESULTS

Topical MC903 treatment induces TSLP-dependent IL-4 production in skin-draining lymph node CD4+ T cells

To characterize TSLP-triggered Th2 priming in vivo, wildtype (WT) Balb/c mice were topically treated with MC903 (1nmol per ear) or with vehicle (ETOH) on ears every other day from day (D) 0 to D4 (Figure 1A). As previously reported (14), TSLP expression was strongly induced in MC903-treated ears at D5 (Figure 1B, left panel). In contrast, TSLP expression in ear-draining lymph nodes (EDLN) was much lower compared to that in ears, and did not show any change upon MC903 treatment (Figure 1B, right panel). We then analyzed the expression of IL-4, IFNγ and IL-17 in EDLN at D1, D3 and D5 upon MC903 treatment. Results showed that IL-4 was increased in EDLN as early as D5 (Figure 1C), whereas the expression of IFNγ and IL-17 did not change (data not shown). To examine whether this induction of IL-4 by MC903 is dependent on TSLP, and is not on the pleiotropic actions of vitamin D3 on immune system (24), TSLP-/- mice (13) were subjected to MC903 treatment. Induction of IL-4 in EDLN at D5 was abolished in MC903-treated TSLP-/- mice (Figure 1D), thus demonstrating that TSLP overexpression leads to IL-4 initiation in skin-draining lymph nodes.

We then performed IL-4 intracellular staining of EDLN cells at D5. Results showed that the $CD4^+$ T cells were the main IL-4 producer (Figure 1E), and that the number of IL-4-producing $CD4^+$ T cells was increased in EDLNs from MC903-treated WT mice (Figure 1F). Moreover, quantitative RT-PCR analyses of purified $CD4^+$ cells from EDLN confirmed that IL-4 mRNA levels were increased in mice upon MC903 treatment (Figure 1G). In addition,

IL-4 induction in EDLN was completely abrogated in MC903-treated RAG1-/- mice (Figure 1H). Altogether, these data demonstrate that skin TSLP promotes IL-4 induction in the draining lymph node $CD4^+$ T cells.

Dendritic cells, but not Langerhans cells, are required for TSLP-promoted IL-4 production in skin-draining lymph node $CD4^+$ T cells

Dendritic cells (DCs) are the bridge between innate and adaptive immune responses through cytokine production and T cell activation (25). To investigate the role of DCs in TSLP-triggered Th2 priming, we first examined the accumulation of DCs ($CD11c^+MHCII^+$) in EDLN. We found that the number of DCs appeared to increase at D3 (Figure 2A), a time earlier than the detection of IL-4 induction (at D5; see Figure 1C), suggesting a possible role of DCs in the initiation of the Th2 polarization. Next, CD11c-$DTR^{Tg/0}$ mice (20) were injected with diphtheria toxin (DT), to generate DC-depleted mice (called thereafter $CD11c^{DEP}$). Although DC depletion was not complete, DT injection led to a significant reduction of the percentage and number of DCs in both ears and EDLN of $CD11c^{DEP}$ mice (Figure 2B). Depletion of DCs did not affect the TSLP induction by topical MC903 treatment (Figure 2C). However, the expression of IL-4 in EDLN was drastically abolished in MC903-treated $CD11c^{DEP}$ mice (Figure 2D), accompanied by a significant decrease of $CD4^+$ T cell number (Figure 2E). Furthermore, we showed that Langerhans cells (LCs) were not implicated in TSLP-induced Th2 polarization, as depletion of LCs in $lang^{DEP}$ mice [DT injected Langerin-$DTREGFP^{+/-}$ (21)] did not lead to any change of TSLP-induced IL-4 expression, or of CD4+ T cell number in EDLN (Figure 2F and G). Thus, these results indicate that dendritic cells, but not Langerhans cells, play an essential role in TSLP-induced Th2 priming.

Basophils are required for TSLP-promoted IL-4 production in skin-draining lymph node $CD4^+$ T cells

Recent studies have unveiled controversial data on the contribution of basophils to Th2 immunity (2, 3, 5-7, 9, 26-29). To investigate the role of basophils in TSLP-induced Th2 polarization, we first examined whether basophils were recruited into EDLN upon MC903 skin treatment. Flow cytometry analyses showed a basophil recruitment [$CD3^-B220^-CD49b^+CD45^{low}Fc\varepsilon RI\alpha^+CD117^-GR1^-$ (30)] (Fig. 3A), starting at D3 and peaking at D5 (Figure 3B), which was abolished in MC903-treated TSLP -/- mice (data not shown). We then depleted basophils in WT Balb/c mice by i.p. injection of MAR-1 antibody (23), followed by MC903 treatment. MAR-1 antibody injection led to the reduction of basophil frequency and number in EDLN of MC903-treated WT Balb/c mice at D5 (Figure 3C), without affecting the number of DCs (Figure 3D) or $CD4^+$ T cells (Figure 3E). Upon MC903 treatment, IL-4 induction in EDLN was abolished in basophil-depleted mice (Fig. 3F). Moreover, the percentage and number of $IL4^+CD4^+$ T cells in EDLN were reduced (Figure 3 G), and IL-4 mRNA levels in purified $CD4^+$ cells from EDLN were largely decreased (Figure 3H). Thus, these results indicate that basophils play an essential role in TSLP-promoted IL-4 expression by $CD4^+$ T cells in skin-draining lymph nodes.

Basophil recruitment in skin-draining lymph nodes requires dendritic cells and CD4+ T cells

To investigate the implication of DCs in the recruitment of basophils in EDLN, we examined MC903-treated $CD11c^{DEP}$ mice. The frequency and absolute number of basophils in EDLN from these mice were significantly lower than in MC903-treated $CD11c^{CT}$ mice (Figure 4A), demonstrating that DCs are required for TSLP-induced basophil recruitment in EDLNs.

We further examined whether $CD4^{+}$ T cells may also play a role in basophil recruitment in EDLN. Indeed, in MC903-treated RAG1-/- mice, basophil recruitment in EDLN was abolished (Figure 4B). Furthermore, adoptive transfer of wildtype CD4+ T cells into RAG1-/- mice led to the recovery of basophil recruitment in EDLN (Figure 4C). All these above data indicate that both dendritic cells and $CD4^{+}$ T cells are essential for TSLP-promoted basophil recruitment in the skin-draining lymph nodes.

