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Mécanismes moléculaires et cellulaires impliqués dans la sensibilisation allergique cutanée et la marche atopique

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Résumé de la thèse

Les maladies atopiques, dont la dermatite atopique et l'asthme, affectent une grande partie de la population mondiale. Elles partagent des caractéristiques communes telles qu'une production d'immunoglobulines E ainsi qu'une réponse immunitaire de type 2 avec une infiltration d'éosinophiles, de basophiles et de lymphocytes Th2. La dermatite atopique peut débuter dès l'enfance, vers 3 mois, et précède souvent d'autres maladies atopiques comme la rhinite allergique, les allergies alimentaires ou encore l'asthme allergique, dans un processus appelé la marche atopique. La prévalence des maladies atopiques, et en particulier de l'asthme, est en augmentation. De plus, l'asthme est la cause de nombreuses hospitalisations et entraine le décès de plus de 200 000 personnes par an, faisant de cette maladie un réel problème de santé publique. Il est donc important de trouver un moyen de bloquer la marche atopique pour prévenir l'apparition de l'asthme.

L'asthme est divisé en deux phases : une phase de sensibilisation lors de la première rencontre avec l'allergène, qui résulte en la mise en place d'une réponse immunitaire mémoire spécifique de l'allergène (lymphocytes B and T mémoires), et une phase de révélation conduisant au phénotype asthmatique. Il a été suggéré que la sensibilisation allergique peut s'effectuer au niveau de la peau des patients souffrant de dermatite atopique. En effet, plus de 50% des patients souffrant d'une dermatite atopique modérée à sévère développeront de l'asthme par la suite. De plus, ces patients présentent une barrière cutanée défectueuse permettant l'entrée de l'allergène, et un microenvironnement inflammatoire propice à la sensibilisation allergique. Cependant, les mécanismes moléculaires et cellulaires impliqués dans la sensibilisation allergique cutanée, et en particulier la manière dont le microenvironnement inflammatoire cytokinique la régule, restent à ce jour mal connus.

Mon laboratoire de thèse a précédemment démontré que la lymphopoïétine stromale thymique (TSLP), une cytokine produite par les kératinocytes, est un facteur essentiel à l'initiation de la réponse immunitaire Th2 au cours de la dermatite atopique et de la marche atopique. Cependant, la dermatite atopique est non seulement caractérisée par une réponse Th2 mais également par une réponse impliquant les lymphocytes auxiliaires folliculaires (Tfh), cette réponse ayant été montrée comme dérégulée chez les patients atteints de dermatite atopique. L'implication et le rôle de TSLP dans la réponse Tfh au cours de la sensibilisation allergique restent mal définis. Dans la première partie de ma thèse, j'ai donc étudié le rôle de TSLP et des cellules dendritiques dans

la différenciation des lymphocytes Tfh au cours de la sensibilisation allergique cutanée.

De plus, la dermatite atopique est une maladie complexe et hétérogène pouvant mettre en jeu plusieurs facteurs. C'est pourquoi dans la deuxième partie de ma thèse, j'ai identifié et étudié le rôle de différents facteurs pro-inflammatoires qui, en plus de TSLP, peuvent promouvoir la sensibilisation allergique cutanée et la marche atopique.

Partie 1 : Etude du rôle de TSLP et des cellules de Langerhans dans la réponse Tfh au cours de la sensibilisation allergique cutanée

Mon laboratoire de thèse a récemment montré que TSLP induisait la différenciation des lymphocytes Tfh dans un modèle de surexpression de TSLP ($TSLP^{over}$) induit par traitement au MC903. De plus, ils ont démontré que les réponses Tfh / lymphocytes B des centres germinatifs (GC B) étaient diminuées dans des souris déplétées en cellules de Langerhans (LC), les cellules dendritiques de l'épiderme. Ceci indique que les LCs promeuvent la différenciation des lymphocytes Tfh induite par TSLP.

Mon objectif était d'étudier le rôle de TSLP et des LCs dans les réponses Th2 / Tfh au cours de la sensibilisation avec un allergène. Mon laboratoire a mis au point un nouveau model expérimental mimant la sensibilisation allergique cutanée. La génération de micropores (LMP) à l'aide d'un laser sur l'oreille des souris induit une barrière cutanée défectueuse, et l'application de l'allergène ovalbumine (OVA) sur la barrière cutanée lésée résulte en la sensibilisation allergique. Ce modèle permet de délivrer l'allergène de manière précise et contrôlée, permettant une importante reproductibilité. Premièrement, nous avons montré que le traitement LMP/OVA induisait la différenciation des lymphocytes Th2 et Tfh, ce qui était entièrement aboli dans les souris *Tslp*$^{-/-}$ indiquant le rôle clé de TSLP dans les réponses Th2 mais aussi Tfh induites par un allergène. Par la suite, nous avons étudié le rôle des LCs dans la sensibilisation allergique. Pour ce faire, nous avons utilisé deux lignées murines : les souris muLangDTR (expression du récepteur humain de la toxine diphtérique sous le contrôle du promoteur murin de la Langerin) qui permettent la déplétion des cellules dendritiques $Lang^+$ (DCs dermales $Lang^+$ et LCs) suite à l'injection de toxine diphtérique (nommées ci-après $Lang^{DEP}$), et les souris huLangDTR (expression du récepteur humain de la toxine diphtérique sous le contrôle du promoteur humain de la Langerin) qui permettent la déplétion spécifique des LCs suite à l'injection de toxine diphtérique (nommées ci-après LC^{DEP}). Suite à l'application de notre protocole LMP/OVA à ces lignées, étonnamment, nous avons observé que

les souris Lang^{DEP} et LC^{DEP} présentaient une augmentation de l'inflammation cutanée et des réponses Tfh / GC B spécifiques de l'ovalbumine comparées aux souris contrôles. Les LCs jouent donc un rôle inhibiteur dans la réponse Tfh au cours la sensibilisation allergique, au contraire du rôle activateur observé dans notre modèle de surexpression de TSLP.

Par la suite, nous avons effectué des analyses transcriptomiques pour comprendre le rôle tolérogène / immunogène des LCs qui dépend du contexte (à savoir LMP/OVA ou MC903). Nous avons fait un RNAseq des cellules dendritiques migratoires Lang^{+} et Lang^{-} à partir des ganglions lymphatiques de souris non-traitées (NT), ou traitées par LMP/OVA ou MC903. Nos analyses ont montré que les DCs Lang^{+} du modèle MC903 étaient transcriptionnellement très différentes des DCs Lang^{+} des souris NT, au contraire des DCs Lang^{+} du modèle LMP/OVA. Pour conclure, nos données indiquent donc que les DCs Lang^{+} du modèle MC903 sont transcriptionnellement actives et promeuvent la réponse Tfh, ce qui n'est pas le cas des DCs Lang^{+} du modèle LMP/OVA qui, elles, inhibent cette réponse.

Perspectives : 1) Nos données de RNAseq suggèrent que la fonction tolérogène des LCs dans le modèle LMP/OVA pourrait avoir lieu directement dans la peau, et non au niveau des ganglions lymphatiques. En effet, ces cellules pourraient limiter la présentation antigénique d'autres populations de cellules dendritiques. Il serait donc intéressant de délivrer de l'OVA couplé à un fluorochrome sur l'oreille des souris LC^{DEP} pour déterminer quelles populations de DCs capturent l'allergène et si elles sont limitées par les LCs. 2) Bien que nous ayons montré le rôle de TSLP dans la différenciation des lymphocytes Tfh, leur rôle n'en demeure pas moins flou. Pour étudier leur fonction, nous sommes en train de générer en collaboration avec l'ICS une nouvelle lignée murine permettant de dépléter ces cellules. En appliquant notre protocole LMP/OVA à cette lignée, nous espérons élucider le rôle des lymphocytes Tfh dans la sensibilisation allergique.

Ce travail a été publié : Marschall P, Wei R*, Segaud J*, Yao W, Hener P, German BF, Meyer P, Hugel C, Ada Da Silva G, Braun R, Kaplan DH, Li M. Dual function of Langerhans cells in skin TSLP-promoted TFH differentiation in mouse atopic dermatitis. Journal of Allergy and Clinical Immunology. 2020;147(5):1778-94.

Partie 2 : Etude du microenvironnement inflammatoire lors de la sensibilisation allergique cutanée et son rôle dans la marche atopique.

La dermatite atopique est une maladie complexe hétérogène pouvant être due à différents facteurs tels que l'environnement, des mutations génétiques ou la progression de la maladie. De ce fait, la sévérité de la DA pourrait résulter en l'exposition aux allergènes à différentes profondeurs cutanées et pourrait donc mettre en jeu différents microenvironnements inflammatoires. Pour étudier ceci, en collaboration avec W. Yao, j'ai mis au point deux modèles de sensibilisation allergique cutanée : l'un mettant en jeu une sensibilisation épicutanée (e.c. HDM, au niveau de l'épiderme) et le second une sensibilisation dermacutanée (d.c. HDM, au niveau du derme). Pour étudier la marche atopique, les souris sont par la suite exposées au même allergène au niveau des voies respiratoires. Nous avons tout d'abord démontré que e.c. HDM et d.c. HDM induisent des microenvironnements cytokiniques différents, lesquels impactent la sensibilisation allergique et le phénotype asthmatique en découlant. En effet, nous avons montré que TSLP est nécessaire pour l'inflammation cutanée, le développement de la réponse Tfh / GC, la production d'IgG1 et IgE spécifiques de HDM, l'inflammation des poumons et l'hyperréactivité des bronches lors de la sensibilisation épicutanée. Au contraire, d.c. HDM induit un recrutement similaire des lymphocytes Th2, des éosinophiles et des basophiles dans la peau, une réponse de Tfh / GC et l'inflammation pulmonaire chez les souris WT et *Tslp*$^{-/-}$. Ces données montrent donc que TSLP est différemment nécessaire lors de la sensibilisation épicutanée et de la sensibilisation dermacutanée.

Nous nous sommes par la suite intéressés aux facteurs pouvant expliquer cette différence de réponse et avons identifié l'IL-1β comme un facteur clé. Premièrement, d.c. HDM, au contraire de e.c. HDM, induit une augmentation de la production de la cytokine IL-1β dans la peau, laquelle est produite par les neutrophiles et monocytes / macrophages recrutés dans le derme. Deuxièmement, la déplétion des neutrophiles et monocytes / macrophages réduit le niveau de l'IL-1β dans la peau. Troisièmement, la déplétion des neutrophiles et monocytes / macrophages lors de la phase de sensibilisation dermacutanée diminue le phénotype asthmatique, lequel est rétabli suite à l'application d'IL-1β lors de la sensibilisation dermacutanée. De plus, nous avons montré que l'administration d'IL-1β lors de la sensibilisation épicutanée (e.c. HDM) augmente la réponse Tfh / GC, la production d'IgG1 et IgE spécifiques de HDM et le phénotype asthmatique, chez les souris WT mais également *Tslp*$^{-/-}$ indiquant que l'IL-1β peut promouvoir la sensibilisation allergique cutanée et la marche atopique indépendamment de TSLP. De plus, nous avons

également démontré que TSLP n'est pas impliqué dans la production d'IL-1β. Nos données démontrent donc que TSLP et IL-1β sont deux facteurs clés indépendants l'un de l'autre et dont l'importance dépend du contexte de sensibilisation allergique cutanée, à savoir, par voie superficielle ou plus profonde.

Perspectives : 1) Notre étude met en lumière l'importance de l'IL-1β et TSLP selon le contexte de sensibilisation qui dépend de la sévérité de la dermatite atopique, ce qui peut se révéler crucial d'un point de vue clinique. En effet, les essais cliniques avec le Tezepelumab, un anticorps anti-TSLP, se sont révélés peu concluants pour les patients souffrant de dermatite atopique modérée à sévère. Ceci pourrait s'expliquer par le fait que le rôle de TSLP chez ces patients est limité et que d'autres facteurs pourraient entrer en jeu, comme l'IL-1β. Notre étude met donc en évidence l'importance des traitements individualisés dans les choix de cibles thérapeutiques pour soigner / prévenir la dermatite atopique et la marche atopique. 2) Nous avons montré l'importance des neutrophiles et de l'IL-1β dans la sensibilisation allergique et la marche atopique. Cependant nous n'excluons pas l'implication d'autres facteurs dans la sensibilisation allergique, en particulier les cytokines IL-36α, γ qui sont produites par les kératinocytes et qui induisent le recrutement des neutrophiles dans la peau. Ces cytokines sont impliquées dans différentes maladies cutanées telles que le psoriasis ou le syndrome de Netherton, lequel est caractérisé par des lésions de type dermatite atopique. Il serait donc intéressant d'étudier le rôle de ces cytokines dans notre modèle pour voir si leur absence peut limiter la sensibilisation allergique et la marche atopique, ce qui en ferait d'intéressantes cibles thérapeutiques.

Abbreviations

A

AAD:	American academy of dermatology
AD:	atopic dermatitis
APC:	antigen-presenting cell
AR:	allergic rhinitis
ASCL2:	achaete scute-like 2

B

BAL:	bronchoalveolar lavage
BATF:	Basic leucine zipper transcription factor ATF-like
Bcl6:	B-cell lymphoma 6
BCR:	B cell receptor
BFP:	blue fluorescent protein
Blimp-1:	B-lymphocyte-induced maturation protein 1
Breg:	regulatory B
BSA:	bovine serum albumin

C

cDC:	conventional dendritic cell
CDSN:	corneodesmosin
CSR:	class switch recombination
CT:	control
cTfh:	circulating follicular helper T
CXCL:	C-X-C motif chemokine ligand
CXCR:	C-X-C motif chemokine receptor

D

DAMP:	danger associated molecular pattern
d.c.:	dermacutaneous
DC:	dendritic cell
dDC:	dermal dendritic cell
DNA:	deoxyribonucleic acid
DOCK8:	dedicator of cytokinesis 8
DT:	diphtheria toxin
DTR:	diphtheria toxin receptor

E

EASI:	eczema area and severity index
e.c.:	epicutaneous
ECP:	eosinophil cationic protein
EDLN:	ear-draining lymph node
EDN:	eosinophil-derived neurotoxin
ELISA:	enzyme-linked immunosorbent assay
EMA:	European medicines agency
EPO:	eosinophil peroxidase
EP4:	prostaglandin E receptor 4
EtOH:	ethanol

F

FACS:	fluorescence-activated cell sorting
FDA:	food and drug administration
FDC:	follicular dendritic cell
FLG:	filaggrin

G

GC:	germinal center

H

HDM:	house dust mite
hDTR:	human diphtheria toxin receptor
H&E:	hematoxylin and eosin

I

ICOS:	inducible T cell costimulator
IGA:	investigator's global assessment
IgE:	immunoglobulin E
IgG1:	immunoglobulin G1
IHC:	immunohistochemistry
IL-1RacP:	interleukin 1 receptor accessory protein
IL:	interleukin
ILC:	innate lymphoid cell
i.n.:	intranasal
i.p.:	intraperitoneal
IRF4:	interferon regulatory factor 4

J

JAK:	Janus kinase

K

KC:	keratinocyte
KLF2:	Krüppel-like factor 2
KLK:	kallikrein
K14:	keratin 14

L

Lang: langerin
LC: Langerhans cell
LCMV: lymphocytic choriomeningitis virus
LEF-1: lymphoid enhancer binding factor 1
LEKTI: lympho-epithelial Kazal-type-related inhibitor
LMP: laser microporation
LN: lymph node

M

MBP: major basic protein
MCPT8: mast cell protease 8
MHC: major histocompatibility complex
Mo-DC: monocyte-derived dendritic cell
mTORC1: mammalian target of rapamycin complex 1
MyD88: myeloid differentiation primary response 88

N

NeoR: neomycine resistance
NET: neutrophil extracellular trap
NF-κB: nuclear factor κB
NT: non-treated

O

OCLN1: occludin 1
OH-Tam: 4-hydroxytamoxifen
OR: odds ratio
OVA: ovalbumin

P

PAMP: pathogen associated molecular pattern
PAR2: protease-activated receptor 2
PD-1: programmed death 1
PRR: pattern recognition receptor
PSGL1: P-selection glycoprotein ligand 1

R

RAR: retinoic acid receptor
RNA: ribonucleic acid
RT-qPCR: reverse transcription – quantitative polymerase chain reaction
RXR: retinoid X receptor

S

S1PR1: sphingosine 1 phosphate receptor 1
S. aureus: Staphylococcus aureus
s.c.: subcutaneous
SAP: SLAM-associated protein
SCORAD: SCORing atopic dermatitis
SHM: somatic hypermutation
SLAM: signalling lymphocyte activation molecule
SLAMF: SLAM family
SNP: single nucleotide polymorphism
SPINK5: serine proteinase inhibitor Kazal-type 5
SRBC: sheep red blood cell
STAT: signal transducer and activator of transcription

T

TCF-1: T cell factor 1
TCR: T cell receptor
TCS: topical corticosteroid
TEWL: trans-epidermal water loss
Tfh: follicular helper T
Tfr: follicular regulatory T
TGF-β: transforming growth factor β
Th: T helper
TLR: Toll-like receptor
Treg: regulatory T
TSLP: thymic stromal lymphopoietin
TSLPR: thymic stromal lymphopoietin receptor

V

VCAM-1: vascular cell adhesion molecule 1
VDR: vitamin D receptor

W

WT: wild type

X

XCR1: X-C motif chemokine receptor 1

INTRODUCTION

1 Atopic dermatitis, asthma and the atopic march

1.1 Atopic dermatitis

1.1.1 Overview of the skin

The skin is the largest organ in the human body with a surface of approximatively 2m². Its thickness depends on the body region. The skin has several functions including thermoregulation, mechanic and metabolic functions, limiting trans-epidermal water loss (TEWL), and protection against the environment. It serves as the first defense barrier against external aggressions such as mechanical or thermal trauma, ultraviolet radiations, or pathogens.

The skin is subdivided in 3 main layers, comprising the epidermis which is the outer layer, the dermis and the adipose tissue. The epidermis is a stratified epithelium further divided in 4 different layers (Figure 1): the stratum corneum (cornified layer), the stratum granulosum, the stratum spinosum and the stratum basale, separated from the dermis by the basement membrane[1].

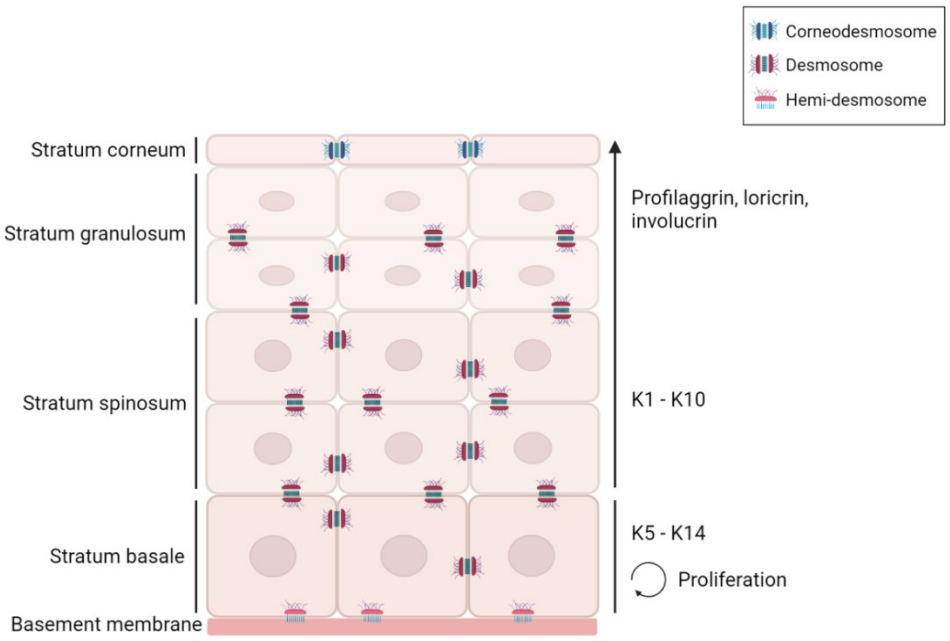

Figure 1. Epidermis organisation in different layers. Created with Biorender.

Epidermis.

The epidermis is mainly composed of keratinocytes that differentiate and migrate from the basal layer towards the stratum corneum, forming a compact and strong physical barrier. The keratinocytes in the basal layer are columnar and are attached to other cells through desmosomes and to the basement membrane through hemi-desmosomes. The basal keratinocytes have the capacity to proliferate and start a carefully regulated cell differentiation program associated with morphological changes, as they migrate towards the suprabasal layers. One observed modification is their expression of keratins which can assemble into intermediate filaments to stabilize desmosomes and resist mechanical stress. The keratinocytes from the basal layer can be identified by their expression of keratin 5 (K5) and K14, which is lost as they enter the stratum spinosum where they start expressing K1 and K10[1].