TSLP promotes IL-3 expression in $CD4^{+}$ T cells, which requires dendritic cells but not basophils

As IL-3 produced by activated $CD4^{+}$ T cells has been reported to play a key role for basophil recruitment into the draining lymph nodes in a helminth infection mouse model (26), we sought to determine whether IL-3 could be implicated in TSLP-triggered basophil recruitment in EDLN. We first analyzed the kinetics of IL-3 expression in EDLNs. Upon MC903 treatment, IL-3 mRNAs were not detected at D0 and D1, but were increased at D3 and peaked at D5 (Figure 5A), which was well correlated with basophil recruitment in EDLNs (see Fig. 3B). IL-3 induction was completely abolished in MC903-treated TSLP-/-, demonstrating its dependence on TSLP (Figure 5B). We further showed that IL-3 was produced by $CD4^{+}$ T cells in EDLN (Figure 5C). In agreement with that, IL-3 expression was not detected in MC903-treated RAG1-/- mice (Figure 5D). These data thus demonstrate that TSLP triggers IL-3 expression in $CD4^{+}$ T cells, which suggest a potential role of IL-3 in recruiting basophils in EDLNs.

We further determined whether DCs are required for IL-3 induction, by comparing IL-3 expression in EDLNs of MC903-treated $CD11c^{DEP}$ and $CD11c^{CT}$ mice. Results showed that depletion of DCs completely abolished the IL-3 induction (Figure 5E), suggesting that DCs

are involved in promoting IL-3 expression by $CD4^+$ T cells. In contrast, basophils appeared to be dispensable for IL-3 induction in EDLN at D5, as depletion of basophils did not result in any change of IL-3 levels in $CD4^+$ T cells (Figure 5F). Thus, these results indicate that in the immune cascade driven by TSLP, dendritic cells but not basophils are upstream of IL-3-expression by $CD4^+$ T cells.

TSLP-induced OX40L expression in dendritic cells may promote IL-3 expression in naïve CD4+ T cells

To investigate how DCs could be involved in TSLP-triggered Th2 priming, we analyzed the expression pattern of costimulatory molecules in purified CD11c+ DCs from EDLN of MC903-treated WT Balb/c mice at D3, a time before IL-4 induction in CD4+ T cells could be detected (i.e. at D5, Fig. 1C). We found that OX40L expression was significantly induced in DCs upon MC903 skin treatment (Fig. 6A). Expression of CD80 and CD86 also exhibited an increase, whereas no change was observed for the expression of CD40, IL12p35 and IL12p40 in purified DCs (Figure 6A).

To test if DCs may activate IL-3 expression in CD4+ T cells through OX40L-OX40 signaling, we employed an in vitro naïve ($CD3^+CD4^+CD62L^+$) T cell culture system. Our preliminary results show that in the presence of an agonistic anti-OX40 antibody, IL-3 expression levels are increased in naïve T cells co-stimulated with anti-CD3, or with both anti-CD3 and anti-CD28 (Figure 6B). Therefore, activation of OX40 signaling induces IL-3 production in naive CD4+ T cells.

DISCUSSION

In this study, we employed our previously established experimental protocol in which TSLP expression in skin keratinocytes is induced by ear topical MC903 treatment, to elucidate the cellular and molecular mechanism underlying TSLP-driven Th2 priming. We provided novel evidences that TSLP-promoted IL-4 initiation in CD4+ T cells in skin-draining lymph nodes (LNs) requires an orchestrated cooperation of DCs, CD4+ T-lymphocytes and basophils.

Our results indicate that DCs are crucial players in TSLP-driven Th2 priming. In contrast to a previous report that TSLP can act *in vitro* directly on CD4+ T cells to induce IL-4 expression (31), we demonstrate here that DCs are required for IL-4 induction in CD4+ T cells *in vivo*. Indeed, IL-4 expression is detected at D5 (but not at D3) in skin draining LNs upon MC903 skin treatment that corresponds well to the time needed for Th2 differentiation. Moreover, our results indicate that DC depletion, IL-3 induction in CD4+ T cells at D3 (which is earlier than IL-4 induction), as well as basophil recruitment in skin-draining LNs, were also abolished. These evidences thus place DCs in a first place in the immune cascade of TSLP driven Th2 priming. Whether and how TSLP promotes DC migration from skin to the draining LNs in absence of exogenous stimuli (e.g. allergen or microbe) remains to be determined. However, a recent report using an *in vitro* system, has suggested that TSLP directly triggers DC migration (32).

Even though DCs are crucial, they are not the only cells required for TSLP-promoted Th2 priming, as depletion of basophils also abolishes IL-4 induction in $CD4^+$ T cells at D5. However, in contrast to DC-depletion, IL-3 expression by $CD4^+$ T cells was not affected by basophil depletion. Of particular interest, we also reveal that basophil recruitment in skin-draining LNs (detected from D3) requires not only DCs, but also $CD4^+$ T cells. Therefore, these data suggest that in the immune cascade of TSLP-driven Th2 priming, basophils are

downstream of DCs and $CD4^+$ T cells expressing IL-3, but are upstream of and required for Th2 priming (see Fig. 7, our proposed model).

The signal(s) driving basophil recruitment in skin-draining LNs remain(s) elusive, although recruitment of basophils to LNs has been reported in several studies (2, 3, 6, 7, 29, 33). A recent study suggests that cooperation between DCs and basophils is required to promote a Th2 response, and that the DC-derived chemokine CCL7 mediates the recruitment of basophils into the draining LNs (9). However in our case, we did not observe any change of CCL7 expression in purified DCs from EDLN at D3 upon MC903 treatment (data not shown). Rather, our data suggest that signal(s) from DC-activated T cells might mediate basophil recruitment. A potential candidate is IL-3. Indeed, we observed IL-3 expression by $CD4^+$ T cells in EDLNs correlates well with basophil recruitment. It has been reported that IL-3 produced by activated $CD4^+$ T cells plays a key role in the recruitment into the draining lymph node following helminth infection with *N. brasiliensis* (26). However, further analyses are required to define the role of IL-3 in the TSLP-induced basophil recruitment into the draining LNs. Interestingly, our preliminary results suggest that DC-derived OX40L induces IL-3 expression in naïve CD4+ T cells through the activation of OX40 signaling. This is supported by a previous study showing that OX40L-expressing epithelial cells induce IL-3 production in T cells, that was inhibited by blocking OX40 signaling by anti-OX40L blocking antibody (34).

Recent studies, using different strategies to deplete basophils and different models, provided controversial data on the contribution of basophils in Th2 responses. Although several studies reported basophil migration into the draining lymph nodes following exposure to protease allergen or helminth infection was crucial for inducing an appropriate Th2 immune response (2-5). This initial hypothesis was doubted by another study reporting that injection of MAR-1

antibody leads to the depletion of a population of inflammatory DCs (FcεRIα^{+} DCs), which are necessary and sufficient for the induction of Th2 immunity against inhaled HDM (6). However, it should be noted that Hammad et al. used a high dose of MAR-1 antibody (100 μg), in contrast to what others (2, 7, 23), and we used (twice daily injection of 5μg for 3 days). Indeed, we did not observe any effect of MAR-1 antibody on the DC number in EDLN at D3 and D5 upon skin MC903 treatment (Figure 3D and data not shown). Moreover, DCs from EDLN of MAR-1 injected mice exhibited similar increases of expression of costimulatory molecules OX40L, CD80 and CD86 at D3 (data not shown). Therefore, it is unlikely that abolished Th2 priming is due to the effect of MAR-1 injection on DCs. Nevertheless, the crucial role of basophils in TSLP-driven Th2 priming is currently further examined by using DT-mediated basophil depletion (35).

How basophils are involved in the initiation of IL-4 expression in $CD4^{+}$ T cells in our model remains elusive. Ablation of the basophils by injection of the MAR-1 antibody abolished IL-4 expression in $CD4^{+}$ T cells at D5. However, unlike DC-depletion, depletion of basophils did not decrease the number of CD4+ T cells at D5, suggesting that basophils do not appear to have a role in T cell proliferation. It could be possible that basophil provide an initial source of IL-4, as previous reported (30, 36), and/or a direct interaction between basophils and T cells is required for promote Th2 priming (2, 4).

Based on those data, we raise a hypothesis for how skin-derived cytokine TSLP drives Th2 priming (Figure 7).

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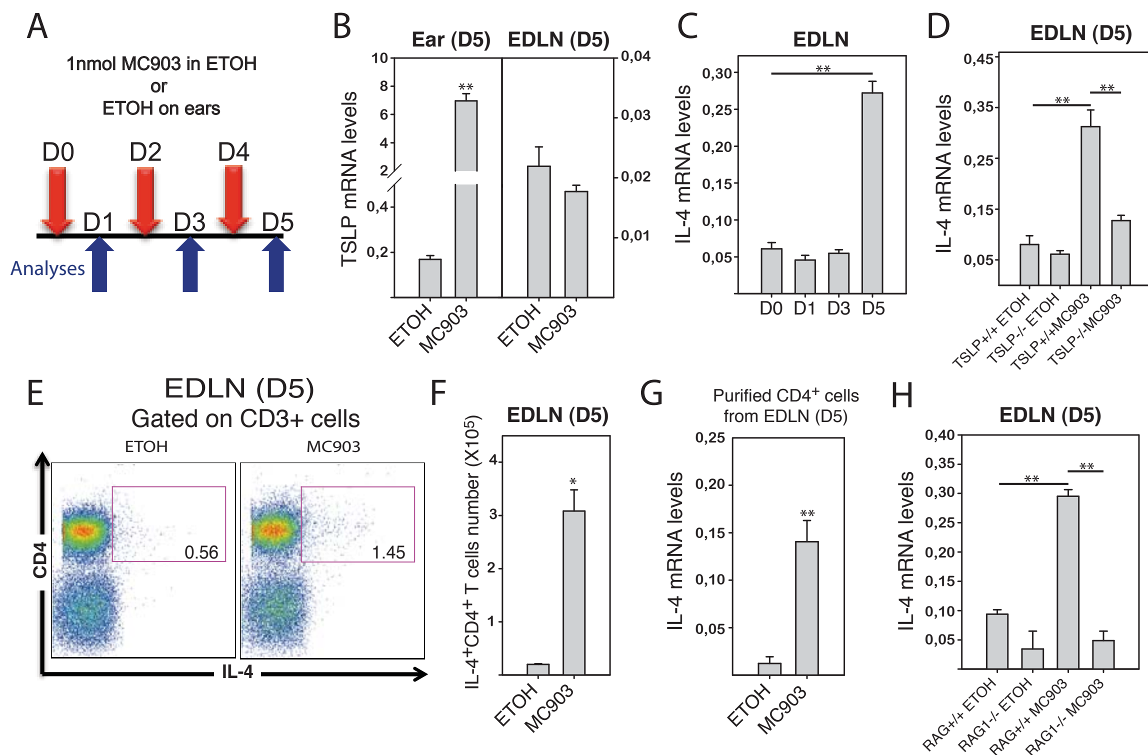

Figure 1. Skin topical MC903 treatment induces TSLP-dependent IL-4 expression in the draining lymph node CD4+T cells.

A) Experimental protocol. Mouse ears were topically treated with MC903 [1nmol in 25 µl of ethanol (ETOH)], every other day from day (D) 0 to D4, and analyzed at D1, D3 or D5. B) Quantitative RT-PCR analysis of TSLP mRNA levels of ears and ear-draining lymph nodes (EDLN) from MC903- or vehicle (ETOH)-treated wiidtype (WT) Balb/c mice at D5. C) Quantitative RT-PCR analysis of IL-4 mRNA levels in EDLN from MC903-treated WT Balb/c mice at different time points after MC903 treatment. D) Quantitative RT-PCR analysis of IL-4 mRNA expression in EDLN of MC903- or ETOH-treated TSLP+/+ and TSLP-/- mice at D5. E) Representative FACS plots of IL-4 intracellular staining of EDLN cells from ETOH- or MC903-treated WT Balb/c mice at D5. F) Number of total IL-4-producing CD4+ T cells in EDLN from ETOH- or MC903-treated WT Balb/c mice at D5. G) Quantitative RT-PCR analysis of IL-4 mRNA levels of purified CD4+ cells from EDLN of ETOH- or MC903-treated WT Balb/c mice at D5. H) Quantitative RT-PCR analysis of IL-4 mRNA expression in EDLN from MC903- and ETOH-treated RAG1+/+ and RAG1-/- mice at D5. *$p<0.05$, **$p<0.005$. Values are mean ± SEM (n≥3 mice per group). Data are representative of two or three independent experiments.