In the stratum granulosum, keratinocytes start to flatten and present a high number of keratohyalin granules, and lamellar bodies (containing phospholipids, ceramides and cholesterol) which are released at the junction between the stratum granulosum and stratum corneum. Keratohyalin granules contain major components of the cornified envelope including keratins, profilaggin, involucrin and loricrin[1].

Finally, the stratum corneum is composed of enucleated keratinocytes called corneocytes. Profilaggrin is cleaved into filaggrin monomers which aggregate keratins into fibrils. Monomers are also degraded in natural moisturizing factors to maintain skin hydration. Different proteins, including periplakin, envoplakin, involucrin and loricrin, are crosslinked to keratins and lipids released from lamellar bodies, forming the cornified envelope which replaces the plasma membrane[1,2]. During cornification, the desmosomes are replaced by corneodesmosomes to reinforce adhesion between adjacent cells. The cornified envelope, embedded in a lipid-rich matrix, forms a “brick and mortar” complex and confers its mechanical resistance to the stratum corneum, as well as its barrier function. Corneocytes are shedded in a process called desquamation to maintain epidermal thickness. Kallikreins (KLK) are serine-proteases produced by keratinocytes and secreted as inactivated precursors[3]. After a step of cleavage, leading to their activation, kallikreins can cleave desmoglein I, desmocollin I and corneodesmosin, the main components of corneodesmosomes, resulting in desquamation[4]. Kallikreins (KLK) are tightly regulated to prevent excessive shedding. Lympho-epithelial Kazal-type-related inhibitor (LEKTI), encoded by *SPINK5*, is expressed in the skin and mainly binds KLK5 and KLK7 at neutral pH preventing their activity[5]. Upon skin acidification, in the outer layers of the stratum corneum,

LEKTI is dissociated from KLK leading to desquamation.

In addition to keratinocytes, the epidermis is also composed of Merkel cells, melanocytes and Langerhans cells (LC). Merkel cells, located in the basal layer, are mechanoreceptors involved in touch perception. Like Merkel cells, melanocytes are located in the basal layer, and they have a photoprotective role. Melanocytes produce melanin in melanosomes and transfer them to keratinocytes to reduce UV-induced DNA damages. LCs, the only dendritic cell (DC) population in the epidermis, represent around 5% of all epidermal cells and assess the environment to detect any danger signal[6].

Dermis.

The dermis is a conjunctive tissue subdivided in 2 layers: a thin and loose papillary layer and a thick reticular layer enriched in collagens and elastin conferring its elasticity to the skin. The dermis is composed of an extracellular matrix, fibroblasts and immune cells such as DCs, mast cells, macrophages, T cells and innate lymphoid cells (ILC). Contrary to the epidermis, the dermis harbors lymphatic and blood vessels, nerves as well as hair shafts.

1.1.2 Epidemiology

Atopic dermatitis (AD) is one of the most common skin inflammatory diseases with increasing prevalence worldwide. AD affects around 10-20% of children[7,8] and 1-3% of adults, albeit AD prevalence is highly dependent on the world regions, as indicated in the ISAAC study where AD prevalence in 6–7-year-olds ranges from 3% in India to 17% in Oceania[9]. AD usually starts in infancy with 60% of AD children developing the disease before 1 year old[7]. 80% of the children outgrow the disease by 8 years, and only 5% of children still present AD 20 years after its initiation[10]. Disease persistence is higher in severe AD patients[10]. People living in urban areas or with family history of atopy are at a higher risk to develop AD[11].

AD can be classified in two categories: extrinsic AD patients which present elevated immunoglobulins E (IgE), and intrinsic AD patients which do not. Around 80% of AD patients present sensitization to allergens with elevated levels of IgE in the blood[12]. Allergic sensitization in AD children increases the risk to develop other atopic diseases including asthma, allergic rhinitis or food allergy at a later stage in life, in a process called the atopic march[13].

1.1.3 Clinical features

AD is a heterogeneous disease characterized by generalized xerosis (skin dryness), pruritus and eczematous lesions which can differ with age and disease stage (Figure 2). Infants usually present acute lesions (oozing vesicles associated to erythema and oedema) widely spread on the trunk, head, cheeks, whereas lesions are concentrated in the flexural areas in children. Adults exhibit chronic lesions characterized by lichenification mainly localized on the feet, hands, flexural areas even though the head / neck is sometimes affected[14].

Figure 2. Skin of healthy patient (A) or non-lesional skin (B), acute (C), subacute (D) and chronic (E) lesions in AD patients. From Weidinger et al.[14]

In addition to asthma, food allergies or allergic rhinitis, other comorbidities are associated with AD, such as skin infections (90% of AD patients are colonized by *S. aureus*, which requires antibiotic treatment to prevent sepsis) and most of all, mental health disorders[14]. Indeed, AD symptoms negatively impact the quality of life of the patients and their families. Itch, which is often linked to sleep loss, as well as the stigma associated to AD lesions in public can lead to reduced self-esteem, social isolation, anxiety and depression[14]. A national survey in the United-Kingdom shows that AD patients have a more frequent utilization of health care services with a higher number of specialists seen, which is also greatly dependent on AD severity[15].

AD is not only impacting the quality of life of the patients, but is also a financial burden for them, and the society[14]. Financial costs for AD include direct and indirect costs comprising hospitalization, medication (from moisturizers to high-cost biological agents), medical fees, absenteeism, and loss of productivity. The financial burden attributed to the patients is highly dependent on the health / insurance system of the country of residence and on AD severity.

The diagnosis of AD is performed by clinical examination as well as medical history review. To unify AD diagnosis, Hanifin and Rajka[16] published in 1980 a first set of criteria based on 27 features. To

be diagnosed, patients need to meet 3 of the 4 main features and 3 of the minor criteria indicated in Table 1. The Hanifin and Rajka set including a lot of criteria, its application and interpretation in a clinical environment was difficult. Moreover, some of the features indicated were non-specific to AD or rare, negatively impacting the diagnosis. Therefore, several sets of criteria have been proposed over the years to palliate these difficulties. Among them, the United Kingdom Working Party simplified the Hanifin and Rajka set to one mandatory and 5 major criteria[17–19], which have later been adjusted to be also applicable to infants. The American Academy of Dermatology (AAD) also divided their criteria into essential features, important features which are observed in the majority of cases, and associated features which can be present in AD patients but are non-specific[20]. An exclusion section was also added to the criteria to eliminate diseases close to AD.

Hanifin & Rajka criteria	**AAD**
Main criteria	**Essential features**
Pruritus	Pruritus
Typical morphology and distribution: 1) Flexural lichenification or linearity in adults 2) Facial and extensor involvement in infants/children	Eczema: 1) Chronic or relapsing history 2) Typical morphology and age-specific patterns
Chronic or chronically relapsing dermatitis	
Personal or family history of atopy	
Minor criteria	**Important features**
Xerosis	Xerosis
Early age of onset	Early age of onset
Immediate (type I) skin test reactivity	Atopy: 1) Personal and / or family history 2) IgE reactivity
Elevated serum IgE	
Ichtyosis/palmar hyperlinearity/keratosis pilaris	
Tendency toward cutaneous infection/impaired cell-mediated immunity	
Cheilitis	**Associated features**

Recurrent conjunctivitis	Ocular / periorbital changes
Nipple eczema	Other regional findings (perioral, periauricular)
Tendency toward non-specific hand and foot dermatitis	Perifollicular accentuation / lichenification / prurigo lesions
Dennie-Morgan infraorbital fold	Keratosis pilaris / hyperlinear palmar / ichtyosis
Keratoconus	Atypical vascular responses
Anterior subcapsular cataracts	
Orbital darkening	**Exclusionary conditions**
Facial pallor/facial erythema	Allergic contact dermatitis
Pityriasis alba	Ichthyoses
Anterior neck folds	Cutaneous lymphoma
Itch when sweating	Psoriasis
Intolerance to wool and lipid solvents	Immune deficiency diseases
Perifollicular accentuation	Scabies
Food intolerance	Seborrheic dermatitis
Course influenced by environmental/emotional factors	
White dermographism/delayed blanch	

Table 1. Sets of criteria proposed by Hanifin and Rajka[16], and by the AAD[20] for AD diagnosis.

Several tools can be used to measure AD severity. The main ones are EASI (Eczema Area and Severity Index), SCORAD (SCORing Atopic Dermatitis) or IGA (Investigator's Global Assessment). The EASI[21] and SCORAD[22] index measure the extent of affected areas and the severity of 4 or 6 clinical signs, respectively (Table 2). In addition, the SCORAD takes into consideration itch and sleep loss to calculate a score from 0 to 103. An EASI score between 1 and 7 corresponds to mild AD, moderate AD patients present a score between 7 and 21 and severe patients are >21. A SCORAD score <25 corresponds to mild AD, a score between 25 and 50 corresponds to moderate AD, whereas a score >50 corresponds to severe AD. The IGA scale measures AD severity based on clinical signs of AD lesions but does not take into consideration the extent of the affected areas for mild or moderate AD. However, to be classified severe, AD should be widespread, in addition

to show marked erythema, lichenification and papulation. Five levels of severity exist: 0 (clear), 1 (almost clear), 2 (mild), 3 (moderate) and 4 (severe)[23].

	EASI	SCORAD	IGA
Measure the extent of affected areas	Yes	Yes	No (except for severe AD)
Clinical signs observed	4 (lichenification – erythema – papulation / infiltration – excoriations)	6 (lichenification – erythema – papulation / oedema – oozing / crusting – excoriations - dryness)	4 (lichenification – erythema – papulation / induration – oozing / crusting)
Subjective symptoms	No	Yes (itch and sleep loss)	No
Score			
Mild AD	1 - 7	<25	2
Moderate AD	7 - 21	25 - 50	3
Severe AD	>21 (72 max)	>50 (103 max)	4

Table 2. AD severity assessment using EASI[21], SCORAD[22] or IGA[23].

1.1.4 Pathogenesis

AD is a heterogenous and complex disease whose etiology is still not completely understood. Two hypotheses have been proposed: the inside-out hypothesis which suggests that AD starts with a dysregulated immune response leading to barrier disruption whereas the outside-in hypothesis is based on skin barrier defects appearing first, which results in immune responses. AD is now recognized as a multifactorial disease with a crosstalk between immunity, barrier disruption, microbiota, environmental factors (such as allergens) and genetics (with genes involved in immunity and skin barrier integrity)[11].

1.1.4.1 Skin barrier disruption

Skin barrier integrity is essential for preventing entry of pathogens / allergens / chemicals but also to prevent excessive TEWL. However, AD patients present a disrupted skin barrier with increased TEWL and facilitated entry of allergens leading to sensitization[11].

Filaggrin

Filaggrin (FLG) is a key constituent of the stratum corneum, not only linking keratins filaments to other proteins to form an impermeable barrier, but also as a natural moisturizing factor to maintain hydration. *FLG* gene is located on chromosome 1q21 in the epidermal differentiation complex, which includes different genes encoding structural proteins. Several loss-of-function mutations have been identified in the *FLG* gene[24–26]. The presence of these variants increases 3 times the risk to develop AD in these subjects, raising FLG as the main risk factor for AD development. In addition, R501X and 2282del4 mutations are associated with early AD onset and allergic sensitization[27,28]. Some genetic variants are segregated by ethnicity, with 3321delA and S2554X mutations found only in asian populations while R501X and 2282del4 are mostly identified in European cohorts[26,29,30]. Reduced FLG levels have been reported in lesional skin of some AD patients, in adequation that not all patients present *FLG* mutation[31]. The importance of FLG has been highlighted by mouse models including the flaky tail mouse ($Flg^{ft/ft}$ $Matt^{ma/ma}$) which has a 1bp deletion mutation[32] in the *FLG* gene and spontaneously develops AD-like lesions as well as increased TEWL[33]. Allergen sensitization through skin and cutaneous inflammation are also exacerbated in these mice. Of note, flaky tail mice, which have been used to study FLG, present a second mutation in the gene *Matt*, located on chromosome 3, and encoding the protein Mattrin expressed in the granular layer of the epidermis[34,35]. Saunders et al.[34] showed that $Matt^{ma/ma}$ (without $Flg^{ft/ft}$ mutation) mice develop spontaneous AD lesions with a Th17 immune response[36] and present exacerbated reaction to allergens. This study highlights the limits of the use of flaky tail mice as a model to investigate FLG role. Indeed, the phenotype observed in these mice and attributed to FLG could be a result of $Matt^{ma/ma}$ mutation. Saunders et al.[37] isolated the $Flg^{ft/ft}$ mutation from flaky tail mice to obtain $Flg^{ft/ft}$ on a Balb/c background (without $Matt^{ma/ma}$) and observed the development of spontaneous AD-like lesions associated to atopy. However, upon further investigation, Muhandes et al.[38] showed that the $Matt^{ma/ma}$ mutation was not eliminated from the genome, which could explain the atopy feature of these mice. Moreover, two studies[38,39] report the generation of *Flg* null mice, which do not develop spontaneous AD lesions, only presenting dry skin, reduced natural moisturizing factors and an altered keratin network, as well as an enhanced response in contact hypersensitivity. These studies might suggest a more limited role for FLG, on its own, than previously thought. However, this does not exclude the role of FLG, in association with other factors, in AD. Indeed *Flg* null mice still present exacerbated response in contact hypersensitivity[39]. Moreover, IL-4, IL-13 and IL-25 downregulate the expression of FLG in vitro[31,40], which could initiate an inflammatory loop.

LEKTI

Corneocyte shedding is a tightly regulated process by kallikreins and their inhibitor LEKTI (Lympho-epithelial Kazal-type-related inhibitor) encoded by the gene *SPINK5* (Serine Proteinase Inhibitor Kazal type 5). LEKTI, expressed in the granular layer of the epidermis, binds KLK5 / KLK7 to prevent their activity and excessive shedding[5]. With increasing pH, KLK5 and LEKTI are dissociated resulting in cleavage of corneodesmosomes and desquamation. Loss of function mutations in *SPINK5* can cause Netherton syndrome, a rare autosomal recessive disease characterized by ichthyosiform erythroderma, hair shaft abnormalities, high levels of IgE, and severe AD lesions[41]. Mutations in *SPINK5* have also been associated with AD in European and Japanese populations[42–44]. Mouse models have shown that *Spink5*$^{-/-}$ mice exhibit loss of cell-cell adhesion resulting in the detachment of the stratum corneum and increased TEWL. The loss of skin barrier function leads to the death of the mice a few hours after birth. Excessive proteolysis of corneodesmosin, desmocollin 1 or desmoglein 1, three components of the corneodesmosomes, have been reported in both patients and mouse[45,46,47]. To investigate which proteases were involved in this process, Furio et al.[48] generated a mouse line in which the coding sequence of the human *KLK5* was inserted under the control of the promoter of the involucrin to induce its overexpression in the stratum granulosum of the skin. The mutant mice presented impaired skin barrier function associated with excessive TEWL at birth. They also developed hair shaft problems and cutaneous inflammation associated with increased thymic stromal lymphopoietin (TSLP), IL-4 and IL-17 expression. Contrary to *Spink5*$^{-/-}$ mice, *Klk5*^{Tg} mice lived through the neonatal period to reach adulthood, indicating that this phenotype is weaker than the one observed in *Spink5*$^{-/-}$ mice. In addition, *Spink5*$^{-/-}$ *Klk5*$^{-/-}$ mice exhibited reduced cleavage of corneodesmosomes resulting in decreased TEWL and no detachment of the stratum corneum, indicating a major role for KLK5 in Netherton syndrome[49,50]. Of note, LEKTI can also inhibits KLK7, however *Spink5*$^{-/-}$ *Klk7*$^{-/-}$ mice did not present any improvement compared to *Spink5*$^{-/-}$ mice. Double knock out of *Klk7* and *Klk5* in *Spink5*$^{-/-}$ mice abolished TEWL and mice survived into adulthood, compared to *Klk5*$^{-/-}$ *Spink5*$^{-/-}$ which died at day 5[50]. Overall, these data indicate a major role for LEKTI, KLK5 and KLK7 in skin barrier integrity and in preventing the entry of allergens in the body, as Netherton syndrome patients present atopic manifestations, including hay fever or asthma. TSLP, produced in *Spink5*$^{-/-}$ mice is a key inflammatory cytokine involved in Th2 immune response and IL-4 and IL-13 production. IL-4 and IL-13 decrease KLK7 expression in vitro[40], initiating an inflammatory loop.

Corneodesmosin

Corneodesmosin (CDSN) is a component of the corneodesmosomes and maintains adhesion between cells. *CDSN* mutations are identified in Peeling Skin Syndrome Type B (PSS-B), an ichthyosiform erythroderma, characterized by AD-like lesions and associated with atopic diseases. In patients, *CDSN* mutation leads to the loss of corneocyte adhesion and increased TEWL[51]. Reduced expression of CDSN has also been reported in AD patients[52] and mouse model[53]. CDSN impairment not only disrupts skin barrier function but also induces Th2 cutaneous inflammation with TSLP expression which shifts overtime to Th17 response in the skin[54].

Tight junctions in the stratum granulosum are involved in cell-cell adhesion therefore participating to structure of the skin, but also regulate paracellular permeability. They are composed of several proteins such as claudin 1, claudin 4, occludin, and zona occludens. Claudin 1 and zona occludens 1 expression is reduced in lesional skin of AD patients[55]. IL-17 negatively regulates claudin 1 and zona occludens 1 in human skin models. The importance of Claudin 1 in skin barrier has been reinforced by *Cldn1*$^{-/-}$ mice which are not viable after 1 day due to excessive TEWL[56].

Lipids are essential to the barrier function of the skin. They are synthesized by keratinocytes and stored into lamellar bodies which are secreted to form the cornified layer. This includes mainly free fatty acids, cholesterol and ceramides. In AD lesional skin, the ceramides are reduced compared to healthy skin[57] and the ceramide composition is altered with more small ceramide species and less larger species[58]. Ceramides can be produced de novo, or following degradation of glucosylceramides by β-glucocerebrosidases, or sphingomyelin can be cleaved in the extracellular space by sphingomyelinases to generate ceramides[59]. Acid and neutral sphingomyelinase activity is reduced in non-lesional and lesional AD skin[60].

1.1.4.2 Epidermal-derived factors driving inflammation

Barrier disruption promotes inflammation by releasing several factors implicated in the immune response or pruritus. The main factors are TSLP, IL-33 and IL-25.

1.1.4.2.1 TSLP

TSLP has been identified in the supernatant of a thymic stromal cell line[61]. It was first shown to promote the development of immature B cells and the proliferation of thymocytes, but is now commonly recognized as a key player in type 2 immune response, even though more recently a role for TSLP in Treg induction has also been proposed[62–64]. TSLP is a cytokine, member of the IL-2 family, and signals through its receptor comprising a heterodimer of TSLPR (encoded by *CRLF2*) and IL-7Rα, leading to the phosphorylation and activation of Janus Kinases (JAK) 1 and 3. JAK1 and JAK3 phosphorylate STAT5A and STAT5B which dimerize to migrate in the nucleus and activate gene transcription. TSLP receptor is expressed by a variety of cells including DCs, type 2 ILCs (ILC2), T cells, B cells, mast cells, eosinophils and basophils[64]. TSLP is mainly produced by epithelial cells from the airways or the intestine, by keratinocytes in the skin, or to some lesser extent by immune cells such as DC or mast cells[64,65]. Several triggers induce TSLP expression, such as pathogens, Toll-like receptor (TLR) ligands, proteases, and chemical or physical injury. TSLP is implicated in several diseases including cancer, asthma and AD[64].

Several single nucleotide polymorphisms (SNP) have been identified in TSLP pathway including in *TSLP*, *CRLF2* and *IL7Rα* genes in European Americans or African Americans, and have been associated to AD risks[66]. Moreover, TSLP is highly expressed in acute and chronic AD lesions[65]. In the skin, mechanical injury, as observed with scratching in AD patients, induces TSLP in keratinocytes as shown with tape-stripping[67], laser-microporation[68] and irritants[69]. In addition to physical injury, TSLP induction can result from genetic defects. *SPINK5* mutation, observed in Netherton syndrome, leads to high levels of TSLP in the skin[49,70], which is also observed in $Cdsn^{KC-/-}$ mice[54]. TSLP induction in Netherton syndrome is through the unregulated activity of KLK5 which cleaves PAR2 to induce NF-κB activation and TSLP expression[70]. TSLP expression in keratinocytes is also controlled by RXR/VDR or RXR/RAR heterodimers and can be induced by vitamin D3 and its analogs[71,72]. Other cytokines, IL-1β and IL-4, stimulate TSLP expression in ex vivo keratinocytes culture[69] and human reconstructed epidermis[73]. Of note, IL-1β promotes TSLP expression more efficiently in human keratinocytes than murine. Several mouse models have been used to investigate the role of TSLP in AD, including overexpression of TSLP in keratinocytes induced by genetic modifications ($Tslp^{Tg}$) or by chemicals such as MC903 (low calcemic analog of vitamin D3) leading to AD development[71,72,74,75]. Barrier-disruption models (through tape-stripping or laser microporation) combined with an allergen are also widely used models as they mimic AD lesions and epicutaneous allergen sensitization observed in patients[67,68]. Using these different models, TSLP has been shown to play a critical role in AD pathogenesis, being required and

sufficient to induce AD-like lesions associated to elevated IgE levels, type 2 inflammation with Th2 cells, eosinophils, mast cells and basophils as well as production of Th2 cytokines IL-4, IL-5 and IL-13. TSLP also induces the recruitment of ILC2 and their production of Th2 cytokines in the skin through TSLP-TSLPR axis[76]. In addition to skin inflammation, TSLP promotes skin allergic sensitization with exacerbated follicular helper T (Tfh) and germinal centre (GC) B cell response, as well as elevated levels of IgE[67,68].

To initiate Th2 and Tfh cell responses, DCs need to be activated and provide different signals (peptide:MHCII, cytokines, co-stimulation) to naive $CD4^+$ T cells to polarize them towards Tfh or Th2 cells. It has been shown that TSLP upregulates CCL17 and CCL22 in DCs, two chemokines involved in Th2 cell recruitment to the skin, as well as HLA-DR and co-stimulatory molecules CD80, CD86, CD40, OX40L leading to the differentiation of naive $CD4^+$ T cells in Th2 cells[65,77] but also Tfh cells in vitro[78]. In vivo, skin TSLP activates DCs to induce Tfh and Th2 cell responses in AD[68], the latter being activated through an immune cascade where OX40L in DCs induces IL-3 expression by T cells leading to the recruitment of basophils in the lymph nodes (LN), promoting in turn the production of IL-4 by T cells[79].

1.1.4.2.2 IL-33

IL-33 is part of the IL-1 cytokine family and signals through its receptor composed of ST2 (*IL1RL1* gene) and IL-1RacP. Upon binding of IL-33, ST2 undergoes a conformational change leading to the recruitment of IL-1RacP to form a heterodimer which will signals through MyD88 in different cell types, including ILC2, Th2 cells, $ST2^+$ regulatory T (Treg) cells, mast cells, basophils, eosinophils, DCs[80]. IL-33 is expressed and stored in the nucleus of mast cells, endothelial cells and epithelial cells of the intestine, lung and skin at homeostasis and is released upon triggers such as mechanical injury, irritants, allergens and infections to promote type 2 immune response[80,81]. Although tissue damage has been shown to induce IL-33 release, the mechanisms driving this release remain unclear, especially whether IL-33 can be released in absence of cell death[80]. Yamagishi et al. propose that IL-33 can be released in a gasdermin D-dependent mechanism which results in the formation of pores in senescent hepatic cells[82]. IL-33 is commonly recognized as pro-type 2 inflammation. Indeed, IL-33 promotes Th2 cell and ILC2 activation as well as their production of Th2 cytokines in several diseases including helminth infection, allergic asthma and AD[80]. IL-33 can synergize with TSLP, IL-2 or IL-7 to induce ST2 expression through STAT5 and GATA3 which in turn lead to IL-5 / IL-13 expression in Th2 cells[83]. IL-33 induces Th2 cytokine expression in ILC2 through

the phosphorylation of GATA3 by p38[84]. In addition to its pro-inflammatory role, it has been reported that IL-33 can exert a tolerogenic role by inducing $ST2^+$ Tregs in the lung[85] or the intestine[86] which limit inflammation. These $ST2^+$ Tregs also express GATA3 in accordance with the fact that GATA3 induces ST2 expression[83].

A SNP in the distal promoter region of *IL1RL1* gene has been identified as a risk factor for AD[87]. Moreover, IL-33 is highly expressed in keratinocytes in AD lesional skin and can be detected in the blood of AD patients where it correlates with xerosis and excoriation[81,88]. IL-33 and ST2 are overexpressed in AD mouse model induced by epicutaneous ovalbumin (OVA) sensitization or in flaky tail mice[81]. In addition to allergens, staphylococcal enterotoxin B, produced by *S. aureus* which colonizes AD lesions, can induce IL-33 expression in the skin[81]. Its role in AD development has been studied in different models. First, transgenic overexpression of IL-33 in keratinocytes leads to spontaneous AD-like lesions characterized by pruritus, hyperplasia, Th2 cytokine production and eosinophils recruitment which is induced through IL-5[89]. IgE^+ mast cells and the release of histamine is increased in the skin of transgenic mice, as well as $IL\text{-}5^+$ $IL\text{-}13^+$ ILC2 infiltration in the skin. The role of IL-33 was then investigated using *Il33*$^{-/-}$ mice or *Il1rl1*$^{-/-}$ which generated conflicting results. Using MC903-induced AD model, Salimi et al.[90] reported that skin inflammation and ILC2 infiltration were reduced in *Il1rl1*$^{-/-}$ mice. Li et al.[91] next showed that IL-33 signals through ST2 and MyD88 to induce skin thickening and Th2 cytokine production. Contrary to these results, two studies[76,92] reported that skin inflammation and ILC2 recruitment was similar in *Il1rl1*$^{-/-}$, *Il33*$^{-/-}$ and wildtype mice. More studies on IL-33 role in AD should be conducted to elucidate these discrepancies.

1.1.4.2.3 IL-25

IL-25, also called IL-17E, is a member of the IL-17 cytokine family which also includes IL-17A, IL-17B, IL-17C, IL-17D and IL-17F. IL-25 signals through a heterodimer of IL-17RB (IL-25R) and IL-17RA[93]. IL-25 is expressed by immune cells such as eosinophils, basophils, T cells, DC, ILC2 but also by epithelial cells in the lung, skin or gastrointestinal tract[93–97]. Pathogens and allergens such as ragweed pollen and house dust mites (HDM) stimulate IL-25 expression[93,95]. This cytokine was first described as a Th2-promoting factor due to the fact that intraperitoneal injection of IL-25 resulted in eosinophilia, elevated IgE, IgG1 and IgA blood levels, as well as mucus production in the lung and exacerbated Th2 cytokines IL-4, IL-5 and IL-13 production[94].

It has since been shown that IL-25 is also involved in skin diseases such as psoriasis or AD. IL-25 and IL-17RB are expressed in AD lesional skin and to a lesser extent in AD non lesional skin[95,97]. Keratinocytes are one major producer of IL-25 in the skin, which not only acts on immune cells, but also directly on keratinocytes in an autocrine manner[53,96]. IL-25 from keratinocytes stimulates the expression of IL-17RB and IL-25 in keratinocytes creating an inflammatory loop. IL-25 intradermal injection induces acanthosis and parakeratosis through the stimulation of proliferation and cell cycle, as well as promotes immune cell infiltration[96]. IL-25 has also been reported to downregulate proteins involved in skin barrier integrity such as FLG, CDSN or OCLN1[53]. In addition to its role on keratinocytes, IL-25 plays an important role for epidermal hyperplasia and recruitment of $CD4^+$ T cells in allergic skin inflammation[53]. IL-25 activates IL-13 production in ILC2 leading to enhanced $CD4^+$ T cells in the skin due to increased CCL17 and CCL22 levels, two cytokines involved in Th2 cell recruitment. However, IL-25 does not seem to be implicated in the infiltration of eosinophils, mast cells and basophils in the skin, nor IL-4 production. In this model of epicutaneous sensitization with OVA, IL-17RB (and therefore IL-25) was not implicated in ILC2 recruitment but in IL-13 production[53]. On the other hand, in an AD model induced by application of MC903, ILC2 recruitment to the skin was dependent on IL-17RB[90] suggesting that different mechanisms might drive ILC2 recruitment to the skin. In addition to act on innate cells, it has been reported that IL-25 could act directly on T cells and enhances Th2 memory cell activity. Indeed, IL-25 enhances IL-4, IL-13, IL-5 production in Th2 memory cells stimulated by TSLP-activated DCs, as well as their proliferation[95].

1.1.4.3 Immune dysregulation

Acute AD lesions are characterized by a type 2 immune response with recruitment of ILC2, mast cells, eosinophils, basophils, Th2 cells to the skin. With chronicity, the immune response changes to a mixed type 1 / type 17 response[14]. I will focus solely on type 2 immune response here.

ILC2

ILC2 do not express T and B cell receptors but express Sca1, c-Kit, IL-7Rα and ICOS. ILC2 require several transcription factors to develop, including GATA3, RORα, TCF-1 and the transcriptional repressor Id2. These cells are implicated in parasite expulsion but also allergic diseases, including AD. AD lesions exhibit high number of ILC2 in human and mice and they can be recruited upon

allergen application[53,76,90]. ILC2 recruitment to the skin is independent of T and B cells but is mediated by alarmins[76]. These cells express ST2, IL-17RB and TSLPR and can therefore respond directly to TSLP, IL-33 and IL-25 which induce their recruitment to the skin and / or activation to produce Th2 cytokines IL-5, IL-13 and to a lesser extent IL-4, exacerbating AD phenotype[76,90]. Indeed, Kim et al. and Salimi et al. showed that depletion of ILC2 diminished inflammation highlighting these cells as key drivers for AD[76,90]. TSLP signalling has been shown to induce STAT5 phosphorylation resulting in GATA3 expression in ILC2[98], while IL-33 and IL-25 have been shown to induce GATA3 expression through p38[84]. GATA3 binds to *Il13*/*Il5* locus to induce their expression as well as ST2 and IL-17RB expression[83,98]. TSLP, IL-25 and IL-33 can act independently or in synergy to induce IL-13 and IL-5[84,98]. IL-5 is implicated in eosinophil recruitment and survival. IL-13 induces CCL17/CCL22 expression and therefore Th2 cells recruitment, tissue remodelling, pruritus and reduced expression of genes involved in skin barrier function and antimicrobial peptides[53,99].

Eosinophils

Eosinophils are innate immune cells involved in protection against parasite infection. They have also been shown to play a deleterious role inducing tissue damage in allergies, including allergic rhinitis, asthma, and AD[1]. Eosinophil mode of action is through their release of deleterious proteins upon stimuli including activation of pattern recognition receptors (PRR). Eosinophils store their toxic proteins in granules in the cytoplasm. Eosinophil peroxidase (EPO) is stored in primary granules while eosinophil-derived neurotoxin (EDN), major basic protein (MBP), eosinophil cationic protein (ECP) and EPO are localized in secondary granules[100]. Granule proteins can either act on their own (e.g. MBP aggregation inducing keratinocyte death[101]) or through association with DNA. Eosinophils release mitochondrial DNA which bind to ECP to form eosinophil extracellular traps[102].

Skin eosinophilia is a key features of AD lesions in mouse and humans. Chronic and acute human AD lesions are infiltrated by a high number of eosinophils associated to elevated levels of toxic granule proteins[103], which can be associated to blood eosinophilia in some patients[100]. Moreover, eosinophil extracellular traps in close proximity to apoptotic keratinocytes have been identified in skin biopsies from positive allergy patch test (mimicking acute AD flares)[104]. TSLP, which is overexpressed in AD lesions[65], promotes extracellular trap formation by eosinophils[102]. Several mouse models of AD showed eosinophils infiltration to the skin and IL-5 expression, including

MC903-induced AD[71], epicutaneous allergic sensitization[67,68] and IL-33[89] or TSLP[75] transgenic mice. IL-5, mainly produced by ILC2 and Th2 cells in AD skin, induce eosinophil survival through its receptor by blocking DNA cleavage and apoptosis[105]. It also enhances activation and trafficking of eosinophils. Indeed, IL-5 blocking with an antibody inhibits eosinophilia in AD skin[89]. Other factors have been proposed to mediate eosinophils trafficking including the complement molecules C5a and C3a, RANTES (CCL5) and eotaxin 1 (CCL11) which binds to CCR3 on eosinophils and has been shown to be expressed in AD-like lesions in mouse[89] and in the blood of AD patients[106].

Basophils

Basophils were identified in 1879 by Paul Ehrlich and named after their affinity for basic stains. In the blood, they represent less than 1% of all leucocytes and therefore have long been thought to play a minor role due to their rarity[107]. However, several studies showed that this cell population plays a role in type 2 immunity in protection against parasites but also in allergies. Basophil expansion has been identified in response to different parasites including (*Trichinella spiralis*, *Nippostrongylus brasiliensis*) as well as in AD and asthma[107]. Indeed, several mouse models of AD showed increased number of basophils in the skin[67,68], but also in the blood and skin of AD patients[107]. Moreover, depletion of basophils reduced IL-4 production and skin inflammation in AD[108,109] highlighting the role of these cells in AD. Several factors can induce basophils activation including cytokines, IgE, complement molecules and allergens[107]. Basophils arise from bone marrow progenitors under the control of GATA2 and C/EBPα[107]. Cytokines also regulate basophil development and activation. IL-3 orientates the differentiation of granulocyte/monocyte progenitors towards basophil lineage-restricted progenitors through STAT5 signalling, leading to basophils expansion[110]. In addition to expansion, IL-3 can activate basophils. Upon *Nippostrongylus brasiliensis* infection, T cells produce IL-3 inducing the recruitment of basophils to the lung and liver and stimulating their production of IL-4 to contribute to parasite elimination[111]. Moreover, IL-3 can enhance IL-4 production in IgE-activated basophils[112]. In addition, TSLP, a pro-type 2 cytokine, has also been shown to induce basophilia through TSLPR expressed on basophils. Compared to IL-3-elicited basophils, TSLP-elicited basophils produce higher levels of IL-4, IL-6, CCL3, CCL4 and CCL12 after IL-33 or IL-3 stimulation[109]. In AD, both IL-3 and TSLP might contribute to basophil response. Indeed, recruitment of basophils to the skin is abolished in allergen-sensitized *Tslp*$^{-/-}$ mice[67,68]. Moreover, Leyva-Castillo et al. showed that the recruitment of basophils in the LN was dependent on IL-3 production by T cells[79]. Activated

basophils produce IL-4 contributing to Th2 cell priming, ILC2 response in skin[113] and eosinophil recruitment through VCAM-1 expression on endothelial cells[114]. Basophils contribute also to pruritus in AD as they release leukotrienes, histamines and proteases (MCPT8, mMCP11) upon IgE-induced activation[115].

Mast cells

Mast cells are morphologically similar to basophils and also contains granules with lipid mediators (leukotrienes, prostaglandins, platelet activating factor), chemokines, cytokines, proteases and histamine. Contrary to basophils, mast cells express c-Kit. Mast cells are more numerous in AD lesions than in healthy skin in human[116] and mouse[71]. Moreover, skin inflammation is reduced in a model of skin allergic sensitization depleted of mast cells[117]. Indeed, sensitization in AD patients leads to elevated levels of allergen-specific IgE and the most common activation pathway for mast cells is through antigen-IgE crosslinking to its high affinity receptor FcεRI on mast cells, leading to the release of the content of their granules such as lipid mediators, chemokines, cytokines, proteases, histamine contributing to itch and inflammation.

Neutrophils

Neutrophils are the most numerous immune cell type in the blood. They have a short lifespan and can be recruited to tissue upon damage or infection. They are implicated in pathogen elimination and wound healing. These cells can release neutrophil extracellular traps which have been shown to promote allergic asthma[118], or they can degranulate and have a strong phagocytosis capacity. They are recruited to the skin through chemokines produced by epithelial and immune cells such as IL-8 (only in humans), CXCL1, CXCL2, CXCL3, CXCL5. Neutrophils have been shown to promote contact dermatitis in response to haptens[119] and promote Th2 allergic airway inflammation[118]. However, less is known for neutrophils in AD. Neutrophils infiltration has been reported in some AD patients[120]. One study showed that neutrophils promotes itch through CXCL10-CXCR3 pathway in neurons, in a TSLP overexpression mouse model induced by MC903[121].

Dendritic cells

DCs are professional antigen-presenting cells (APC) which are implicated in T cell activation and

polarization. First, DCs assess their environment to detect danger which is then internalized in a vesicle through endocytosis. Then the antigen is processed to be presented on MHCI to $CD8^+$ T cells for endogenous antigens, or MHCII to $CD4^+$ T cells for exogenous antigens. In addition, cross presentation allows exogenous antigens to be loaded on MHCI to be presented to $CD8^+$ T cells. DCs are retained in the skin through cell-cell adhesion. LCs are attached to keratinocytes through E-cadherin. Upon activation DCs upregulate CCR7 to respond and migrate towards CCL19 and CCL21 in LNs. To activate and polarize naive T cells, DCs provide 3 signals: peptide:MHC complex, costimulatory signal and cytokines.

In the skin, mouse DCs are divided into LC which is the only DC population in the epidermis, and dermal dendritic cells (dDC). dDCs are further divided in $Lang^+$ cDC1, $CD11b^+$ cDC2, $CD11b^-$ $CD24^-$ double-negative DCs, monocyte-derived DCs and plasmacytoid DCs[122].

cDC1 express Langerin (CD207), CD11c, XCR1, CD24, CD103, Clec9A, and require IRF8, BATF3, Id2 for their development, as well as Flt3-Flt3L signalling[122]. It is important to note that human cDC1 do not express langerin. cDC1 are involved in Th1 and $CD8^+$ T cell response and have the ability to cross present different type of antigens to $CD8^+$ T cells.

$CD11b^+$ cDC2 express CD11c, SIRPα, CCR2, CD11b, MGL2, and IRF4 which is required for their migration to the LNs. These DCs also require Flt3-Flt3L signalling and PU.1, RelB and RBP-J for their development. $CD11b^+$ cDC2 have been reported to induce Th2 response. Indeed, specific ablation of *Irf4* in DCs lead to reduce Th2 cytokines in response to papain or parasite[123]. Moreover, $CD11b^+$ cDC2 promote HDM-induced lung inflammation with IL-5, IL-13 production, eosinophil recruitment and mucus production[124]. On the other hand, specific ablation of *Irf4* in DC did not impact Th1 immune response to viral infection.

LCs are the only DCs in the epidermis at homeostasis. LCs express CD11b, CD11c, Langerin, SIRPα, and Epcam. Their development does not involve Flt3-Flt3L signalling but requires several transcription factors including PU.1, RUNX3 and Id2 in addition to cytokines IL-34 and TGF-β[6]. IL-34, produced by keratinocytes, is an important factor for development and maintenance of LCs through CSF1R as LCs are absent in *Il34* deficient mice[125]. In addition, TGF-β, produced by keratinocytes and LCs, retains LCs in the epidermis. LCs have been reported to induce Th17 response and Tregs[6]. Indeed, LCs induce resident memory Treg proliferation under steady state[126].

The role of DCs in AD has been suggested by *Rag1*$^{-/-}$ mice as skin inflammation and sensitization was reduced in these mice, indicating an important role for B and T cells and therefore for DCs

which induce their differentiation[67]. Moreover, TSLP, which is highly expressed in AD lesion, induces activation of DCs which promote Th2 cell differentiation[65]. In addition, TSLP has been shown to activate DCs in vivo to induce Th2 cell differentiation through a DC – T – basophil – T cascade implicating OX40L signalling[79]. AD lesions present activated LCs and recruitment of inflammatory dendritic epidermal cells in the epidermis of AD patients, which express high levels of FcεRI[65,127].

LCs, being the first line of DCs in the skin, have attracted a lot of attention, however their role remains controversial in AD and skin sensitization. LCs can uptake antigens by elongating their dendrites through tight junctions of keratinocytes and internalize antigens, such as ovalbumin, by endocytosis through langerin[128]. Tight junctions reorganize around dendrites to prevent permeability, however if impaired, like claudin 1 expression in AD, it could impact both the skin barrier and LC function. Different models showed activation of LCs. First, tape-stripping induces both activation and migration of Langerin (Lang)$^+$ DCs (LCs and cDC1) and Lang$^-$ DCs to the LNs[129]. Moreover, Elentner et al. showed that MC903 induces LC activation with upregulation of CD40, CD86 and CCR7 while Lang$^+$ and Lang$^-$ dDCs remain inactivated. In addition, LCs and Lang$^+$ dDCs migrate to the LN in response to MC903. Depletion of both LCs and Lang$^+$ dDCs reduced Th2 response and skin inflammation highlighting a promoting role for LCs (albeit a role for Lang$^+$ DCs can not be excluded) in AD[130]. Nakajima et al. reported that LCs promote also allergic skin sensitization with OVA through tape-stripped skin as depletion of LCs reduced OVA-specific IgE and IL-4 production[131].

On the other hand, LCs have also been reported to promote proliferation of resident memory Tregs in the skin[126] and to mediate epicutaneous immunotherapy to ovalbumin through the induction of LAP$^+$ Tregs in LNs and spleen[132]. Moreover, Deckers et al. showed that Th2 response following HDM skin sensitization (on intact skin) and intranasal challenge is reduced in *Flt3l*$^{-/-}$ mice (absence of all cDCs) or in mice missing IRF4 in DCs (deficiency of cDC2)[133]. The depletion of LC leads to an increase in IL-5 and IL-13 production suggesting a tolerogenic role for LC while cDC2 promote Th2 response. In agreement with this study, we showed that LCs exert a tolerogenic role during OVA skin sensitization by inhibiting not only Th2 but also Tfh cell response and that LCs and Lang$^+$ dDCs remain inactivated in LNs, while Lang$^-$ dDCs are in an activated state[68]. One difference between our study and the one of Nakajima is the delivery of the allergen (patch or not), and the mouse background (C57BL/6 vs Balb/c) which might explain these discrepancies about the role of LCs. Moreover, we showed that the role of LCs in AD is dependent on the context. Indeed, while

LCs are tolerogenic during allergic skin sensitization, we showed that LCs promote Tfh cell response in TSLP overexpression model induced by MC903 which is characterized by high level of TSLP compared to OVA-induced AD. Therefore, the skin microenvironment might be one factor switching LC function from tolerance to a promoting role.