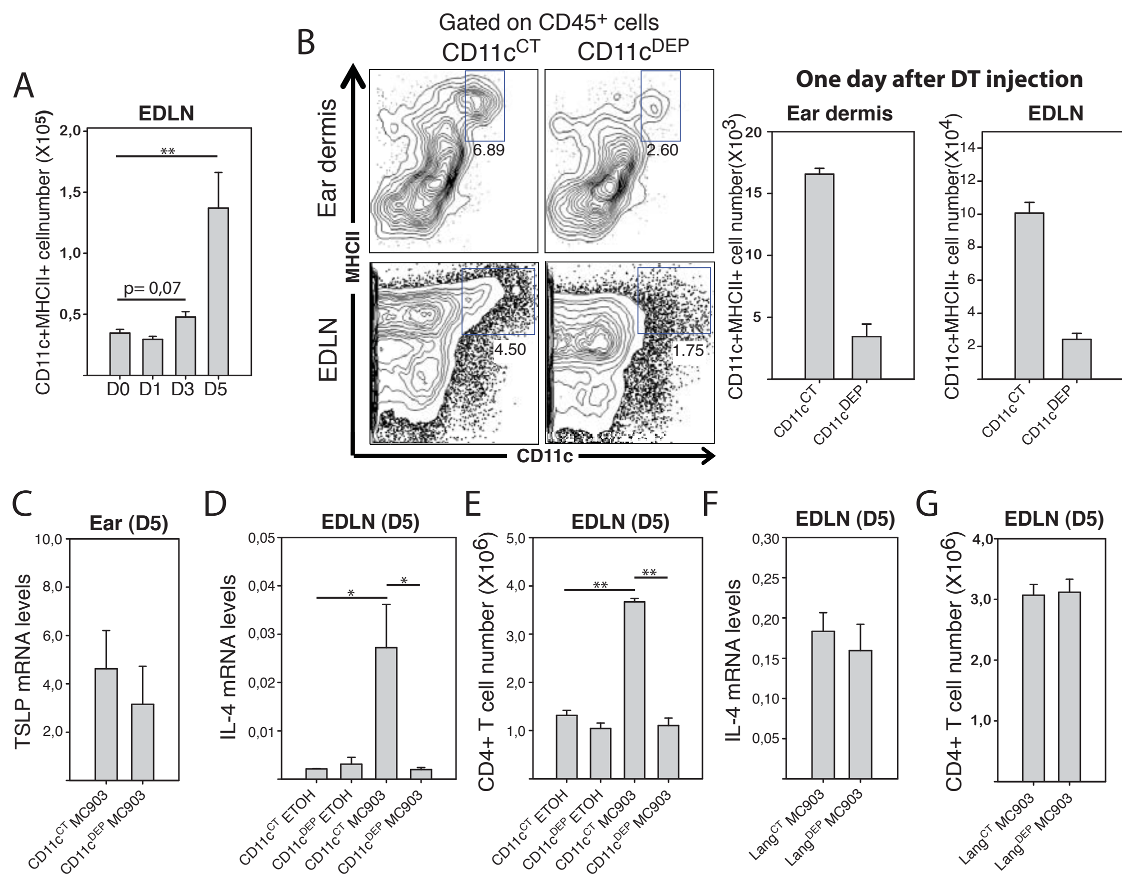

Figure 2. CD11c+ dendritic cells, but not langerin+ Langerhans cells, are required for skin TSLP-promoted IL-4 expression in $CD4^+$ cells.

A) Dendritic cell [DCs (CD11c+MHCII+)] number in ear-draining lymph nodes (EDLN) of MC903-treated wildtype (WT) Balb/c mice. B) Frequency and total cell number of DCs (CD11c+MHCII+) in ear dermis and EDLN of $CD11c^{Dep}$ (diphtheria toxin (DT)-injected CD11c-$DTR^{Tg/0}$ mice; in C5BL/6 background) and $CD11c^{CT}$ control mice (DT-injected CD11c-$DTR^{0/0}$ littermates) at one day after DT treatment. C) Quantitative RT-PCR analysis of TSLP mRNA levels in MC903-treated ears from $CD11c^{Dep}$ and $CD11c^{CT}$ mice at D5. D) Quantitative RT-PCR analysis of IL-4 mRNA levels in EDLN of MC903- or ETOH-treated $CD11c^{Dep}$ and $CD11c^{CT}$ mice at D5. E) Total CD4+ T cell number in EDLN of MC903- or ETOH- treated $CD11c^{Dep}$ and $CD11c^{CT}$ mice at D5. F) Quantitative RT-PCR analysis of IL-4 mRNA levels in EDLN from $Lang^{Dep}$ (DT-injected Langerin-$DTREGFP^{+/-}$ mice; in Balb/c background) and $Lang^{CT}$ control mice (DT-injected Langerin-$DTREGF^{-/-}$ littermates), upon MC903 treatment at D5. H) Total CD4+ T cell number in EDLN of MC903-treated $lang^{Dep}$ and $Lang^{CT}$ mice at D5. *$p<0.05$, **$p<0.005$. Values are mean ± SEM (n≥3 mice per group). Data are representative of two independent experiments.

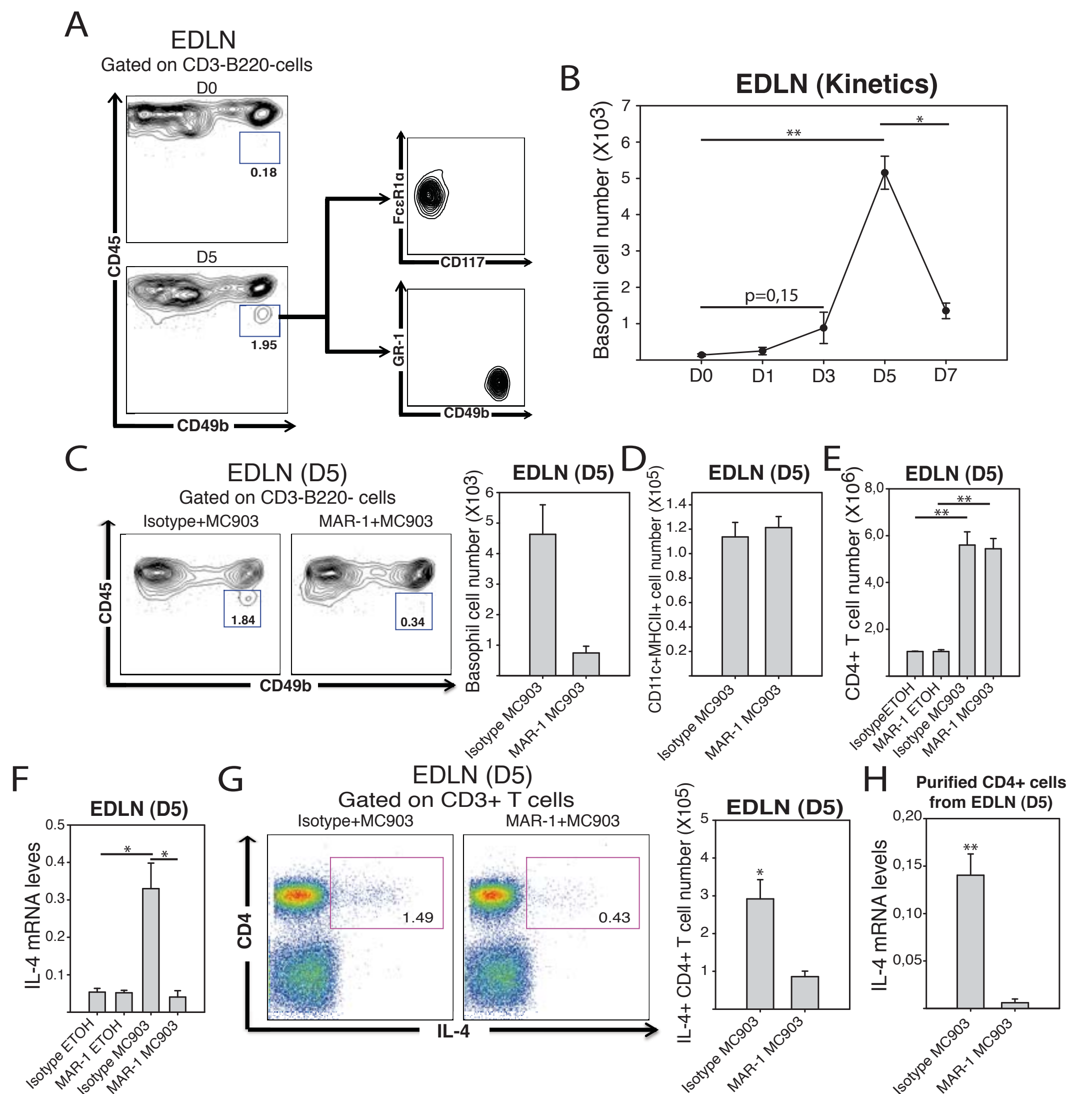

Figure 3. Basophils are required for skin TSLP-promoted IL-4 expression in CD4+ T cells.

A) Representative FACS plots of basophils (characterirized as $CD45^{low}CD49^{+}Fc\varepsilon RI\alpha^{+}CD117^{-}GR1^{-}$) in ear-draining lymph nodes (EDLN) of MC903- or ETOH-treated wildtype (WT) Balb/c mice at D0 and D5. B) Total basophil number in EDLN of MC903-treated wildtype Balb/c mice at various time points. C) Frequency and total number of basophils in EDLN of Isotype- or MAR-1-injected WT Balb/c mice upon MC903 treatment at D5. D) Dendritic cell (CD11c+MHCII+) number in EDLN from Isotype- or MAR-1-injected WT Balb/c mice upon MC903 treatment at D5. E) Total CD4+ T cell number in EDLN of Isotype- or MAR-1-injected WT Balb/c mice upon MC903 or ETOH treatment at D5 F) Quantitative RT-PCR analysis of IL-4 mRNA expression in EDLN of Isotype- or MAR-1-injected WT Balb/c mice upon MC903 or ETOH treatment at D5. G) Frequency and total number of $IL\text{-}4^{+}CD4^{+}$ T cells in EDLN of isotype- or MAR-1-injected WT Balb/c mice upon MC903 treatment at D5. H) Quantitative RT-PCR analysis of IL-4 mRNA levels in purified CD4+ cells from ear-draining LNs of isotype- or MAR-1-injected WT Balb/c mice upon MC903 treatment at D5. *$p<0.05$, **$p<0.005$. Values are mean ± SEM (n≥3 mice per group). Data are representative of two independent experiments.

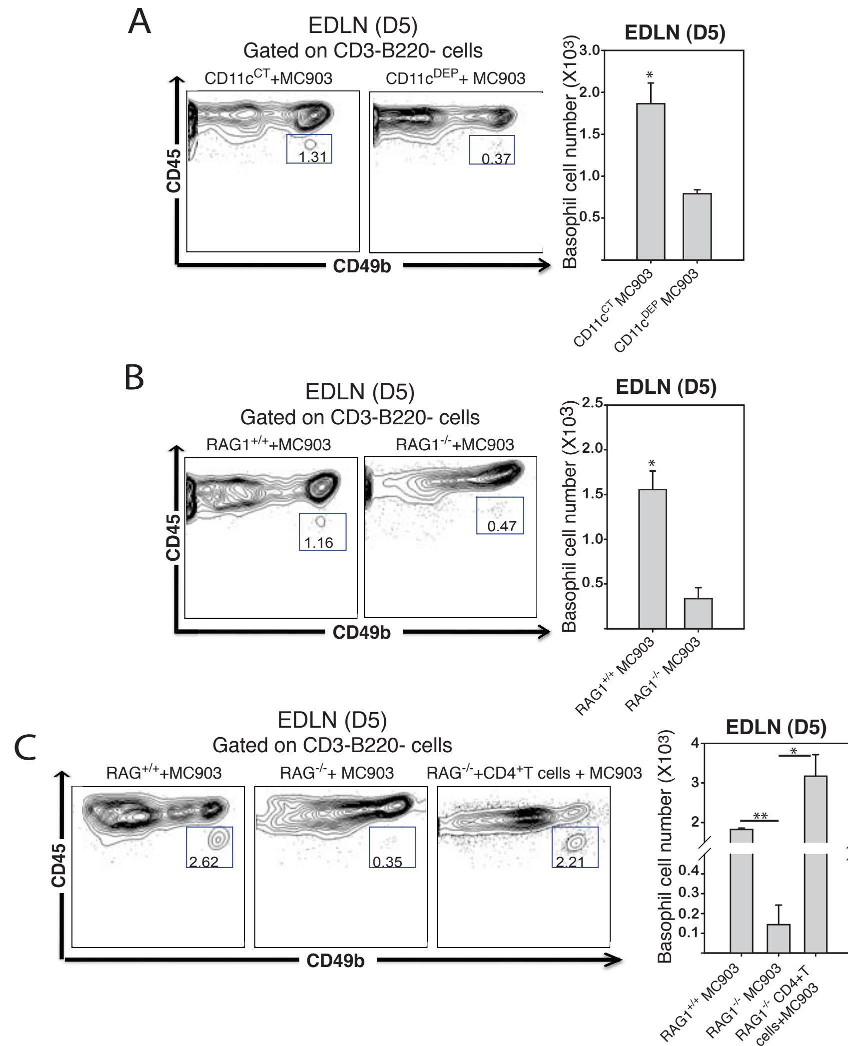

Figure 4. TSLP-induced basophil recruitment in skin draining lymph nodes requires dendritic cells and CD4+ T cells.