Th2 cells

Th2 cell differentiation is dependent on activation by DC and IL-4 cytokine in the microenvironment. IL-4 signalling activates STAT6 leading to GATA3 expression which is the key transcription factor of Th2 cells. GATA3, in coordination with STAT5, activate Th2 cell program with transcription of Th2 cytokine locus[134]. AD skin lesions present increased number of Th2 cells which produce IL-4, IL-13 and IL-5 contributing to inflammation and pruritus. Indeed, skin inflammation is reduced, and particularly eosinophil and basophil recruitment as well as IgE production in T cell-deficient mice sensitized to ovalbumin[67,135]. TSLP induces Th2 cells and Th2 cytokine production in AD and their expression of CCR4 which is the receptor for CCL17[136]. After activation in the LNs, Th2 cells are recruited to skin lesions through CCL17 and CCL22 produced by DC[65]. Both CCL17 and CCL22 levels are elevated in serum of AD patients and correlated with disease severity[137,138]. Th2 cells have also been shown to express IL-31 which signals through IL-31RA in sensory nerves to induce pruritus and nerve growth in the skin[139,140].

Tfh cells and B cells

B cells and Tfh cells coordinate humoral responses to produce antibodies. Tfh cells are involved in GC formation where they provide critical help to antigen-specific B cells, promote the selection of high-affinity B cells and class switching to IgE. As previously stated, IgE contributes to AD through FcεRI crosslinking on basophils and mast cells to induce degranulation. AD patients often present sensitization to one or several allergens[13]. Szabo et al. reported that circulating Tfh cells are dysregulated in AD children and that their frequency correlates with AD severity[141]. We showed recently that TSLP induces Tfh and GC B cells during AD in mice[68]. Tfh cell differentiation is initiated by DCs in LNs, however the DC population involved seems to be context-dependent. Indeed, we found that LCs promote Tfh cell differentiation in a model of TSLP overexpression induced by MC903, whereas these cells inhibit Tfh cell response in OVA-induced AD, suggesting a differential role for LCs depending on the context[68]. In addition to Tfh cells, regulatory B (Breg) cells have been

reported to be reduced in extrinsic AD patients but not in intrinsic patients and negatively correlates with Tfh cells, in adequation that extrinsic AD patients present high levels of IgE[142]. Bregs have different modes of actions to exert their suppressive functions, including through contact or secretion of soluble factors (IL-35, TGF-β, IL-10)[143]. IL-10 produced by Bregs can induce Tregs or inhibit effector T cells and DC maturation. BCR engagement in B cells is crucial for Breg development and their production of IL-10. Another pathway implicated in IL-10 production by B cells is TLR and CD40-STAT3 activation, which has been reported to be dysregulated in Bregs of AD patients[142].

1.1.5 Treatments

AD treatments vary depending on severity (mild, moderate, severe). The first line of treatment is topical treatments, followed by systemic or even biological treatments if AD is uncontrolled and patients do not respond to the previous drugs.

The first recommendation for AD is therapeutic education by practitioners. Several educational programs showed improvement in the "educated" groups compared to the control groups[144–146] suggesting that all patients (and the parents for children) should be educated to understand their disease and therefore improve quality of life. Answering patients' questions and education about knowledge of the disease, psychological and social burden, skin care, nutrition, adherence to treatment and triggers should be implemented. Among the triggers, avoidance of allergens should be one focus as AD children are often sensitized to different allergens, which encounters can exacerbate their lesions.

Topical treatments

Moisturizers. AD skin is characterized by xerosis and skin barrier impairment, therefore to restore the skin barrier and maintain hydration, the first line of treatment relies on the use of moisturizers[147], containing humectants (urea or glycerol, for hydration) and occludents (preventing TWEL and evaporation). The formula should not contain irritants (such as fragrance) or present potential allergens. The usual application is 2 or 3 times a day, which can put a financial burden on patients, subsequently limiting their use.

Topical corticosteroids. When moisturizers are not sufficient to control AD, different class of

topical corticosteroids (TCS) can be prescribed in children or adults, depending on AD severity. TCS are classified from group I, corresponding to mild potency, to high potency group IV TCS[147] (which is inversed in the US: group I: high potency, group VII: low). Class II TCS are recommended in children or adults with mild AD, whereas class II or III TCS are recommended in moderate AD[147], however patient age, disease activity and body areas need to be taken into consideration when choosing the correct TCS. If used correctly, TCS have few side-effects apart from skin atrophy, however respecting the correct dosage for each body site and monitoring side-effect appearance is important.

Calcineurin inhibitors. Two different calcineurin inhibitors are used in clinic: tacrolimus and pimecrolimus. Tacrolimus is available as an 0,03% or 0,1% ointment, whereas pimecrolimus is a 1% cream[148]. Several studies showed that patients treated with tacrolimus twice daily present AD improvement with decrease pruritus and symptoms (oedema, erythema…) compared to the control group[149–151]. The main side-effects are a sensation of skin burning and pruritus, which resolve after a few days. The molecular mechanism of action is well described. Calcineurin is a calcium-dependent protein phosphatase implicated in T cell activation. Upon TCR binding on T cells, calcineurin is activated and dephosphorylates NFAT, which migrates into the nucleus to act as a transcription factor, leading to IL-2 production and T cell activation. Tacrolimus and pimecrolimus have been shown to bind to the protein FKBP12 in the cytosol to form a complex which will in return inhibits calcineurin[152,153], abolishing IL-2 production and T cell activation, acting therefore as immunosuppressive agents[148].

Phophodiesterase 4 inhibitors. Crisaborole is an 2% ointment approved for mild to moderate AD in the US. This drug is no longer authorized in the EU after the company asked for withdrawal of the marketing authorization.

Phototherapy

Phototherapy can be used in moderate to severe AD. It mainly uses NB-UVB (narrow-band UVB 311-313nm) or UVA1 (340-400nm)[147]. Phototherapy is commonly used in association with topical treatments (TCS and moisturizers). Treatment is 3 to 5 times a week and lasts 4 to 8 weeks in general, which can be prohibitive for some patients.

Systemic treatments

If topical treatments or phototherapy are not effective for severe AD patients, systemic immunosuppressive drugs can be considered.

Cyclosporin A is considered the first line of treatment in severe patients requiring systemic treatment[154]. However, patients need to be monitored during treatment as this drug can induce renal toxicity. It is an immunosuppressive drug with a similar mechanism of action as tacrolimus. It binds to cyclophilin to form a complex which will inhibit calcineurin and block subsequently the production of cytokines and T cell activation[152,153].

Other systemic drugs consist of azathioprine, which is a purin analog inhibiting DNA production and therefore T and B cell proliferation, while methotrexate and mycophenolate mofetil block purine synthesis[155]. Oral corticosteroids can be used but are usually restricted to short-time treatment of flares due to their side-effects.

JAK1/2 inhibitors. Many cytokines involved in AD pathogenesis signal through JAK/STAT after binding to their receptors. Among them are TSLP, IL-4, IL-5, IL-13 and IL-31. After activation, JAK phosphorylates STAT to induce the transcription of different genes implicated in inflammation[156]. Several drugs have been developed to inhibit JAK1/2 and therefore inflammation and AD. Baricitinib, which inhibits both JAK1 and JAK2, has been approved by the European medicines agency (EMA) in adult patients with moderate to severe AD. More recently, the EMA and the Food and Drug Administration (FDA) approved abrocitinib, an inhibitor of JAK1.

Biological treatments

Dupilumab. Dupilumab (Dupixent) is the first human monoclonal antibody approved for AD. Dupilumab targets IL-4Rα, the shared subunit of IL-4 and IL-13 receptors, inhibiting both IL-4 and IL-13 signaling and therefore limiting skin inflammation. Dupilumab has been approved for adults with moderate to severe AD in March 2017 by the FDA followed by the EMA in September. It has since also been approved for children > 6 years old (2020) and more recently for 6-months to 5-years old children (June 2022 for FDA – under review by EMA). Dupilumab can be used in association with TCS. Two randomized, placebo-controlled phase 3 trials[157], SOLO 1 and SOLO 2, enrolling 671 and 708 moderate to severe AD patients respectively, showed that 37% of participants treated with 300mg of dupilumab weekly or every other week (after an initial dose of

600mg for both groups) reached the primary end point (IGA score = 0 (clear) or 1 (almost clear)), compared to 10% in the placebo group. No difference was observed between weekly or every other week treatment groups. The efficacy of dupilumab associated to medium-potency TCS was investigated in the LIBERTY AD CHRONOS phase 3 trial[158]. 39% of patients treated with dupilumab (every week or every other week) and TCS reached an IGA score of 0 or 1 at week 16, compared to 12% in the placebo and TCS control group. These results were similar overtime at week 52. Conjunctivitis incidence was higher in the dupilumab and TCS groups.

Tralokinumab. While targeting the IL-4/IL-13 axis has shown great improvement for AD, several studies showed that IL-13 might have the preponderant role in AD. IL-13 is expressed in AD lesions at higher levels than IL-4 and is correlated to disease severity. IL-13 induces fibrosis, immune cell infiltration in the skin, as well as reduced expression of genes involved in barrier integrity. Therefore, this cytokine has been selected as a target for blocking with tralokinumab, an anti-IL-13 human monoclonal antibody. ECZTRA 1 and ECZTRA 2 phase III clinical trials[159] tested the efficacy of tralokinumab in 802 and 794 adult patients. Patients were treated with an original injection of 600mg/ml, followed by 300mg/ml injection every other week for 16 weeks. The percentage of patients reaching IGA 0 or 1 in tralokinumab-treated group is higher than in placebo-treated group at week 16. Two other clinical trials (ECZTRA 3[160] and ECZTRA 7[161]) tested the efficacy of the combination of tralokinumab and TCS compared to TCS alone and showed that 56% and 88% of the patients treated with the drug combination achieved EASI 75 at week 16, compared to 35% and 69% in the TCS only groups. The association of tralokinumab with TCS was well tolerated. Tralokinumab has since been approved for moderate to severe AD in adults by the EMA and the FDA in 2021.

Several other antibodies are in development with different targets. Among them are IL-36R (spesolimab, phase IIa), IgE (omalizumab), IL-13 (lebrikizumab, phase III,) and TSLP antibodies (tezepelumab, phase IIb)[162]. Tezepelumab is an anti-TSLP approved for asthma treatment. A phase IIb clinical trial (NCT03809663) to test its efficacy in AD patients was terminated because this antibody did not reach the targeted efficacy. No further trials are ongoing. As AD patients are often sensitized to different allergens and present high levels of IgE in the blood, therefore targeting IgE with omalizumab was tested. However, clinical trials with omalizumab present conflicting results about its efficacy[154]. On the other hand, lebrikizumab, another IL-13 antibody, is more promising and entered phase III clinical trials (NCT04146363, NCT04178967), after succession of phase II. Several other antibodies are in development, which could represent future

therapies for moderate and severe AD patients[162].

1.2 Asthma

1.2.1 Overview of the airway

The respiratory tract is divided in upper airways and lower airways. The upper airways are further divided in nasal cavity, pharynx (nasopharynx, oropharynx, laryngopharynx) and larynx. The lower airways include the trachea, the bronchi, the bronchioles which terminate by alveoli, the site of gas exchange in the lungs[163]. The human right lung is composed of 3 lobes (superior, middle, inferior) while the left is only composed of two (superior and inferior). They are covered by the visceral pleura and are separated from the parietal pleura, which is on the inner surface of the chest cavity, by the pleural space.

The conducting airways include the trachea and bronchi, which are surrounded by by C-shaped rings of cartilage, and the bronchioles. It is a pseudostratified epithelium composed of basal cells, ensuring regeneration, ciliated cells, and secretory cells. Among secretory cells, goblet cells and submucous glands produce mucins. MUC5AC and MUC5B are secreted to form the mucus gel which is pushed towards the glottis by the coordinated movement of the ciliated cells. The mucus entraps pathogens to allow their clearance by the mucociliary elevator. Other cells such as tuft cells, neuroendocrine cells, ionocytes, club cells also line the airway epithelium[163].

The bronchioles give rise to the alveoli. The alveolar epithelium is mainly comprised of type I pneumocytes which are in close proximity to capillaries, allowing gas exchange, and type II pneumocytes secreting surfactant proteins and lipids to reduce surface tension during expiration[163].

All these cells, in addition to smooth muscle cells and immune cells maintain barrier integrity and lung function, which can be altered upon external or internal aggressions, such as pollutants, smoke, allergens, viruses, bacteria, resulting in several diseases.

1.2.2 Epidemiology

Asthma is a chronic airway inflammatory disease which affected approximatively 260 million

people in 2019 and caused 455 000 deaths worldwide from WHO estimates. Although asthma prevalence is increasing worldwide, its prevalence is heterogenous between the countries, with 7% of 6-7 years old children affected in the Indian region, to 10% in Africa to more than 20% in Oceania[9]. These numbers raise asthma as the most common chronic disease in children. During childhood, asthma is more common in young boys than girls, which reverses during adolescence to become[164] equal or more frequent in girls than boys starting around 13-14 years old[9]. Asthma usually starts during childhood. However, it can also affect adults due to persistent asthma or adult onset, which is suggested to be more severe than childhood asthma, albeit the prevalence of severe asthma in adults and children varies considerably among the studies[165,166]. Severe asthma is defined as impossibility to achieve disease control albeit the use of corticosteroids.

1.2.3 Clinical features

Asthma is characterized by airway hyperresponsiveness (AHR), bronchial obstruction, shortness of breath, chest tightness, wheezing and coughing. Symptoms can vary in intensity and frequency and be absent during several weeks or months while patients can also exhibit flare-ups.

Asthma is often associated with allergen-sensitization exhibited by high allergen-specific IgE levels[167]. Allergic asthma patients exhibit a history of atopy and are often affected by other atopic diseases such as AD, food allergies, rhinitis or conjunctivitis[9] which are risk factors for asthma development. Among other comorbidities of asthma, we can find obesity, chronic obstructive pulmonary disease, gastro-esophageal reflux, airway infections and mental health problems (panic attacks, stress) which negatively impact the quality of life of asthma patients.

From a histological point of view, asthma is characterized by goblet cell hyperplasia, fibrosis, hypertrophy of muscle cells and immune cell infiltration.

Diagnosis. No standardized procedures exist to diagnose asthma however different tests are used to identify asthma. Physical examination as well as an overview of the patient's history and his family are the first components of diagnosis. An history of atopy can be one indicating factor. Next, several tests can be performed: spirometry test to measure airflow obstruction[168]. If constriction is apparent with spirometry, results need to be confirmed with a broncho-dilatator reversibility test to see whether airway obstruction return to normal after treatment with a broncho-dilatator as usually seen in asthma patients. A negative spirometry test does not necessarily mean a negative diagnosis for asthma, as this test can present normal results during a stable asthma

disease course. Therefore, physicians can perform an AHR test with a bronchoconstrictor (methacholine, allergens, histamine) to confirm the diagnosis[168]. Asthma patients should present bronchoconstriction upon stimulation. Bronchoconstriction can also be assessed following exercise instead of bronchoconstrictor delivery. In addition to lung function tests, a FeNO test can be used to support diagnosis as it measures the exhaled nitric oxide, a biomarker for airway inflammation. Smoking and corticosteroids are known to reduce FeNO[169], therefore low levels of FeNO do not exclude asthma.

1.2.4 Pathogenesis

Asthma is a heterogenous disease which was subdivided depending on the different phenotypes: age of onset, triggers, comorbidities. It has since been classified as type 2 $(T2)^{high}$ endotype with eosinophil infiltration in the respiratory tract or $T2^{low}$ endotype which is characterized by type 1 / type 17 response with neutrophils[170]. My focus here will be the $T2^{high}$ endotype which includes allergic and non-allergic asthma. The allergic phenotype is characterized by early-onset and sensitization with high IgE levels in the blood.

Asthma is a multifactorial disease which includes genetic, immune and environmental factors. Other atopic diseases are a risk factor for asthma. Indeed, children with AD or food allergy have an increased risk to develop asthma[13]. It has been showed that sensitization can occur through AD skin and that food sensitization increases the risk to develop sensitization to inhaled allergens such as house dust mites, pollens, mold, animal dander, cockroach etc. In addition, active and passive smoking, viral infections and air pollution (ozone, nitrogen dioxide, particles) can exacerbate inflammation through oxidative stress and tissue damage[171]. In addition to environmental factors, several genes involved in immunity, skin barrier function and airway remodelling, have been associated with a risk to develop asthma, including *IL4*, *IL4Rα*, *FLG*, *ADAM33*, *HLA-DQ*, *IL33*[80], *TSLP*[64], *IL1RL1*, *ORMDL3*[171].

Asthma is commonly characterized by inflammatory infiltrates in the airways of both innate and adaptive immune cells, the latter being specific of the antigen. The epithelium is an important protective barrier, preventing entry of immune triggers such as viruses, bacteria, allergens, ozone, smoke, pollutants etc[171]. Epithelial cells play a key role in asthma by maintaining the barrier function and assessing the environment with a variety of PRR to detect danger-associated (DAMP) and pathogen-associated molecular patterns (PAMP), including all TLRs located at the plasma

membrane or in endosomes, RIG-I-like receptors which detect self or exogenous RNA, and NOD-like receptors. Activation of these receptors results in the production of different chemokines and cytokines such as TSLP, IL-25 and IL-33 in response to different stimuli (allergens, epithelial damages or pollutants). Several allergens have protease activity, such as HDM, which can cleave tight junctions, stimulate protease-activated receptors or activate TLR4, inducing the production of TSLP, IL-33 and IL-25[171]. Diesel exhausted particles activate Aryl hydrocarbon Receptor (AhR) to translocate to the nucleus to bind *IL33*, *TSLP* and *IL25* promoter leading to their expression in epithelial cells[172]. These cytokines and ST2 are elevated in asthmatic patients and correlate to disease severity[64,95,171]. Moreover, polymorphisms in *IL25*, *IL33* and *TSLP* genes are associated with asthma[64,171]. They initiate type 2 response in asthma by activating DCs (with upregulation of co-stimulatory molecules) which then migrate to the LNs to present the complex peptide:MHCII to naive $CD4^+$ T cells, leading to their differentiation. DCs are required for Th2 cell response and mice depleted of DCs fail to develop airway inflammation[173]. More precisely, it has been shown that $IRF4^+$ $CD11b^+$ DCs are required for Th2 and Tfh cell differentiation in LNs following allergen sensitization[174,175]. While Th2 cells contribute to allergic inflammation through IL-5, IL-13 and IL-4, Tfh cells produce IL-21 and IL-4 contributing to B cell class switching and IgG1 / IgE production which can then bind to basophils and mast cell to induce their degranulation or to DCs to participate to antigen-presentation[176].

In addition to promote adaptive immunity, TSLP, IL-25 and IL-33 act on ILC2 to produce IL-5 and IL-13, the latter promoting fibrosis, mucus production, and airway hyperresponsiveness[177,178]. In addition, ILC2 can also target DCs to promote Th2 cell response. In a model of papain-allergic inflammation, it was shown that ILC2, in response to IL-33, produce IL-13 which induces the upregulation of prostaglandin E receptor 4 (EP4) on DCs[179]. Prostaglandin E2 signals through EP4 to increase the sensitivity of DCs to CCL21 and therefore their migration towards the LNs[179]. ILC2 also induce CCL17 and CCL22 expression by DCs promoting Th2 cell recruitment in the lung[180]. In addition to their role on Th2 cell response, ILC2 are also implicated in Tfh cell development, which provide help to B cell to differentiate in memory B cells or plasma cells, and therefore in immunoglobulin production[181]. Indeed, in peanut allergy, IL-13 produced by ILC2 induces Tfh cell differentiation through DCs, leading to IgE production.

IL-5, produced by ILC2 and Th2 cells, mediates eosinophil differentiation, survival and activation[176]. Their infiltration to the lung is mediated by IL-5, eotaxin 1 (CCL11) and eotaxin 3 (CCL24) produced by epithelial and endothelial cells[182]. Activation of eosinophils induce their degranulation,

releasing MBP, EPO, EDN, ECP and lipid mediators (leukotrienes) leading to tissue damage and remodeling. In addition, MBP and leukotrienes induce bronchoconstriction[182].