A) Frequency and total number of basophils in ear-draining lymph nodes (EDLN) of MC903-treated CD11c^{Dep} and CD11c^{CT} mice at D5. B) Frequency and total number of basophils in EDLN of MC903-treated RAG+/+ and RAG1-/- mice at D5. C) Frequency and total number of basophils in EDLN of MC903-treated RAG1+/+, RAG1-/- and CD4+ T cell transfered RAG1-/- mice at D5. *$p<0.05$, **$p<0.005$. Values are mean ± SEM (n≥3 mice per group).

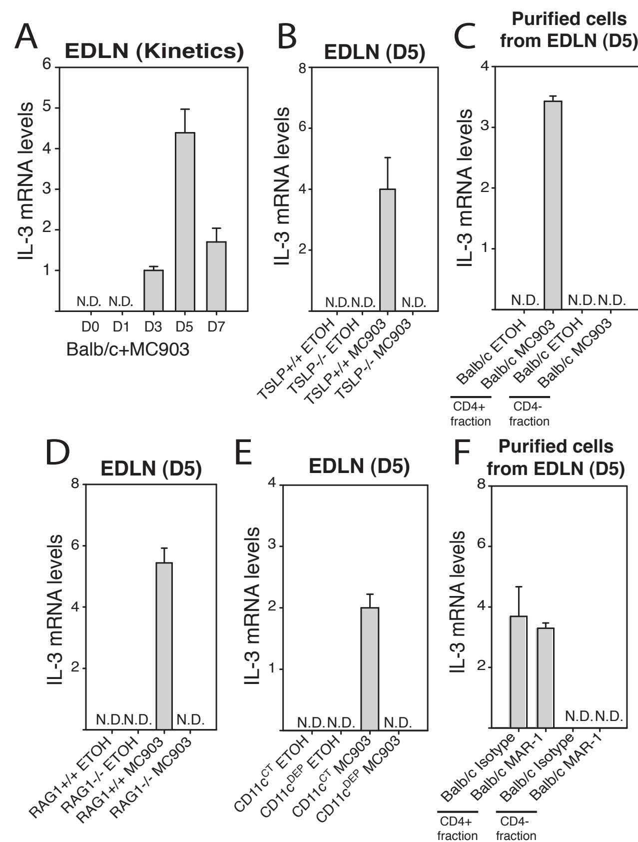

Figure 5. TSLP induces IL-3 expression in CD4+ T cells, which requires dendritic cells but not basophils. Quantitative RT-PCR analysis of IL-3 mRNA levels in A) EDLN from MC903-treated wildtype (WT) Balb/c mice at various time points, B) EDLN of MC903- or ETOH-treated TSLP+/+ and TSLP-/- mice at D5, C) purified CD4+ and CD4- cells from EDLN of MC903- or ETOH-treated WT Balb/c mice at D5 , D) EDLN of MC903 or ETOH treated RAG1+/+ and RAG1-/- mice at D5, E) EDLN from MC903- or ETOH-treated CD11c^{Dep} and CD11c^{CT} mice at D5, and F) purified CD4+ and CD4- cells from EDLN of isotype- or MAR-1-injected WT Balb/c mice upon MC903 or ETOH treatment at D5. N.D., not detected. Values are mean ± SEM (n≥3 mice per group).

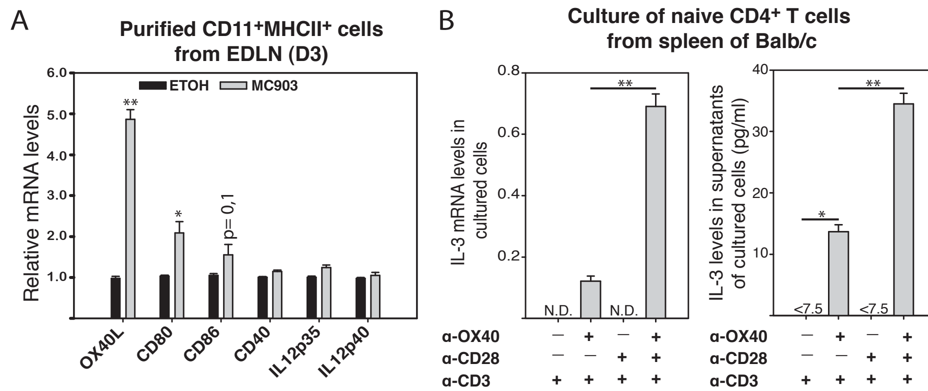

Figure 6.TSLP-induced OX40L expression in dendritic cells promotes IL-3 expression in naive CD4+ T cells.

A) Quantitative RT-PCR analysis of costimulatory molecules in purified dendritic cells ($CD11c^+MHCII^+$) from ear-draining lymph nodes (EDLN) of MC903- or ETOH-treated wildtype (WT) Balb/c mice at D3. B) Purified naive $CD4^+$T cells ($CD3^+CD4^+CD62L^+$) were cultured with different combinations of anti-CD3, anti-CD28 and anti-OX40 antibodies for 72 hours, and analyzed for IL-3 mRNA levels in cultured cells (left panel) and secreted IL-3 protein levels (right panel) N.D., not detected. *$p<0.05$, **$p<0.005$. Values are mean ± SEM (n≥3 mice per group).