Mast cells are key players in allergic inflammation. It has been reported that they infiltrate airway smooth muscle of asthmatic patients promoting airway hyperresponsiveness through the release of IL-9 and other mediators[183]. Mast cells, as well as basophils, can crosslink IgE produced by B cells through FcεRI leading to degranulation upon allergen recognition[176]. They can also be activated independently of IgE, through TSLP or IL-33[64,80,109]. The granules of basophils and mast cells contains histamine, prostaglandins (PGD2) and leukotrienes inducing damage to the tissue and bronchoconstriction. In addition, basophils produce IL-4 contributing to Th2 polarization[79].

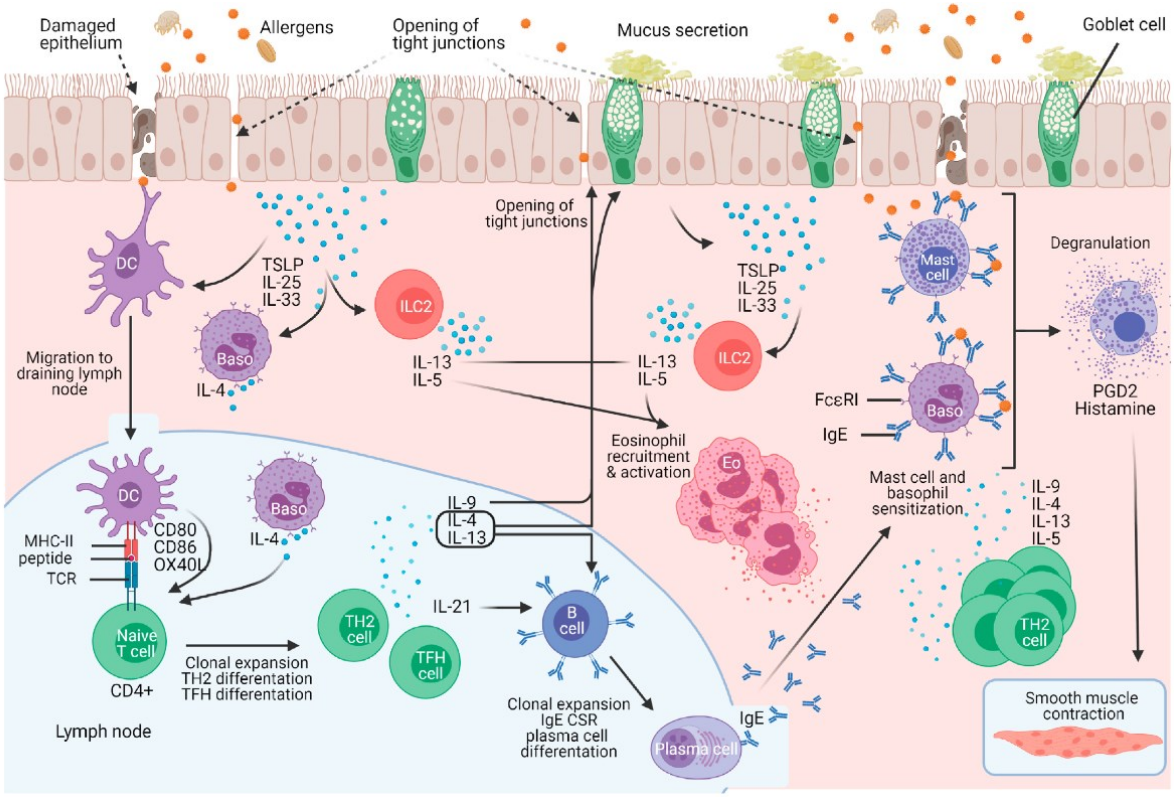

Figure 3. Pathophysiology of type 2 asthma. Upon aggression by external stimuli (allergens), the epithelial barrier releases proinflammatory cytokines TSLP, IL-33 and IL-25 which will activate DCs, ILC2, basophils and mast cells. DCs will migrate to the lymph nodes to activate naïve T cells and induce their differentiation towards a Th2 phenotype through antigen presentation, costimulatory signals and cytokine production. DCs will also polarize naïve T cells towards Tfh lineage which will help B cells for affinity maturation (class switch recombination, somatic hypermutation and selection), plasma cell and memory cell differentiation in germinal centres. IgE produced by plasma cells can crosslink FcεRI on mast cells and basophils, which will induce their degranulation upon binding with the allergen, leading to tissue damages and bronchoconstriction. ILC2 and Th2 cells recruited to the lung will release IL-5, IL-13 and / or IL-4. IL-5 recruits eosinophils in the airways. IL-13 and / or IL-4 induce tissue damages, mucus production and bronchoconstriction. DC: dendritic cell. Tfh: follicular helper T. CSR: class switch recombination. Eo: eosinophil. Baso: basophil. PGD2: prostaglandin D2. From Komlosi et al.[171]

1.2.5 Treatments

Asthma severity is defined according on the treatments that are required to achieve asthma control. The goal of asthma treatment is to achieve symptom control and to reduce the risk of exacerbation. The treatment used depends on asthma control and needs to be re-assessed frequently. Treatments are divided in controllers, which are used daily and on the long term to achieve control, and the relievers which are used when needed to quickly relieve bronchoconstriction. The standard medication are low-to-high dose of inhaled corticosteroids and long-acting β-agonists[184]. If symptoms are not relieved with this medication, long-acting muscarinic antagonists, leukotriene receptor antagonists or theophylline can be used, as well as biological agents[185]. The main relievers are short-acting β-agonists.

Biologic treatment includes omalizumab, reslizumab, benralizumab, dupilumab and Tezepelumab[186]. Omalizumab (humanized anti-IgE) was approved in 2003 for severe and persistent asthma with high IgE levels. IgE are produced by B cells and bind FcRI and FcRII on different cell types, including basophils and mast cells to induce degranulation. Targeting IgE in allergic asthma reduced the number of patients presenting asthma exacerbations compared to the control group in a phase III clinical trial involving 525 people[187]. Three humanized antibodies targeting IL-5 axis were developed: mepolizumab (approved in 2015) and reslizumab (approved in 2016) binds to IL-5 whereas benralizumab (approved in 2017) is an anti-IL-5Rα which is IL-5 receptor. IL-5 is a key cytokine for eosinophil survival[105], therefore blocking it prevents eosinophilia, supporting the use of these antibodies in eosinophilic asthma. Dupilumab, an anti-IL-4Rα, has been approved in 2018 to limit Th2 inflammation[186]. In December 2021, FDA approved for adults and children >12 years old an anti-TSLP, Tezepelumab, which showed that patients had fewer asthma exacerbations when treated with tezepelumab, compared to the placebo group[188].

In case of mild to moderate allergic asthma, allergen-specific immunotherapy is an option after confirming allergen sensitization[189]. The goal is to induce immunological tolerance by delivering increasing doses of allergens to the patient to decrease AHR in response to the targeted allergen.

1.3 Atopic march

Atopic diseases include AD, food allergy, allergic rhinitis and asthma. They are characterized by a type 2 immune response with high IgE levels. The atopic march is the sequential evolution from one atopic disease to another one (Figure 4). It usually starts with AD in infants to progress later to food allergy, allergic rhinitis and / or asthma in children. Atopy is the predisposition for one individual to develop IgE in response to allergens. It is considered the link between all atopic diseases however the course of the atopic march is also influenced by environmental and genetic factors. Several longitudinal studies support the concept of atopic march, which have been reinforced by mouse studies.

Figure 4. Atopic march. Evolution of atopic dermatitis, food allergy, asthma and rhinitis over the years. From Barnetson et Rogers[190]

1.3.1 Longitudinal studies

1.3.1.1 AD and food allergy

Several studies show that AD is a risk factor for food sensitization and food allergy, which is also impacted by AD severity. In a US national survey involving more than 70 000 children below 18 years old, 15.1% of children with AD have food allergies, compared to 3.6% in the non-AD population[15]. Moreover, AD severity is associated with food allergy presence: 27% of severe AD have food allergy opposed to 14.1% for mild/moderate AD children. AD severity is not only associated with food allergy but also with its severity. Among the children with severe AD which have food allergy, 48.6% present severe food allergy, whereas only 23.3% of mild-moderate AD

patients develop severe food allergy.

These results have been confirmed by other studies. In a population-based cohort of 4 453 children (HealthNuts study, Australia), Martin et al. showed that 21.5% of children with AD develop food allergy whereas 4.1% of healthy children develop food allergy (odds ratio, OR=6.2)[191]. Moreover, the most common food allergen is egg followed by peanut, although cow's milk was not tested as an allergen. Moreover, food sensitization, assessed by skin prick test, is not only associated with AD (OR= 6.18) at 3 months old but also with AD severity, with an OR= 3.9 for SCORAD <20 and OR= 25 when SCORAD>20 (corresponding to severe AD)[192].

In addition to AD severity, onset and persistence of AD are also risk factors for food allergy. The T-CHILD study in Japan showed that having eczema during the first year of life increases 3 times the risk to have food allergy at 3 years old[193]. Moreover, early-onset AD increases the risk to develop food allergy as the odds ratio to develop food allergy at 3 years old for children with AD at 1 or 2 months old is 7, whereas OR= 2 when AD starts between 5 and 12 months.

In a high-risk birth cohort of 373 children, 6.1% of children present late-onset AD (after 2 years old). 16.6% of children had early-onset AD (before 2 years old), of which 58% did not exhibit AD at 7 years old (non-persistent AD), while 41.9% still presented AD at this age (persistent AD). Early-onset AD was associated with sensitization to food allergens at 2 years old, independently of persistence of AD[194]. Even though food sensitization was observed, at 2 years old early-onset AD was not associated with food allergy. However, at 7 years old, early-onset persistent AD was associated with food allergy (OR= 13.4). On the contrary, late onset AD was not associated with food allergy.

The Canadian Healthy Infant Longitudinal Development Study assessed 2 311 children for allergic sensitization and atopic diseases[195]. Children were considered sensitized if they exhibited a positive skin prick test to one out of 10 allergens. Without considering sensitization status, AD at 1 year old increased the risk of food allergy at 3 years old (OR= 4.61), as observed in previous studies. However, risks are varying greatly depending on sensitization and AD status. Indeed, children with AD but not sensitized present an OR of 2.5, whereas sensitized AD children have an OR of 33.79. Sensitized children without AD exhibit an OR of 13.76. These results indicate that while AD is a risk factor, the main risk is coming from sensitization.

The main allergens found in longitudinal studies for food allergens are egg followed by cow's milk[192,194], as in Japan where 50% and 15% of allergic children are sensitized to these allergens

respectively[193]. Overtime, the percentage of egg or milk-sensitized children decreases while the percentage of children with peanut sensitization increases[196].

1.3.1.2 AD and allergic rhinitis / asthma

In addition to be a risk factor for food allergy, AD is also a risk factor for asthma and allergic rhinitis (AR) which is an inflammation of the nasal mucosa. Silverberg et al.[15] showed in a UK national survey in 2007 that 19.8% of AD children presented asthma symptoms in the last year, compared to 7.9% of non-AD children. Allergic rhinitis was also associated with AD with 34.4% of AD children exhibiting AR, compared to 14.3% of healthy children. These results were supported by other studies. Tran et al.[13] (Canada) found that AD at 1 year old increases 2-fold the risk to develop asthma and 4-fold for AR, while Von Kobyletski et al.[197] (Sweden) reported an OR of 3.07 for asthma and 2.63 for AR.

AD severity is also a risk factor for asthma and AR. Silverberg et al[15]. showed that AD severity was impacting asthma prevalence with 32.2% of severe AD children having asthma, while only 19% for mild or moderate AD patients. However, in this study, AD severity did not increase AR incidence. On the other hand, Von Kobyletski et al.[197] reported that AD severity was a risk factor for both asthma and AR. In addition to impact asthma and AR incidence, AD severity also increases the odds for asthma and AR severity[15]. Among the children with severe AD which have asthma, 36.1% present severe asthma, whereas only 5.5% of mild-moderate AD patients develop severe asthma. Severe AR is present in 29.1% of severe AD children with AR, compared to 4.6% of mild-moderate AD patients.

The time of AD development in infants has also been reported to be a risk factor for asthma and AR. Von Kobyletski et al.[197] showed that children developing AD before 1 had a 3-fold risk to have asthma or AR, whereas this risk became null if AD occurs after 1. In a high-risk cohort, Carlsten et al.[194] showed that AD developed before 2 (early-onset) was not associated with AR and asthma at 2 years old, but was associated with asthma at 7, especially if AD was persistent (OR=7.48). Only early-onset persistent AD was associated with AR at 7 years old, whereas non-persistent early-onset AD was not associated with an elevated risk for AR. Late-onset AD was not associated with asthma, nor with AR.

In addition, sensitization to food allergens occurs earlier than sensitization to inhaled allergens. It has been shown that food sensitization, especially multiple food sensitization, is a risk factor for

inhalant allergen sensitization[196,198]. Sensitization is another risk factor for AR and asthma. Tran et al.[13] showed that AD but also allergic sensitization at 1 year old, if taken independently, are associated with asthma and AR development at 3 years old. Sensitized AD children present a higher risk of asthma (OR= 7.04) than non-sensitized non-AD patients. The risk is lower for sensitized non-AD children (OR= 2.87) and absent in non-sensitized AD patients (OR=0.46). Concerning AR, sensitized AD children present an OR of 11.75, sensitized non-AD children have an OR of 5.35 and non-sensitized AD patients exhibit an OR of 4.53. AD associated with sensitization is therefore a strong risk factor for AR and asthma.

1.3.2 Mouse studies

The molecular and cellular mechanisms driving the atopic march are still incompletely understood. It has been proposed that sensitization to allergens could occur through the defective skin barrier of AD patients which is associated to a pro-inflammatory microenvironment prone to sensitization. Sensitization results in allergen-specific memory B and T cells, which can lead to the symptoms upon the reencounter with the same allergen (challenge phase) in the airways (for asthma) or gastrointestinal trat (for food allergy). Several mouse studies support skin allergic sensitization and the atopic march.

First, it has been shown that intraperitoneal injection of OVA/alum leads to allergic sensitization (induction of IgE), as well as asthma after intranasal challenge. Skin TSLP, produced by keratinocytes in AD lesions, exacerbates sensitization and the asthmatic phenotype[199,200].

Intraperitoneal sensitization being artificial, mouse model with allergic sensitization occurring through barrier defective skin have been developed to mimic more closely the atopic march. Allergen delivery on barrier-defective skin induced by tape-stripping or laser microporation leads to sensitization with elevated levels of IgE and Tfh - Th2 responses[67,68]. After intranasal OVA challenge, contrary to non-sensitized mice, OVA-sensitized mice develop asthma with recruitment of eosinophils and basophils, mucus and Th2 cytokine production, indicating that skin sensitization predisposes to airway inflammation. It has been shown that airway inflammation is dependent on antigen-specific T and B cell responses. Moreover, as previously stated, skin TSLP is required for sensitization as Tfh and Th2 responses are abolished in *Tslp*$^{-/-}$ mice[68]. In adequation with this, TSLP ablation in keratinocytes also abolishes the asthmatic phenotype after challenge[67]. Moreover, TSLP overexpression induced by MC903 or intradermal injection of TSLP promotes asthma[67,201].

Overall, this highlights the key role of TSLP not only for sensitization but also for the atopic march. TSLP acts on Lang$^-$ DCs to promote Tfh and Th2 cells response[68], however which cells are the main contributors to the atopic march remains to be investigated.

Cutaneous sensitization also predisposes to food allergies[202]. In addition to promote asthma, skin TSLP has also been shown to promote food allergy. Two studies[203,204] showed that overexpression of TSLP in skin during cutaneous OVA sensitization led to high IgE levels, Th2 immune response, mast cell infiltration in the intestine and diarrhea, a key feature of food allergy, following intragastric OVA challenge. Moreover, it has been shown that while TSLP is crucial during the sensitization phase, TSLP is dispensable during challenge. Diarrhea was dependent on T cell response induced by TSLP-expressing DCs. Moreover, it has been suggested that TSLP could act directly on basophils to drive food allergy as their depletion resulted in diminished gastrointestinal inflammation and the transfer of TSLP-elicited basophils promotes food allergy[203].

In addition to TSLP, Han et al. showed that IL-33 also promotes cutaneous sensitization, asthma and food allergy[205–207]. In contrary to TSLP, IL-33 is not only important during sensitization but also during intragastric challenge. IL-33-ST2 signaling enhances mast cell degranulation leading to anaphylaxis.

2 Follicular helper T cells

Follicular helper T cells are a subpopulation of CD4$^+$ T cells expressing Bcl6, PD-1, CXCR5 and ICOS. They are implicated in GC formation and humoral response by promoting survival and differentiation of B cells and providing help for affinity maturation[208]. Therefore, Tfh cells are mainly located in secondary lymphoid organs, even though circulating Tfh cells have also been reported[209].

2.1 Tfh cell differentiation

Tfh cell differentiation is a multistep process starting in the T cell zone to carry on in the B cell zone (Figure 5). APCs mediate Tfh cell differentiation through different interactions and signals. The first APCs to be involved are DCs which interact with naive T cells to initiate Tfh cell differentiation

which is then mediated through T – B interactions in the B cell zone, giving rise to GC Tfh cells.

The differentiation program of Tfh cells involves different cytokines, proteins and transcription factors which have been extensively reviewed[210,211]. Among the main transcription factors, we can find Bcl6 (which inhibits Blimp-1), STAT3, TCF-1, LEF-1, ASCL2, c-Maf, Batf and IRF4.

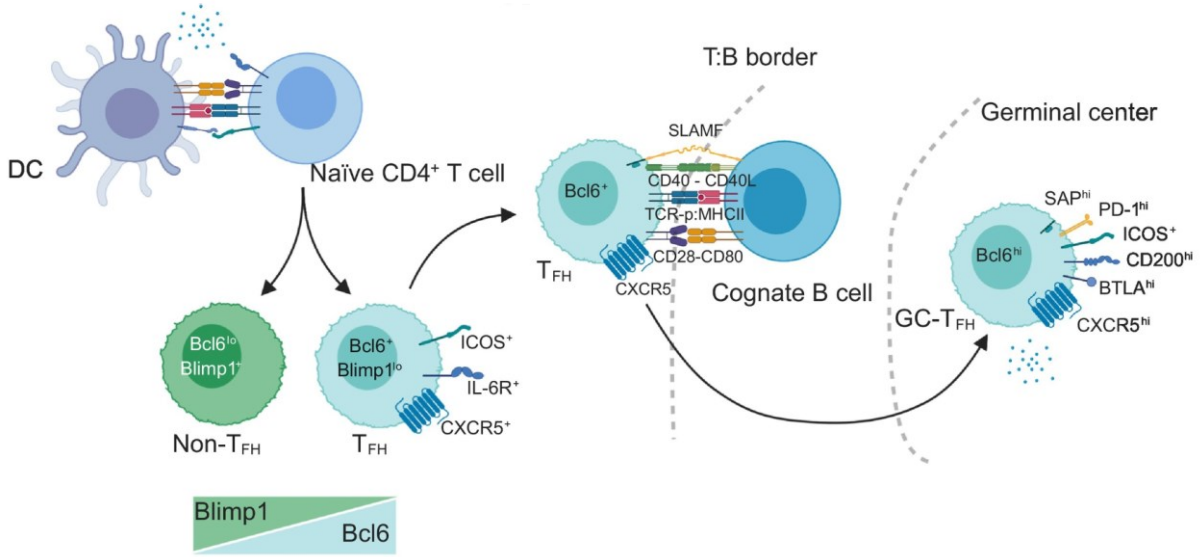

Figure 5. Tfh cell differentiation. Tfh cell differentiation is initiated by dendritic cells in secondary lymphoid organs. DCs polarize naïve T cells towards Tfh lineage with 3 signals: peptide:MHCII, costimulatory molecules, and cytokines such as IL-6, leading to the expression of the transcription factor Bcl6. In Tfh cells, Bcl6 represses Blimp-1 which is known to inhibit Tfh cell differentiation whereas Blimp-1 inhibits Bcl6 expression in non-Tfh cells. Tfh cells, which express CXCR5, migrate towards the T:B border where they interact with cognate B cells to pursue their differentiation. Tfh cells will enter the B follicle, participating to the formation of germinal centres and helping B cell for affinity maturation. Tfh cells inside GCs are called GC-Tfh and express high levels of Bcl6, PD-1 and CXCR5. DC: dendritic cell. Tfh: follicular helper T. Bcl6: Blimp-1: IL-6R: interleukin 6 receptor. SLAMF: signalling lymphocyte activation molecule family. SAP: SLAM-associated protein. PD-1: programmed cell death-1. BTLA: B and T lymphocyte-associated protein. ICOS: inducible T cell costimulatory. TCR: T cell receptor. p:MHCII: peptide:major histocompatibility complex II. GC: germinal centre. From Choi et Crotty[210].