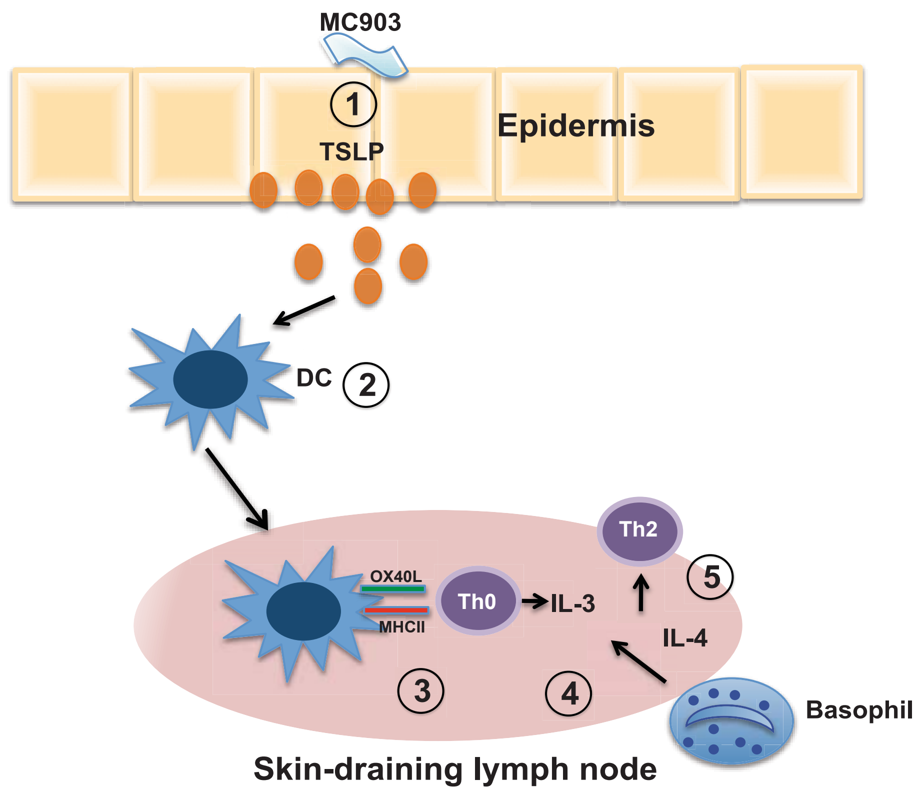

Figure 7. Proposed mechanism for skin TSLP-driven Th2 priming.
Topical MC903 treatment induces TSLP production in epidermal keratinocytes (1), which targets dermal DCs and triggers their maturation and migration to the draining lymph nodes (2). TSLP-targeted DCs express OX40L and stimulate CD4+ T (Th0) cell activation and production of IL-3 (3), which attracts basophils secreting Th2 promoting molecules (e.g. IL-4) (4) that complete the Th2 differentiation of activated CD4+ cells (5).

DISCUSSION

Atopic march refers to the natural history of allergic diseases, which is characterized by a typical sequence of sensitization and manifestation of symptoms in different tissues. Commonly, the clinical manifestation of AD appears early in life and precedes the development of airway allergic diseases, such as allergic rhinitis and asthma (Spergel and Paller, 2003). AD has been proposed as an entry point for subsequent allergic diseases (Bieber, 2010; De Benedetto et al., 2012), which suggests that effective management of AD could prevent the development of respiratory allergic diseases. However, little is known about the natural course of AD and the potential succession of atopic phenotypes in humans. The objectives of my thesis were: 1) to better understand the role of TSLP derived form skin keratinocytes in the atopic march and 2) to dissect TSLP-initiated immune cascade leading to AD pathogenesis. To reach these objectives we used mouse models of atopic diseases developed in the laboratory in combination with various deficient-mouse lines.

Part 1.- Role of the keratinocytic TSLP in the atopic march.

To investigate the role of the keratinocytic TSLP in the atopic march, we developed a mouse model that faithfully mimics the progression observed in human patients in combination of $TSLP^{iep-/-}$. First, the allergen sensitization was achieved through barrier-impaired skin without exogenous adjuvants, not through a systemic sensitization with use of exogenous adjuvant used in previous studies (Demehri et al., 2009; Zhang et al., 2009). Second, the allergen-sensitization was achieved by topical allergen-application, not in a patch as previously reported (patch method) (Spergel et al., 1998). Third, the production of TSLP was endogenous in physiological levels, not in artificial manner with a high systemic levels (Demehri et al., 2009; Zhang et al., 2009). Fourth, we used a mouse line with selective ablation of TSLP in adult epidermal keratinocytes, not a TSLPR-knockout mouse line (He et al., 2008).

Our results demonstrate that keratinocytic TSLP expression was essentially required, not only for developing an allergic skin inflammation, but also for mounting a systemic immune response against the allergen upon epicutaneous sensitization. In contrast, previous report, using TSLPR-deficient mouse line, established a role of

TSLP in the effector phase of allergic inflammation, induced by patch method, as TSLPR mice presented a decreased skin inflammation with normal secretion of Th2 cytokines by allergen-stimulated splenocytes (He et al., 2008).

Our results demonstrate that keratinocytic TSLP production simultaneously to the allergen sensitization, was required to generate an asthma-like lung inflammation after intranasal challenge. Interestingly, another recent study reported that TSLP-deficient mice intradermally-injected with a combination of TSLP and allergen develop an airway inflammation following intranasal challenge with the same allergen (Han et al., 2012). Thus, these results indicate that the skin-derived TSLP is an important factor in the atopic march.

In conclusion, our study reveals that keratinocyte-derived cytokine TSLP induced by tape stripping is crucial for promoting skin inflammation and allergen sensitization, which contribute for the progression from AD to asthma. Thus, blocking the expression or/and activity of keratinocytic TSLP could be helpful for treat skin allergic inflammation and prevent the progression to asthma. However, the function of TSLP in skin homeostasis remains unclear. Actually in our study, we noticed that small but significant IL-17 secretion by skin-draining lymph node cells, upon anti-CD3 stimulation in $TSLP^{iep-/-}$ mice (without any treatment). It suggests that keratinocytic TSLP may, in some way, affect the production of IL-17 in draining lymph node cells under stimuli. Recent report demonstrates that IL-17A and IL-17F, not only prevent but also inhibit the production of TSLP in skin explants of healthy and moderate AD patients (Bogiatzi et al., 2012). Therefore, a possible crosstalk may exist between keratinocytic TSLP and IL-17 producing cells. The combined use of mouse line with selective ablation of TSLP in adult epidermal keratinocytes with models of IL-17 related skin diseases [e.g. Imiquimod-induced psoriasis-like skin inflammation (van der Fits et al., 2009)] might be useful to dissect this crosstalk.

Part 2. Dissection of TSLP-induced Th2-mediated skin inflammation.