2.1.1 Dendritic cell – T cell interactions

Like for all Th cells, Tfh cell differentiation is initiated in secondary lymphoid organs by DCs. Upon receiving a danger signal through its PRR, DCs internalize the source of danger leading to presentation of its peptides on MHCII. Activated DCs migrate from tissue through lymphatic vessels in a CCR7-dependent manner in direction of the T cell zone of the secondary lymphoid organs where CCL21 and CCL19 are produced by stromal cells. DCs are therefore localized in close proximity to naive T cells allowing their interaction to induce Tfh cell differentiation. In addition

to be adequately localized, DCs are the most numerous APC at this time, raising them as primary initiators of Tfh cell differentiation, instead of B cells.

Indeed, Goenka et al.[212] showed that Tfh cell differentiation is abrogated in DC-depleted mice. However, restriction of peptide:MHC presentation to DCs demonstrates that DCs initiate Tfh cell differentiation (expression of CXCR5, ICOS and Bcl6) but are not sufficient to induce complete differentiation. Particularly, these Tfh cells do not acquire GL7 expression, indicating the absence of GC Tfh cells. DCs are thus required for initiation of differentiation but are not sufficient to induce GC Tfh and GC B cell response.

2.1.1.1 DC populations

Different DC subpopulations have been reported to induce Tfh cell differentiation[213], which might be explained by the fact that antibody production is implicated in a variety of immune responses, all mediated by different DCs.

The most described DC population for Tfh cell differentiation is cDC2. This population has been shown to induce Tfh and GC B cells in response to *Listeria monocytogenes*[214], rabies vaccination[215], sheep red blood cell (SRBC)[214] or OVA immunization[174,216]. Moreover, reduced humoral response and Tfh cells in aged mice are associated with impaired antigen-presentation and costimulation by cDC2[217]. Sakurai et al. reported that in vitro, lung cDC2 from OVA-immunized mice induce Tfh cell differentiation[216]. Using different mouse models in which DCs are impaired, Krishnaswamy et al.[174] showed that migrating cDC2 are driving Tfh cell differentiation in response to intranasal immunization. Indeed *Dock8*$^{DC-/-}$ and *Irf4*$^{DC-/-}$ mice, in which migration and development of cDC2 is impaired respectively, presented abrogated Tfh and GC B cell response, as observed in *Ccr7*$^{-/-}$ mice, in which migration of all DCs is impaired. In addition, *Batf3*$^{-/-}$ mice, which lack cDC1, exhibited similar Tfh response as WT mice, indicating that cDC2, but not cDC1, are involved in Tfh cell differentiation. It has been proposed that cDC2 are ideally localized to promote Tfh cell differentiation. Indeed, cDC2, expressing EBI2 and CXCR5, migrate to the T:B border[174] which is the site of the initiation of Tfh cell differentiation[218]. Moreover, ablation of EBI2 in cDC2, which results in the altered localization of cDC2 to the centre of the T cell zone instead of their outer localization, leads to decreased Tfh cell differentiation, supporting the importance of DC localization for Tfh cell response[219]. In contrast, cDC1 which highly express CCR7, remain in the centre of the T cell zone[174].

The role of cDC1 in Tfh cell response is more controversial[174,220,221]. While some studies show that Tfh cells can develop normally in *Batf3*$^{-/-}$ mice[174], it was also reported that targeting antigens to cDC1 through Clec9A induces Tfh cell differentiation[222,223]. Targeting DEC-205 on cDC1 induces limited humoral response, which can be enhanced in presence of an adjuvant. Yao et al. showed that addressing the antigen to cDC1 through Langerin leads to Tfh cell response, albeit to a lesser extent than LCs[224].

Concerning LCs, several studies report their promoting role in Tfh cell differentiation. Yao et al. demonstrated that targeting antigens to LCs via Langerin in the skin induced Tfh cell response[224]. In addition, Levin et al.[220] showed that, in a model of intradermal immunization to HIV p24-coated nanoparticles, Tfh cell response is abrogated when the site of immunization is removed after injection which prevents skin DCs migration to the LNs. Moreover, using LangDTR mice in which depletion of LCs and cDC1 is achieved upon diphtheria toxin injection, and taking advantage of the differential time needed to recover cDC1 (1 week) and LCs (2 weeks), they investigated specifically the role of LCs and demonstrated that Tfh and GC B cell responses are reduced when LCs are depleted. In a model of cutaneous infection with *L. major*, Zimara et al.[221] showed that in LC-depleted mice, Tfh cell and GC B cell number, GC formation and IgG1 production were diminished. The number of T:B conjugates was similar in LC-depleted and WT mice indicating that conjugate formation was not impaired. However, in absence of LCs, T cells isolated from conjugates failed to induce GC B cell differentiation or produce IL-4 which is a key cytokine for IgG1 class switching. In agreement with that, we showed that upon activation, LCs migrate to the LNs to induce Tfh cell differentiation and IL-4 production, as well as GC B cell differentiation and IgG1 production, in a mouse model of AD induced by TSLP overexpression[68]. On the other hand, in a model of skin OVA sensitization, we showed that LCs did not induce Tfh cell response, which was instead mediated by other DCs, indicating that different factors including the microenvironment and the nature of the antigen can polarize different DC subpopulations for Tfh cell priming[68,221].

Monocyte-derived dendritic cells (mo-DCs) have been reported to also promote Tfh cell differentiation. In a model of subcutaneous immunization, Chakarov et al.[225] showed that while cDCs can prime Tfh cell differentiation, mo-DCs can enhance Tfh cell response through IL-6 production induced by CpG-B adjuvant.

2.1.1.2 DC signals

In the T cell zone of secondary lymphoid organs, DCs provide 3 signals to naive T cells to induce Tfh cell differentiation: 1) antigen presentation through peptide:MHCII complex, 2) costimulation molecules and 3) cytokines.

Peptide:MHCII – TCR

Recognition of peptide:MHCII complex by T cells is required for Tfh cell priming. It has been shown that both DC – T and T – B interactions through peptide:MHCII and TCR are required for Tfh cell differentiation. Indeed the ablation of MHCII specifically in DCs or in B cells impairs Tfh cells and humoral response[226]. In addition, antigen-presentation by DCs is sufficient to induce the initiation of Tfh cell differentiation[212]. The strength of TCR signaling plays an important role in naive T cell polarization. However, studies report conflicting results regarding TCR strength and Tfh commitment. Indeed, strong TCR signaling has been shown to promote Tfh cell differentiation in models of pigeon cytochrome C[227] or *Listeria monocytogenes* immunization[228] whereas other groups reported that low TCR signaling induces Tfh cells[229–231]. Bartleson et al.[232] proposed that polarization might not result from the strength of TCR signaling upon activation but mainly from tonic TCR signaling in response to self-antigens at basal state. T cells that underwent weak tonic signaling differentiate into Tfh cells upon *Listeria monocytogenes* antigen recognition, which is not the case for strong tonic signaling T cells.

Costimulatory molecules

In addition to antigen presentation, DCs provide costimulatory signals to T cells. CD80 (B7.1)/CD86 (B7.2) expressed by DCs upon activation are well known costimulatory molecules which interact with CD28 on T cells. Their ablation in DCs abrogates Tfh / GC B cell responses and IgG1 production[226].

ICOSL – ICOS signaling has been proposed as a key pathway for Tfh cell differentiation and humoral response, however, ICOSL is not only expressed by DCs but also by B cells making it difficult to know whether it plays a role for the initiation of Tfh cells or for their maintenance. Gain of function of ICOS, due to a point mutation in *Rc3h1* gene which encodes the post-transcriptional repressor of ICOS, Roquin-1, results in uncontrolled Tfh and GC B cell responses with elevated

autoantibodies in mice[233]. Moreover, ICOS deficiency in humans leads to immunodeficiency with hypogammaglobulinemia and recurrent infections due to impaired Tfh cells and GC formation[234,235]. This phenotype is also observed in *Icos*$^{-/-}$ mice[234]. Analyses of Tfh cells in *Icos*$^{-/-}$ mice at an early stage (3 days post LCMV infection) indicate that ICOSL – ICOS signaling is required for Tfh cell polarization by DCs and that it induces Bcl6 leading to CXCR5 expression[236].

Similarly to ICOSL, OX40L is expressed not only by DCs but also by B cells. OX40L is part of the TNF superfamily and binds OX40 on T cells which can enhance TCR signaling through PI3K-Akt pathway[237]. It has been shown that in vivo blocking of OX40L, expressed by DCs and B cells in close proximity to T cells, or ablation of OX40 impairs Tfh response[238]. Moreover, in vitro TSLP-activated human DCs promote Tfh cell differentiation and their production of IL-21 and CXCL13 through OX40L signaling[78]. Jacquemin et al.[239] reported that Tfh cell frequency, which are critical for systemic lupus erythematosus (SLE), is correlated to CD11c^{+} OX40L^{+} APC percentage in SLE patients. In addition, culture of naïve T cells with OX40L and anti-CD3/CD28 beads results in high expression of Tfh markers such as CXCR5, PD1, Bcl6, IL-21 while CCR7, and Blimp-1, which is a negative regulator of Tfh cell differentiation, are reduced.

Cytokines

The last signal from DCs polarizing Tfh lineage is cytokines. In mice, IL-6 has been highlighted as the main factor driving Tfh cell differentiation. Indeed, IL-6 promotes IL-21 production by T cells through STAT3. In turn, IL-21 can act on T cells in an autocrine manner to augment Tfh cell differentiation and humoral response[240–244]. In addition, IL-6 has been reported to limit Tfh cell responsiveness to IL-2 and subsequently promoting Tfh cell response[245]. IL-2 is a negative regulator of Tfh cell differentiation. Indeed IL-2 blocking augments Tfh and GC B cell responses in vivo while IL-2 delivery diminishes them[246,247]. IL-2 signals through its receptor, composed of CD25 (IL-2Rα), IL-2Rβ and IL-2Rγ, to promote Blimp-1 expression in T cells through STAT5, leading to Bcl6 repression and therefore inhibition of Tfh cell differentiation[246–248]. In adequation with that, effector T cells express high level of CD25 whereas Tfh cells are CD25^{low} [236]. Moreover, CD25 deficiency in T cells results in increased Tfh cell polarization[248]. DCs have been reported to quench IL-2 to promote Tfh cell differentiation[249].

While the role of IL-6 for mouse Tfh cells has been extensively studied, its role for human Tfh cells is more confused[208]. In humans, Tfh cell differentiation is mainly driven by IL-12. IL-12 is a

heterodimer formed by p35 and p40 subunits, and signals through its receptor composed of IL-12Rβ1 and IL-12Rβ2[250]. IL-12R expression is increased on T cells upon TCR activation and can be further upregulated by CD28 or IL-12 signaling[250]. It has been shown that children deficient for IL-12Rβ1 present a decrease in Tfh cell number[251]. Moreover, IL-12, produced by DCs, can stimulate in vitro Tfh cell differentiation with expression of CXCR5, ICOS, IL-21, Bcl6, c-Maf, and Batf. IL-23 can also induce Tfh cell differentiation and IL-21 production in human T cells but is less potent than IL-12. IL-12 and IL-23-induced Tfh cell differentiation through STAT3 and STAT4 can be further enhanced by TGF-β[251–253]. Of note, TGF-β represses IL-21 and ICOS in mice[252]. In addition, activin A can potentiate IL-12-mediated Tfh induction through SMAD2/3 phosphorylation[254].

The integration of all these signals from the DCs by naive T cells results, within the first two cell divisions[236], in a specific transcriptional program with repression of Blimp-1 and expression of Bcl6 leading to the acquisition of a Tfh phenotype: $Bcl6^+$ $CXCR5^+$ $ICOS^+$ $PD1^+$ $CD4^+$ T cells.

2.1.2 T cell – B cell interactions

Following their activation and polarization by DCs, Tfh cell migration to the T:B border is mediated by different molecules such as CXCR5 and CCR7. CXCR5 has been shown to be required but is not sufficient for Tfh cell migration towards CXCL13-rich B cell follicles[255]. In addition to upregulating CXCR5, expression of Bcl6 in Tfh cells also downregulates CCR7 and PSGL1 to diminish Tfh cell responsiveness to CCL19 and CCL21 which are highly expressed in the T cell zone of the lymphoid organs[256]. On the contrary to Tfh cells, B cells upregulate CCR7 to migrate to the outer follicle. Both Tfh and B cells upregulate EBI2, which ligand is found at the border[257].

Once at the T:B border, Tfh cells need to interact with cognate B cells to further differentiate and initiate GC reaction, acquiring a GC Tfh phenotype. In GCs, GC Tfh cells, which exhibit higher expression of Bcl6, CXCR5 and PD1, continue to interact with GC B cells to contribute to affinity maturation. While DCs initiate Tfh differentiation, it has been shown that the absence of B cell impairs GC-Tfh formation and maintenance[212,258,259]. This next step of differentiation requires the formation of T:B conjugates with long-lasting interactions implicating several molecules. T cell interactions with B cells are restrained by antigen specificity through the recognition of peptide:MHCII by the TCR. This interaction is crucial as ablation of MHCII in B cells leads to a reduced Tfh cell population[226]. The formation of the immune synapse requires signalling lymphocyte activation molecule (SLAM) family members on B and T cells. SLAM family (SLAMF)

includes SLAM, 2B4, Ly9, CD84, NTB-A, Ly108 and CRACC which are transmembrane molecules with a cytoplasmic domain associated to SLAM-associated protein (SAP, encoded by *Sh2d1a*). SLAMF members form homophilic interactions, except for 2B4 which binds CD48. *Sh2d1a*$^{-/-}$ and *Cd84*$^{-/-}$ mice present impaired Tfh cell response and fewer GC compared to WT mice. SAP, CD84 or Ly108-deficient T cells are unable to form long-lasting contacts with B cells to provide help, leading to reduced GC formation[260,261].

It has been shown that CD40L-CD40 signalling is crucial for humoral responses. Indeed, *Cd40l*$^{-/-}$ or *Cd40*$^{-/-}$ mice present impaired Tfh and GC B responses with reduced class-switched immunoglobulins[262,263]. CD40 can be expressed by different cells population, including DCs and B cells. Watanabe et al. showed that mice defective for CD40 specifically in B cells exhibit similar phenotype as *Cd40*$^{-/-}$ mice, whereas CD40 absence in DCs do not impact the phenotype[226]. In addition to be important for Tfh and GC B cell differentiation, CD40L is also required for the maintenance of Tfh / GC B cells as blocking CD40L once these responses are already established leads to reduction in Tfh and GC B cell numbers[259,264]. Among the other costimulatory signals, CD28 and CD80/CD86 has been reported to contribute to Tfh cell differentiation using *Cd28*$^{-/-}$ mice or blocking antibodies. However, whether its expression on B cells is required remains controversial[226,259,262,265,266]. Yusuf et al.[259] found that CD28 signalling is crucial for Tfh maintenance during autoimmunity but not for SRBC immunization.

ICOS-ICOSL axis has been shown to be important not only in T and cognate B cell interactions but also for T and bystander B cell interactions[262,264,267,268]. Bystander B cells do not present cognate peptides to Tfh cells, but they express high level of ICOSL and PD-L1 to interact with Tfh cells in a costimulatory-independent manner. ICOS-ICOSL signalling activates PI3K pathway leading to Tfh cell migration to B follicle and GC[268]. High expression of these molecules is of importance to overcome the inhibition of PI3K by PD1-PD-L1 axis which prevents motility[269]. In addition, it has been shown that ICOS represses the transcription factor KLF2, resulting in the upregulation of CXCR5, and downregulation of CCR7, PSGL1 and S1PR1 and therefore promoting Tfh cell localization to GCs[270].

2.2 Tfh role

Tfh cells are well-known key players in humoral response by participating in GC formation and providing help to B cell for affinity maturation in the GCs. Affinity maturation (which includes

somatic hypermutation (SHM) and selection) and class switch recombination lead to high affinity plasma cells and memory B cells. In addition, Tfh cells also participate in the extrafollicular B cell response.

Extrafollicular response

The extrafollicular B cell response gives rise to low affinity short-lived plasma cells compared to GC plasma cells which have undergone affinity maturation. This response occurs earlier than GC response which allows to limit pathogen dissemination while high affinity long-lived plasma cells are generated in the GCs. After antigen recognition by the B cell receptor (BCR), this response, like GC response, is crucially dependent on Tfh – B cell interactions at the T:B border and IL-21 production[271]. Following these interactions, B cells pursue either towards an extrafollicular response or towards a GC response. The fate of B cells is dependent on BCR affinity. Low affinity B cells engage towards GC reaction, whereas high affinity B cells continue towards an extrafollicular response leading mainly to the production of IgM[267,272] even though class switch recombination has also been reported in extrafollicular B cells at low level[273].

GC response

Instead of pursuing an extrafollicular response, after contacts at the T:B border, B cells can migrate to the center of B follicles with Tfh cells where they initiate GC formation. Their migration is driven by downregulation of EBI2 and upregulation of CXCR5, both under the control of Bcl6 which is a key transcription factor for GC Tfh and GC B cell development[274]. In GC B cell, Bcl6 represses genes involved in plasma cell differentiation, damage sensing, cell cycle arrest and apoptosis to allow B cells to undergo SHM[275].

GCs are tightly organized structures divided in two zones: the dark zone where GC B cells undergo proliferation and SHM, and the light zone where GC B cells undergo selection through interactions with GC Tfh and follicular dendritic cells (FDC) to select GC B cells with the highest affinity. GC B cell migration from the light zone to the dark zone is dependent on CXCL12 produced by reticular cells in the dark zone and CXCR4 expressed by GC-B cells, whereas dark zone to light zone migration depends on CXCR5 and CXCL13[274]. This controlled migration is therefore important for SHM and selection.

In the dark zone, GC B cells undergo proliferation and SHM which inserts point mutations in the immunoglobulin variable region to generate BCR with different affinities[274]. Newly mutated BCR are produced and displayed at the surface of GC B cells. Moreover, former peptide:MHCII are degraded and replaced by new MHCII before to enter the light zone where FDCs present the antigen to GC B cells. GC B cells can recognize and uptake the antigen through their BCR to load it on their MHCII and present it to GC Tfh cells. Then, a step of selection occurs through contacts between GC B and GC Tfh cells. Subsequently these cells can then either re-enter the dark zone for a new round of SHM to potentially increase their BCR affinity (a process called "cyclic re-entry"), or they can differentiate into plasma cells or memory cells[208] (Figure 6). Therefore, in GCs, GC Tfh cells contribute to affinity maturation of GC B cells, and their differentiation in plasma cells or memory B cells.

During selection, GC B cells compete for GC Tfh help to survive or differentiate[208]. This selection is based on BCR affinity which information is transmitted to GC Tfh through TCR engagement. Indeed, high affinity GC-B cells uptake more antigens resulting in increased peptide:MHCII presentation and therefore stronger TCR signalling in GC Tfh cells associated to strong costimulatory signals. Consequently, the lowest affinity GC B cells are unable to present peptide:MHCII efficiently, depriving them of GC Tfh help which results in cell death[274].

Positive selection requires brief contacts called "entanglements" between GC-Tfh and cognate GC B cells with the formation of an immune synapse[276,277]. While these contacts are shorter than the ones at the T:B border, they remains longer than contacts with non-selected GC B cells[277]. Among the molecules involved, we can find CD40 - CD40L and ICOSL - ICOS in addition to peptide:MHCII - TCR. Entanglements are crucially dependent on ICOSL/ICOS signalling and it induces Ca^{2+} influx leading to the upregulation of CD40L at the cell surface of GC Tfh cells, which in turn upregulates ICOSL on GC B cells creating a feedback loop for positive selection[276,278]. Of note, human GC Tfh cells, but not mouse, release dopamine upon interaction with cognate GC B cells which induces ICOSL translocation to the membrane in these cells and subsequently CD40L in GC Tfh cells, promoting the area of contact between these cells and therefore the help provided[278]. CD40L/CD40 and ICOSL/ICOS axes are crucial for GC response as their blocking abolishes pre-established GC B cells[264].

In addition to costimulatory molecules, GC Tfh cells secrete first IL-21 and then IL-4 which play critical roles for plasma cell differentiation and class switching[279]. *Il21*$^{-/-}$, *Il21r*$^{-/-}$ and *Il4*$^{-/-}$ mice exhibit lower plasma cells and class switched antibodies, in particular IgG1[280–283]. Moreover, IL-4

and IL-21 are important for proliferation of GC B cells[279], in adequation with the fact that they induce Bcl6 expression[284]. IL-4 has also been reported to limit apoptosis in GC B cells by post-transcriptionally repressing the proapoptotic protein Bim[285].

The cell fate of GC B cells towards cyclic re-entry, plasma cell differentiation or memory B cell differentiation has been shown to be dependent on BCR affinity and is therefore dependent on GC Tfh cells[208]. GC Tfh cells integrate BCR affinity through the strength of peptide:MHCII – TCR signalling, which reflects GC B cell affinity. BCR affinity also modulates costimulatory signals and cytokines, therefore modulating the help provided by GC Tfh cells to GC B cells. However, the exact mechanisms driving polarization remain incompletely understood.