TSLP is able to promote Th2 responses *in vivo* (Ziegler and Artis, 2010), but the immune cascade remained to be elucidated. We demonstrate here, using MC903-induced TSLP-dependent AD mouse model, that TSLP induces early innate skin basophilia, which is enhanced by adaptive immunity at a later stage. In addition, Th2 priming promoted by TSLP is dependent of the cooperative contribution of dendritic cells, $CD4^+$ T cells and basophils.

Part 2A. TSLP promotes innate and adaptive skin basophil recruitment.

Recent studies established that mouse basophils play an important role during allergic inflammation and helminth infection, however, little was known about the factors implicated in their activation, migration and function.

Our results demonstrate that TSLP promotes an innate basophil skin recruitment. How TSLP induces innate basophil recruitment remains unclear. Several keratinocyte-derived molecules, such as eotaxin-1, eotaxin-2, G-CSF, GM-CSF, RANTES, IL-33, MCP2, MCP3 and TSLP, possibly attract basophils to the skin (Chirumbolo, 2012; Siracusa et al., 2011). It is also possible that TSLP-activated innate cells in the skin (mast cells, dermal dendritic cells or others) express basophil chemoattracting molecules. The use of high-through screening techniques will be helpful to investigate the molecules implicated in TSLP-induced innate basophil recruitment. In addition, the use of cell-deficient mice (mast-cell-, eosinophil-, NKT cell-, γδ cell-deficient mice, etc) would be helpful to determine the source of the molecules implicated in the TSLP-induced innate basophil skin recruitment.

We demonstrate that innate basophil skin recruitment was enhanced by adaptive immunity at later stage (D5 and D11), most probably by $CD4^+$ T cells, as reconstitution of RAG1-/- mice with these cells restore the basophil skin recruitment at D5. How $CD4^+$ T cells contribute to the basophil recruitment to skin remains to be elucidated. IL-3, an important factor for basophil activation, expansion, migration and survival (Kim et al., 2010; Lantz et al., 1998; Lantz et al., 2008; Ohmori et al., 2009; Shen et al., 2008), is a potential candidate. Expression of IL-3 was detected in

wildtype (RAG1+/+) mice but not in RAG1-/- mice. To determine the implication of IL-3 in the later “adaptive” basophil recruitment to skin (D5 and later), the use of IL-3 deficient mice in combination with MC903-induced AD-like skin inflammation will be helpful.

Our results suggest that adaptive immunity may contribute to the full activation of skin basophils. TSLP-triggered skin basophils from MC903-treated wildtype (RAG1+/+) control mice exhibited higher CD200R and lower CD11b expression, compared with RAG1-/- mice. Moreover, whether these phenotypic changes lead to functional difference remains to be clarified. Purification of skin basophils from MC903-treated RAG1-/- and wildtype control mice in combination with cell culture techniques and/or expression pattern analysis will be useful to determine if TSLP-triggered skin basophils exhibit functional difference in presence of adaptive immunity.

Part 2B. Skin TSLP-induced Th2 priming requires an orchestrated cooperation of dendritic cells, CD4+T cells and basophils.

The mechanisms leading to Th2 cell differentiation *in vivo* remain controversial. Recent reports suggest that basophils are key cells inducing Th2 responses. However, other reports suggest that dendritic cells, and not basophils, are the cell implicated in the induction of Th2 responses. Finally, other reports suggest a cooperation between DCs and basophils to promote a Th2 response. We reported that TSLP-promoted Th2 priming in skin-draining lymph nodes (LNs) involves an orchestrated cooperation of DCs, $CD4^{+}$ T-lymphocytes and basophils.

We demonstrate that DCs from skin draining LNs express high levels of OX40L upon MC903 treatment, and that in vitro activation of OX40 signaling induces IL-3 expression in naive $CD4^{+}$ T cells. This suggests a link between TSLP-targeted DCs and the production of IL-3 by activated $CD4^{+}$ T cells. Whether topical MC903 treatment promotes OX40 expression in CD4+ T cells remains to be determined.

Our results demonstrate that topical MC903 treatment promotes TSLP-dependent basophil recruitment in the draining lymph nodes. How basophils are attracted to

draining LNs in our model remains to be determined. However, we demonstrate that reconstitution of RAG1-/- mice with $CD4^+$ T cells restores the basophil recruitment to draining lymph node induced by topical MC903 treatment. $CD4^+$ T cell-derived IL-3 is an interesting candidate, as expression of IL-3 in $CD4^+$ T cells from draining LNs exhibited similar kinetics as basophil migration. The use of adoptive transfer of IL-3 deficient CD4+ T cells in RAG1-/- mice will allow to determine if IL-3 is the CD4+ T cell-derived signal inducing basophil recruitment in draining LNs upon MC903 treatment.

How basophils are involved in the initiation of IL-4 expression in $CD4^+$ T cells in our model remains still elusive. Various mechanisms have been proposed, such as providing the initial source of Th2 promoting cytokines (IL-4 and/or TSLP) (Sokol et al., 2008), or for the direct interaction with $CD4^+$ T cells (Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009). Expression analysis of purified basophils from draining LN of MC903-treated mice will be helpful to identify the basophil-derived molecule(s) involved in the initiation of Th2 polarization in our system.

Our data lead to a model where DCs, $CD4^+$T cells and basophils cooperate to fulfill Th2 polarization trigger by TSLP. Whether this model represents a general mechanism for Th2 immune responses remains to be investigated.

Whether TSLP functions in different models (allergen-induced or MC903 induced skin inflammation) are similar needs to be determined. Our results suggest that the TSLP-driven immune cascade will be similar in both models. Our results demonstrate that TSLP influences the expression of OX40L expression in DCs, the production of IL-4 in CD4+ T cells, and the eosinophil and basophil skin infiltration in both models. However, in allergic induced skin inflammation the innate basophil skin recruitment and IL-3 expression in the skin and in the draining LNs were not evident. This suggests that TSLP levels [100 pg/mg of total protein (in allergic induced skin inflammation) vs 40000 pg/mg of total protein(in MC903 induced skin inflammation)] might play an important role. Indeed, different doses of MC903 lead to different basophil and eosinophil recruitment of the skin, that correlate with the local TSLP levels in the skin at D11.

In conclusion my PhD work provides new knowledge in the cellular and molecular mechanisms implicated in atopic diseases involving TSLP, and should provide new insight for the development of therapeutic options of these diseases.

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