It has been shown that high affinity GC B cells are preferentially differentiated into plasma cells by GC Tfh cells, requiring strong peptide:MHCII-TCR, ICOSL-ICOS and CD40L-CD40 signalling which induces IL-4 and IL-21 production[276,277,286]. IL-21 and CD40L cooperate to induce Blimp-1 expression through STAT3 and IRF4[287]. CD40L signalling leads to high IRF4 concentration which upregulates Blimp-1, therefore inhibiting Bcl6[287,288]. While Bcl6 is the main transcription factor for GC B cells, Blimp-1 and IRF4 drives plasma cell differentiation and high affinity class switched antibody secretion[289]. In contrast to high affinity GC B cells, low affinity GC B cells pursue a memory B cell pathway through induction of the transcriptional repressor Bach2[290]. Ablation of Bach2 inhibits memory B cell differentiation. Moreover, blocking CD40L increases Bach2 expression in a dose-dependent manner supporting the fact that receiving limited help from GC Tfh cells, resulting from low BCR affinity, leads to memory B cell development[290]. In addition to promoting plasma cell and memory B cell differentiation, GC Tfh cells can guide GC B cells towards cyclic re-entry to undergo further round of SHM, proliferation and selection. To this aim, GC Tfh cells induce c-Myc expression which controls the number of cell divisions[291,292], and mTORC1 expression through CD40L/CD40 axis in GC B cells[293]. Their expression is dependent on antigen capture and therefore BCR affinity leading to the expansion of high affinity GC B cells.

It has been suggested that in addition to BCR affinity, temporality might also drive GC B cell fate as memory B cells start to appear before plasma cells[294].

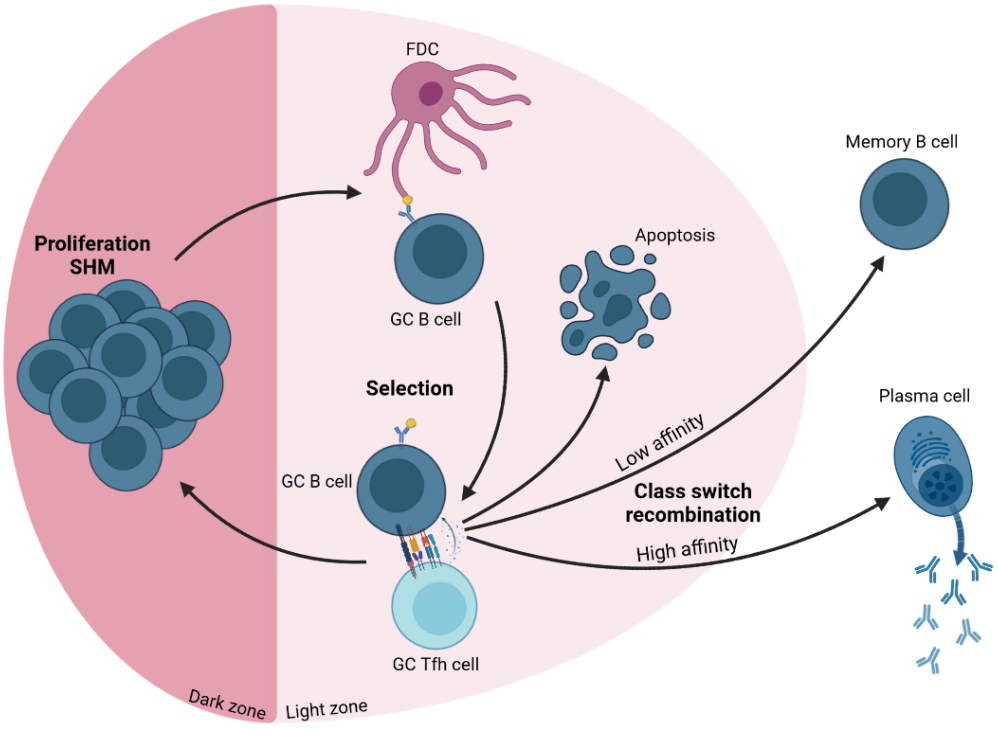

Figure 6. Germinal centre response. *Upon entering the germinal centre (GC), GC B cells undergo proliferation and somatic hypermutation (SHM) in the dark zone. In the light zone, there is a step of selection. Upon recognition through BCR, GC B cells uptake the antigen displayed by follicular dendritic cells (FDC) and present it to GC Tfh cells to undergo selection. GC B cells which do not receive survival signals from GC Tfh cells due to poor BCR affinity undergo apoptosis. GC B cells receiving help from GC Tfh cells can re-enter the dark zone for further proliferation and SHM rounds, or they can go through class switch recombination and differentiate in plasma cells or memory B cells. BCR affinity modulates the help received from GC Tfh cells, which orientates cell fate: low affinity GC B cells differentiate in memory B cells whereas high affinity GC B cells in plasma cells.*

2.3 Tfh cells in allergy

Allergies, such as food allergies, asthma, AD and allergic rhinitis, have long been considered as Th2-driven diseases due to their Th2 inflammation. Moreover, they are also characterized by high IgE levels, and it was first thought that Th2 cells were mediating IgE production by providing help to B cells. However, since then, Tfh cells have been discovered and raised as providers of critical help to B cells for affinity maturation, class switch recombination and memory or plasma cell differentiation instead of Th2 cells[208]. Therefore, it has been suggested that Tfh cells might be key players in allergies.

Human data about Tfh cells originate mainly from circulating Tfh (cTfh) cell analyses as this population is more accessible than Tfh from tissue. cTfh cells, which are in the blood, are the counterpart of Tfh cells in the secondary lymphoid organs and are able to provide help to B cells. Morita et al.[209] identified, among $CD4^+$ $CXCR5^+$ cells, three cTfh cell populations based on their

expression of CXCR3 and CCR6: CXCR3^{+} CCR6^{-} Tfh1 cells which produce IFNγ, CXCR3^{-} CCR6^{+} Tfh17 cells which secrete IL-21, IL-17A and IL-22, and CXCR3^{-} CCR6^{-} Tfh2 cells which produce IL-21 and IL-4. Szabó et al.[141] showed that cTfh cell percentage is increased in AD children, but not in AD adults, compared to healthy controls, which is correlated with AD severity. As AD is heterogenous with some patients presenting high levels of IgE and others who do not, Jiang et al.[142] divided the patients according to IgE levels and showed that cTfh cells were increased in extrinsic AD but not in intrinsic AD, in agreement with the notion that Tfh cells are important for IgE production. cTfh2 cell number is also increased in allergic rhinitis[295–298] and asthma[299] patients and correlates with IgE levels[299,300]. In addition to the quantity of cTfh cells, the quality of these cells is also dysregulated. cTfh cells from allergic rhinitis patients produce increased quantity of IL-4, resulting in higher IgE production by B cells[295]. Follicular regulatory T (Tfr) cells and Bregs play an important role for controlling Tfh cell response through different mechanisms such as IL-10, CTLA-4, granzyme B and TGF-β[301]. Both Breg and Tfr cells have been shown to be dysregulated in allergic rhinitis, asthma and AD with decreased frequency but also potency to inhibit Tfh cells[142,296–298]. Allergen immunotherapy, which reprograms Th2 immune response towards Th1 and induces tolerance, has been shown to promote Tfr cells number and their suppressive activity in allergic rhinitis which is associated with reduced Tfh cells and a better outcome[295,297,298].

Our knowledge about Tfh cells in allergy cells is mainly coming from mouse models. Tfh cells, associated to IgE and IgG1 production, are induced upon skin sensitization, food allergy or asthma however the driving mechanisms are still incompletely understood[68,302,303]. In peanut allergy, IL-1α and/or IL-1β, but not TSLP, seem to be key player(s) for Tfh cell differentiation[303]. On the other hand, we showed that TSLP was crucially required for OVA-induced IL-4^{+} Tfh and GC B cell responses, as well as IgE and IgG1 production during skin sensitization[68]. Moreover, the transfer of TSLP+OVA-activated DCs, but not OVA-activated DCs, induce Tfh cell differentiation and lung inflammation following intranasal OVA challenge[299]. cDC2 have been reported to be the main players in IgE production and/or Tfh cell differentiation in asthma and skin sensitization[133,174,216]. During allergic skin sensitization, LCs have also been suggested to induce IgE production[131] however the results remain controversial as we showed that depletion of LCs augments IgE and IgG1 levels and Tfh cell number[68].

The role of Tfh cells in immunoglobulin production is well established, however their role in Th2 inflammation is more ambiguous. Using HDM-sensitized *Cd4*-Cre *gp130*$^{fl/fl}$ mice, which are defective for IL-6 signalling in T cells and therefore lack Tfh cells, Noble et al.[304] showed decreased

IgE and IgG1 levels in the blood, however eosinophils number and Th2 cytokines were unchanged in the BAL. Of note, this mouse line also presents an impaired Th17 immune response, which resulted in reduced number of neutrophils in the airway. Kobayashi et al.[302] showed that, OVA and *Alternaria* intranasal delivery resulted in Tfh and GC B cell differentiation, IgE and IgG1 production, which were all abolished in *Cd4*-Cre *Bcl6*$^{fl/fl}$ mice, which lack Tfh cells. Contrary to the humoral response, Th2 airway inflammation was not diminished and Th2 cytokine production was increased in LNs. However, *Cd4*-Cre *Bcl6*$^{fl/fl}$ mice present a Th2 bias at steady state and therefore these results should be considered with caution. In peanut allergy, *Cd4*-Cre *Bcl6*$^{fl/fl}$ mice present a defective IgE and IgG1 response but do not exhibit a drop in body temperature due to anaphylaxis, as observed in WT mice[303]. On the other hand, Ballesteros-Tato et al.[305] showed that HDM induces IL-4^{+} and IL-21^{+} Tfh cells in the airways, which later give rise to Th2 cells pointing out the plasticity of Tfh cells.

Tfh cytokines are essential for antibody production and especially class switch recombination. In allergy, Tfh cells mainly produce IL-4 and IL-21. IL-4 has been shown to promote IgE class switch[306], while IL-21 induces class switch recombination to IgG1 and represses class switch recombination to IgE. *Il21r*$^{-/-}$ mice present reduced IgG1 levels but increased IgE levels[281,307]. Moreover, delivery of IL-21 decreases IgE levels[308], which abrogates the anaphylactic response to peanut[309]. IL-21 induces Id2 which is known to repress Pax5 and E2A, two transcription factors leading to IgE class switch recombination[306,309]. On the other hand, the transfer of IL-21^{+} Tfh cells leads to increased eosinophilia in HDM-induced asthma[307]. The opposite role of IL-21 in different contexts might be one explanation for the dichotomous role of Tfh cells in Th2 inflammation.

RESULTS

PART 1:
TSLP-driven Tfh cell differentiation in allergic skin sensitization

Background

AD is a skin inflammatory disease which affects up to 20% of children and 3% of adults worldwide. AD patients exhibit eczematous skin lesions, pruritus and xerosis, and moreover are often associated with other atopic diseases including allergic rhinitis, asthma and food allergy which negatively impact their personal and work life, representing an important economic burden for the society. AD is characterized by Th2 immune response, elevated IgE levels, as well as a defective skin barrier due to environmental and genetics factors[310]. It has been recognized that AD children are prone to develop allergen sensitization due to their skin with barrier defects and proinflammatory cutaneous microenvironment, which leads to other atopic diseases in a process called the "atopic march". As there is no cure in sight, it is therefore urgently needed to get a better understanding of the pathogenesis of AD and allergic skin sensitization.

TSLP, produced by keratinocytes in human AD lesions[65], has emerged as a key cytokine in AD pathogenesis. My lab has shown that TSLP overexpression, induced by low calcemic vitamin D3 analog MC903, triggers an AD-like phenotype by promoting skin inflammation and Th2 cell response, which are all abolished in *Tslp*$^{-/-}$ or *Tslp*$^{ep-/-}$ (in which TSLP is ablated in keratinocytes) mice, indicating that TSLP produced by keratinocytes is required and sufficient to induce AD[71,72,79]. Moreover, my lab also reported that TSLP promotes Th2 allergic sensitization through tape-stripped skin and thereby triggers the atopic march[67].

Despite that the role of Th2 cells has long been recognized in AD pathogenesis, more and more evidence is suggesting a role for Tfh cells in allergic diseases including AD[141,302,303]. Tfh cells are a subset of CD4^{+} T cells characterized by CXCR5, PD1 and Bcl6 expression, and involved in GC formation and providing help to B cells to differentiate into memory B cells or plasma cells[208], which have been found to be dysregulated in AD patients and are associated with allergen-specific IgE and disease severity[141]. Despite these studies, the role and regulation of Tfh cells in AD remain poorly understood.

When I joined the lab, the previous PhD students P. Marschall and R. Wei were investigating the cellular and molecular mechanisms driving Tfh cell differentiation in AD, using a TSLP-overexpression (TSLP^{over}) mouse model induced by MC903. Their data showed that MC903 treatment on WT mouse skin induced Tfh cell differentiation and their production of IL-4, which were all abolished in *Tslp*$^{-/-}$ mice. Their results also showed that when Lang^{+} DCs were depleted, using Lang^{DEP} mice in which all Lang^{+} DCs are depleted upon DT injection, Tfh / GC B response was

diminished in MC903-treated mice. Furthermore, by taking advantage of the differential recovery time required for LC and Lang^{+} dDC repopulation after depletion, they showed that it was LCs which are implicated in Tfh cell differentiation in MC903-induced AD model.

However, the MC903 AD mouse model is based on highly overproduced TSLP and does not implicate exogenous allergens. In order to explore whether these findings on the role of TSLP and LCs also apply to allergen sensitization-induced Tfh / GC B cell response, the objectives of my project were to use a pathophysiological model for allergic skin sensitization and the atopic march to investigate the function of TSLP and LCs in Tfh and GC B cell response.

To this aim we established a new experimental mouse model in which mouse ear is subjected to laser-microporation (LMP) that disrupts skin barrier and allows the topical application of the protein allergen ovalbumin (OVA), resulting in cutaneous allergic sensitization and Tfh / GC B cell response, as well as an asthma phenotype upon the reencounter with OVA in the airways. First, I investigated the role of TSLP in OVA-induced Tfh cell differentiation. Using *Tslp*$^{-/-}$ mice, I demonstrated that TSLP is crucially required for Tfh and GC B cell response. Next, to investigate the role of DCs and more specifically LCs in skin allergic sensitization, I used Lang^{DEP} and LC^{DEP} mice, in which all Lang^{+} DCs or specifically LCs are depleted upon DT injection, respectively. Surprisingly, in contrast to the findings in MC903 AD model, my results indicated an inhibitory role for LCs in OVA-induced Tfh / GC B cell response. Finally, to gain insights in the dichotomous role of LCs in MC903- or OVA-induced AD, we performed transcriptomic analyses of migratory Lang^{+} DCs from LNs of MC903 or LMP/OVA-treated mice.

This work has been published in Journal of Allergy and Clinical Immunology (2020) and is presented as follows.

Dual function of Langerhans cells in skin TSLP-promoted T_{FH} differentiation in mouse atopic dermatitis

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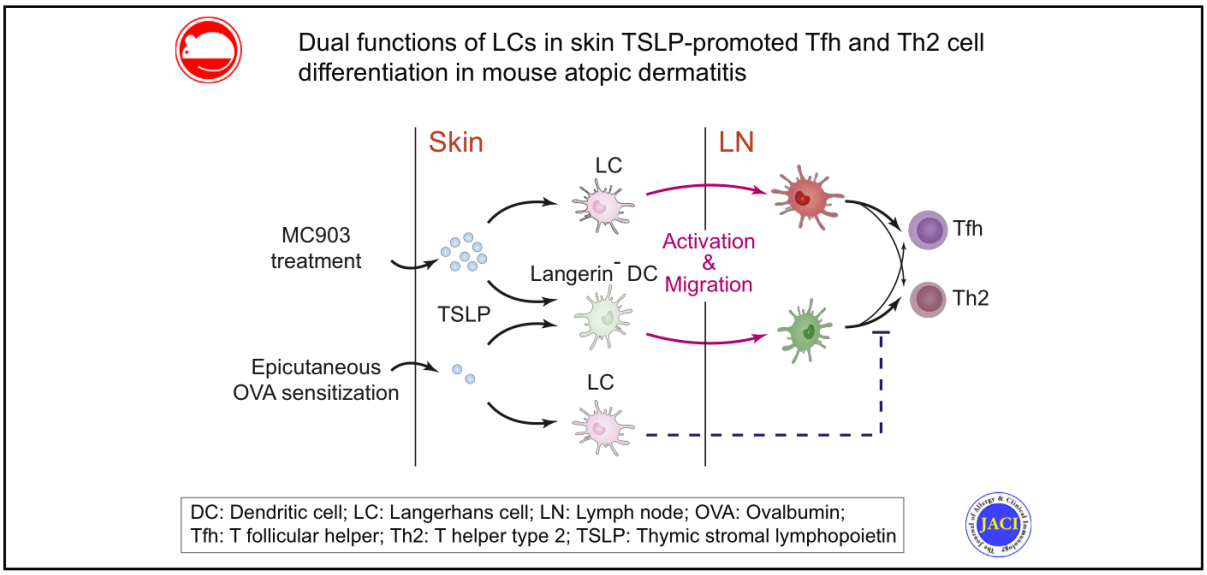
GRAPHICAL ABSTRACT



Background: Atopic dermatitis (AD) is among the most common chronic inflammatory skin diseases, usually occurring early in life, and often preceding other atopic diseases such as asthma. T_H2 has been believed to play a crucial role in cellular and humoral response in AD, but accumulating evidence has shown that follicular helper T cell (T_{FH}), a critical player in humoral immunity, is associated with disease severity and plays an important role in AD pathogenesis.

Objectives: This study aimed at investigating how T_{FH}s are generated during the pathogenesis of AD, particularly what is the role of keratinocyte-derived cytokine TSLP and Langerhans cells (LCs).

Methods: Two experimental AD mouse models were employed: (1) triggered by the overproduction of TSLP through topical application of MC903, and (2) induced by epicutaneous allergen ovalbumin (OVA) sensitization.

Results: This study demonstrated that the development of T_{FH}s and germinal center (GC) response were crucially dependent on TSLP in both the MC903 model and the OVA sensitization model. Moreover, we found that LCs promoted T_{FH}

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differentiation and GC response in the MC903 model, and the depletion of Langerin$^+$ dendritic cells (DCs) or selective depletion of LCs diminished the T_{FH}/GC response. By contrast, in the model with OVA sensitization, LCs inhibited T_{FH}/GC response and suppressed T_H2 skin inflammation and the subsequent asthma. Transcriptomic analysis of Langerin$^+$ and Langerin$^-$ migratory DCs revealed that Langerin$^+$ DCs became activated in the MC903 model, whereas these cells remained inactivated in OVA sensitization model.
Conclusions: Together, these studies revealed a dual functionality of LCs in TSLP-promoted T_{FH} and T_H2 differentiation in AD pathogenesis. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: *Atopic dermatitis, TSLP, dendritic cells, Langerhans cells, T_{FH}, T_H2, allergen sensitization, mouse*

Atopic dermatitis (AD) is among the most common chronic inflammatory skin diseases, and it affects up to 20% of children and 3% of adults worldwide, with increasing prevalence in the industrialized countries during the last 30 years.[1] AD is characterized by chronic cutaneous inflammation, T_H2 response and hyper-IgE. Patients suffering from AD often present genetic risk factors in the form of mutations affecting the skin barrier structure or the immune system.[2] Onset of AD usually occurs early in life and may lead to allergen sensitization, which can trigger the progression from AD to other atopic diseases such as asthma/allergic rhinitis, in a process called "atopic march."[3,4]

It has been recognized that T_H2 response is critically implicated in the pathogenesis of AD. Previous studies from us and others using mouse models have established a central role of the cytokine TSLP expressed by epidermal keratinocytes in promoting T_H2 response and driving the pathogenesis of AD.[5-8] In addition to T_H2 response, humoral immune response is another key feature of AD, with increased serum IgE and IgG_1 levels associated with AD, which contribute to AD pathology and the atopic march.[9,10] For a long time, T_H2 has been believed to play a crucial role both in cellular response and humoral response, for example, helping B cells to produce immunoglobulins. However, such knowledge has been challenged with the identification of follicular helper T cell (T_{FH}), which emerges to be a critical player in humoral immunity and T-cell memory.[11]

In lymphoid organs, T_{FH} differentiation process is believed to begin with an initial dendritic cell (DC) priming of naive CD4$^+$ T cells, which undergo a cell-fate decision with the acquisition of master transcription factor Bcl6 expression and chemokine receptor CXCR5 expressed on cell surface to become early T_{FH}s, of which CXCR5 promotes their migration from the T-cell zone to the B-cell follicles.[12,13] The full differentiation and maintenance of T_{FH}s implicate the T_{FH}-B-cell interaction, leading to germinal center (GC) T_{FH}s, which are phenotypically defined by their high expression of CXCR5 and PD-1.[14] It has been shown that T_{FH}s coordinate generation of the GC, initiate help for antigen-specific B cells, and promote selection of high-affinity B cells and differentiation into either memory B cells or long-lived plasma cells.[15] Recent studies have identified T_{FH}s as an important source of IL-4, a master regulator in type 2 immunity, which was previously thought to be produced by T_H2s, for providing critical B-cell help by its anti-apoptotic and IgE and IgG_1 class switch effects.[16] In addition, it was reported that T_{FH}s produce IL-4 in a GATA3-independent manner,[17] suggesting distinct mechanisms employed by T_{FH}s and T_H2s in the regulation of IL-4.

Abbreviations used

AD:	Atopic dermatitis
BAL:	Bronchoalveolar lavage
cDC1:	Type-1 conventional DC
cDC2:	Type-2 conventional DC
CT:	Wild-type control
D0:	Day 0
DC:	Dendritic cell
DEG:	Differentially expressed genes
DT:	Diphtheria toxin
DTR:	Diphtheria toxin receptor
EDLN:	Ear-draining lymph node
GC:	Germinal center
GFP:	Green fluorescent protein
GFP^{neg} (GFP^{pos}):	GFP$^-$ (GFP$^+$) cells from Lang^{GFP} mice
huLang:	Human Langerin
Lang^{DEP}:	Depletion of Langerin$^+$
LC:	Langerhans cell
LMP:	Laser-assisted skin microporation
LN:	Lymph node
migDC:	Migratory dendritic cell
Neg_MC (Pos_MC):	GFP^{neg} (GFP^{pos}) migDCs from MC903-treated Lang^{GFP} mice
Neg_NT (Pos_NT):	GFP^{neg} (GFP^{pos}) migDCs from NT Lang^{GFP} mice
Neg_OVA (Pos_OVA):	GFP^{neg} (GFP^{pos}) migDCs from LMP/OVA–treated Lang^{GFP} mice
NT:	Nontreated
OVA:	Ovalbumin
OX40L:	Alias for TNFSF4
PD-1:	Alias for PDCD1
T_{FH}:	Follicular helper T cell
TS:	Tape stripping
TSLP^{over}:	Overproduction of TSLP
WT:	Wild type

Since their initial identification, the biological functions of T_{FH}s and their mechanisms of action in the onset and development of diseases have been studied in autoimmuity, infectious diseases, immunodeficiencies, and vaccination.[18] Less is known about T_{FH}s in the context of AD and other atopic diseases, but more and more evidence has suggested that T_{FH}s are associated with disease severity and T_{FH}s play an important role in the pathogenesis.[19-21] In humans, alteration of circulating T_{FH}s is correlated with severity of the disease in children with AD,[22] or with the comorbid association of allergic rhinitis with asthma,[23] and allergen-specific T_{FH} counts are correlated with specific IgE levels and efficacy of allergen immunotherapy.[24] In mice, it has been reported that T_{FH}s are important for house dust mite–induced asthma[25] or peanut allergy.[26]

Despite of the accumulating evidence showing the importance of T_{FH}s in atopic diseases, how T_{FH}s and humoral responses are generated and regulated in AD remained to be investigated. In this study, by employing 2 experimental AD mouse models—one triggered by the overexpression of TSLP in mouse skin through topical application of MC903,[6,7,27] and the other one induced by epicutaneous allergen ovalbumin (OVA) sensitization—we demonstrated that skin TSLP plays a crucial role in driving/promoting T_{FH} differentiation and GC response, in addition to its recognized role in promoting T_H2 response. Moreover,

we investigated the role of skin DCs in mediating the T_{FH} differentiation. We uncovered a dual functionality of epidermal Langerhans cells (LCs) in TSLP-promoted T_{FH}/T_H2 differentiation in AD pathogenesis, and we further explored the molecular insights by transcriptomic analyses, thus shedding new light onto the long-standing controversy of LCs in skin immunity.

METHODS

Details on the methods used in this study are described in the Methods section in this article's Online Repository (available at www.jacionline.org). Topics discussed include experimental mice, MC903 topical application, epicutaneous OVA sensitization and airway challenge, depletion of Langerin$^+$ DCs or LCs in mice, cell preparation for flow cytometry analyses, surface staining for flow cytometry analyses, lymph node (LN) cell culture and antigen stimulation, RNA sequencing, bronchoalveolar lavage (BAL) cell analyses, ELISA, histopathology, immunohistochemistry staining, RNA *in situ* hybridization, and statistics.

RESULTS

Topical MC903 treatment induces TSLP-dependent T_{FH} differentiation and GC response

We have previously reported that topical treatment with MC903, a low calcemic analog of vitamin D3, induces the overproduction of TSLP (TSLP^{over}) and the pathogenesis of AD.[6,7] To examine the T_{FH} differentiation and GC response in the MC903-induced AD model, BALB/c wild-type (WT) mouse ears were topically treated every other day from day 0 (D0) to D10 with MC903 and ear-draining lymph nodes (EDLNs) were analyzed at D0, D7, and D11 (Fig 1, *A*). Results showed that the frequency and number of CXCR5$^+$ PD-1$^+$ T_{FH}s were both increased in MC903-treated WT mice at D7 and further augmented at D11 (Fig 1, *B*). We next examined the expression of IL-4, a key signal provided by T_{FH}s to sustain B-cell maturation, by taking use of *Il4/Il13* dual reporter 4C13R$^{Tg/0}$ mice, in which AmCyan and dsRed are expressed under the control of IL-4 and IL-13 regulatory elements, respectively.[28] In agreement with a previous report,[29] CXCR5$^+$ PD-1$^+$ T_{FH}s in EDLNs express IL-4 (AmCyan) but not IL-13 (dsRed) (see Fig E1, *A* in this article's Online Repository at www.jacionline.org), and the IL-4 expression by T_{FH}s was augmented in MC903-treated 4C13R$^{Tg/0}$ mice at both D7 and D11 (Fig 1, *C*). Together, MC903 treatment induces not only T_{FH} differentiation but also the production of IL-4 by T_{FH}s.

To examine whether the induction of T_{FH}s in the MC903 model is triggered by TSLP, mice lacking TSLP (*Tslp*$^{-/-}$)[6] were subjected to MC903 treatment. Results showed that these mice, compared with WT mice, exhibited highly diminished T_{FH} frequency and number at D7 and D11 (Fig 1, *B*). By breeding *Tslp*$^{-/-}$ with 4C13R$^{Tg/0}$ to generate *Tslp*$^{-/-}$4C13R$^{Tg/0}$ mice, we showed that MC903-induced IL-4 expression in T_{FH}s was abrogated in the absence of TSLP (Fig 1, *C*). In agreement with the recognized role of TSLP in T_H2 differentiation, we showed that the MC903-induced IL-4– or IL-13–expressing CXCR5$^-$ CD4$^+$ non-T_{FH}s (representing T_H2s) were also abrogated in *Tslp*$^{-/-}$ mice (Fig E1, *B*). These results indicate that the overproduction of TSLP triggers not only T_H2 differentiation, but also T_{FH} differentiation and IL-4 expression by these cells.

Next, we examined the GC response in MC903-treated BALB/c WT mice. The number of GC B cells, identified as GL-7$^+$ CD95$^+$ B cells, exhibited an increase in MC903-treated WT mice at D11, but not at D7 (Fig 1, *D*). Such increase was abrogated in MC903-treated *Tslp*$^{-/-}$ mice (Fig 1, *D*). This was confirmed by immunofluorescence staining for GCs (see Fig E2, *A* in this article's Online Repository at www.jacionline.org). In addition, both IgG$_1^+$ and IgE$^+$ B cells exhibited an increase in their numbers in MC903-treated WT mice at D11, which was also abrogated in MC903-treated *Tslp*$^{-/-}$ mice (Fig 1, *E*). Of note, we observed that most of the IgG$_1^+$ B cells were GL-7$^+$ CD95$^+$ (Fig E1, *C*), suggesting that these cells harbor a GC phenotype; however, this was not the case for IgE$^+$ B cells (Fig E1, *C*).

Taken together, these data indicate that the overproduction of TSLP triggers T_{FH} differentiation and GC response.

Depletion of Langerin$^+$ DCs or LCs diminishes the TSLP^{over}-triggered T_{FH}/GC response

LCs reside in the epidermis as a dense network of immune system sentinels, in close proximity to keratinocytes. We then asked whether LCs mediate the TSLP^{over}-triggered T_{FH}/GC response. To this aim, we first employed Langerin-DTR knock-in mice (Lang^{DTR}) in which Langerin$^+$ cells, including LCs and Langerin$^+$ dermal DCs, express the human diphtheria toxin receptor (DTR) and can thus be depleted on injection of diphtheria toxin (DT).[30] Lang^{DTR} mice and their WT control (CT) littermates were intraperitoneally injected with DT at D-2, D0, and every 4 days to maintain the depletion of Langerin$^+$ cells (named Lang^{DEP} and CT, respectively), and were subjected to topical MC903 treatment (Fig 2, *A*). Results showed that the TSLP^{over}-triggered T_{FH} differentiation was largely diminished in Lang^{DEP} mice (Fig 2, *B*). The expression of IL-4 (AmCyan) by T_{FH}s was also reduced in Lang^{DEP}/4C13R$^{Tg/0}$ mice (Fig 2, *C*). Accordingly, GC B-cell number was lower and IgG$_1^+$ (however not IgE$^+$) B-cell number was significantly decreased (Fig 2, *D*). Therefore, these results indicate that Langerin$^+$ DCs play an important role in mediating the TSLP^{over}-induced T_{FH}/GC response.

As LCs and Langerin$^+$ type-1 conventional DCs (cDC1s) were both depleted in Lang^{DEP} mice, we next examined whether LCs mediate T_{FH} differentiation by depleting selectively LCs using 2 strategies: one used the differential recovery time between LCs and Langerin$^+$ cDC1s after DT-induced depletion as previously reported[31] (see Fig E3, *A* and *B* in this article's Online Repository at www.jacionline.org), and the other employed human Langerin-DTR (huLang^{DTR}) mice in which DT injection efficiently depletes LCs but not Langerin$^+$ cDC1s[32] (Fig E3, *C* and *D*). In both cases, we showed that the selective depletion of LCs led to a decrease in frequency and number of T_{FH}s, suggesting an important role for LCs in TSLP^{over}-triggered T_{FH} differentiation.

Epicutaneous OVA sensitization induces a TSLP-dependent T_{FH} differentiation and GC response

We have previously reported that TSLP plays a crucial role for promoting skin sensitization to allergens, using an experimental mouse protocol in which OVA sensitization through tape-stripped (TS) skin leads to an allergic AD inflammation, accompanied by T_H2 response and an increased production of OVA-specific IgG$_1$

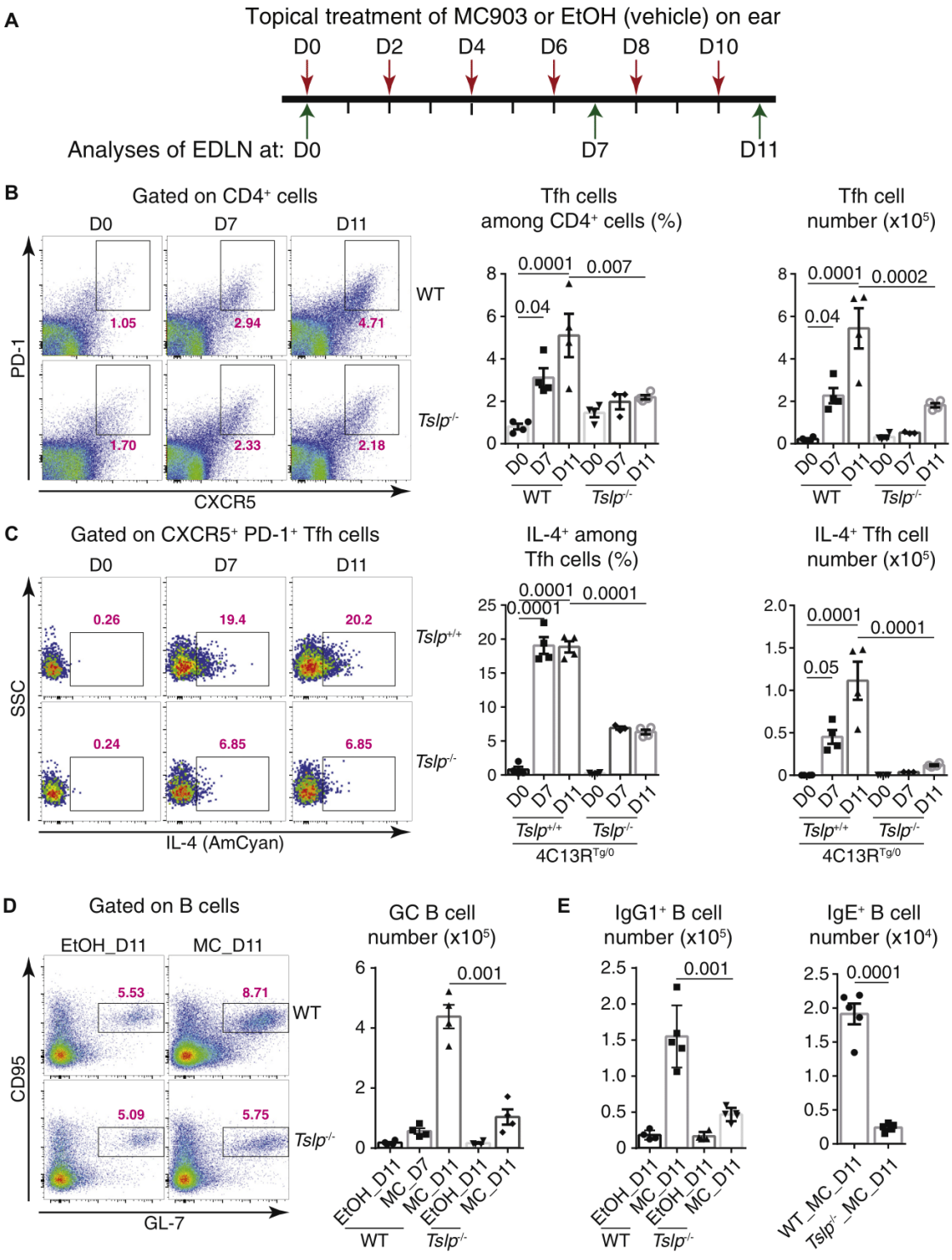


FIG 1. Overproduction of TSLP in the skin triggers T_{FH} differentiation and GC response in MC903-induced AD mice. **A,** Experimental protocol. Mouse ears were topically treated with MC903 or ethanol ([*EtOH*]; as vehicle control) every other day from D0 to D10 and EDLNs were analyzed at D0, D7, and 11. **B,** Frequency and number of CXCR5$^+$ PD-1$^+$ T_{FH}s in EDLN from MC903-treated BALB/c WT and *Tslp*$^{-/-}$ mice. **C,** Frequency of IL-4 (AmCyan) $^+$ in T_{FH}s and cell number of IL-4$^+$ T_{FH}s in EDLNs. **D** and **E,** Number of CD95$^+$ GL-7$^+$ GC, IgG_1^+, and IgE$^+$ B cells in EDLNs. Values shown are means ± SEMs. **B-D,** One-way ANOVA with Tukey's multiple comparison *post hoc* test. **E,** Unpaired *t*-test with Welch's correction. Data are representative of 3 independent experiments with similar results. *SSC,* Side scatter.

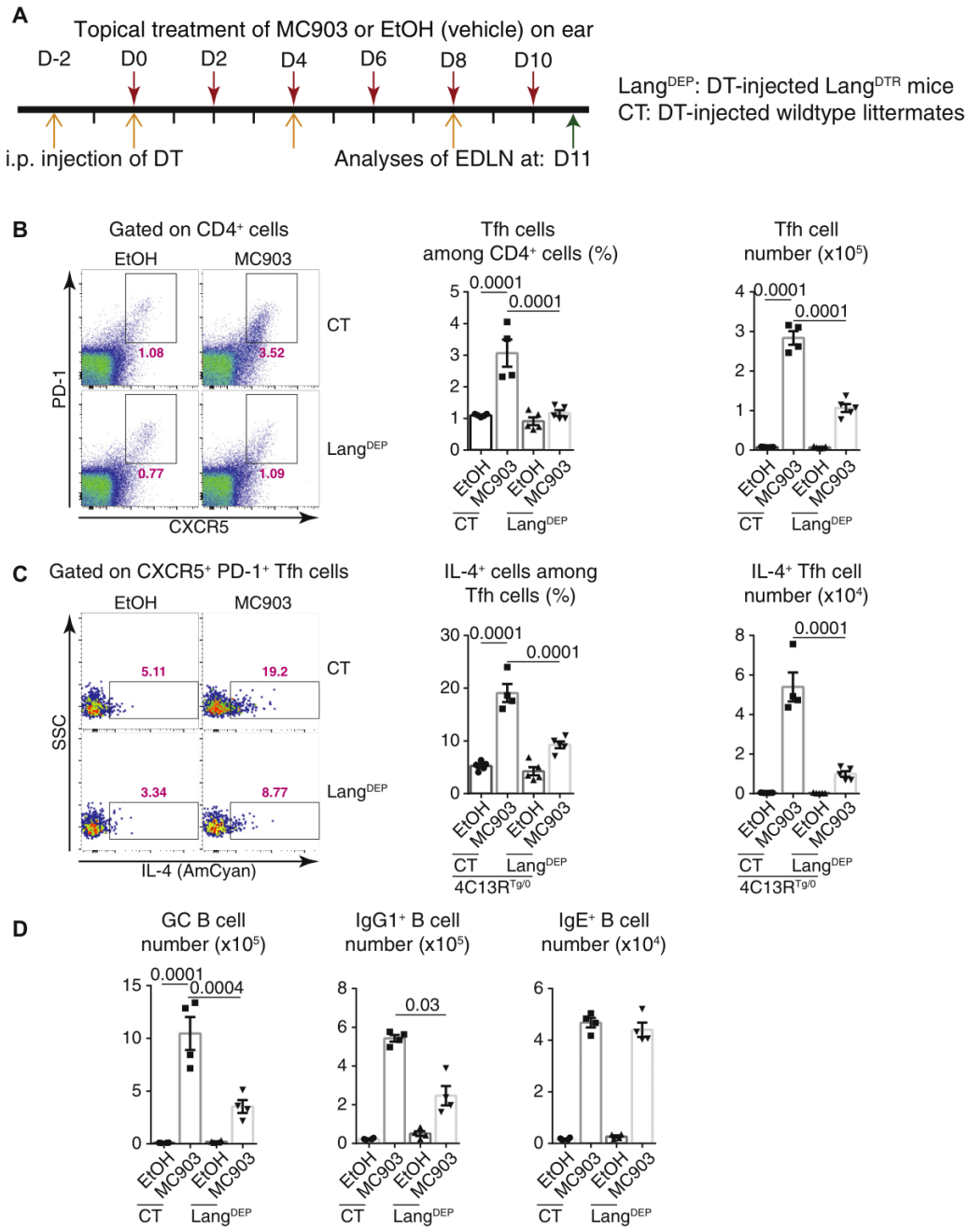
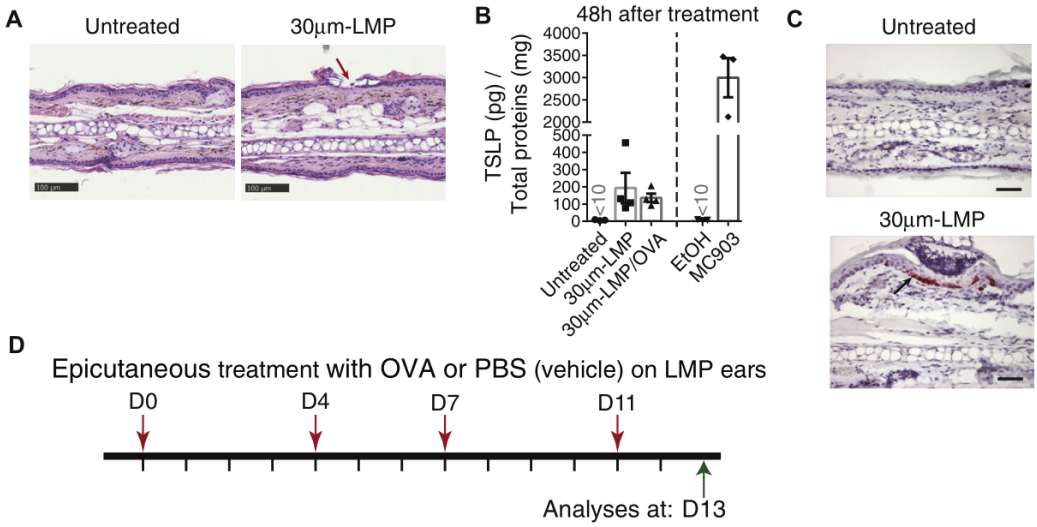
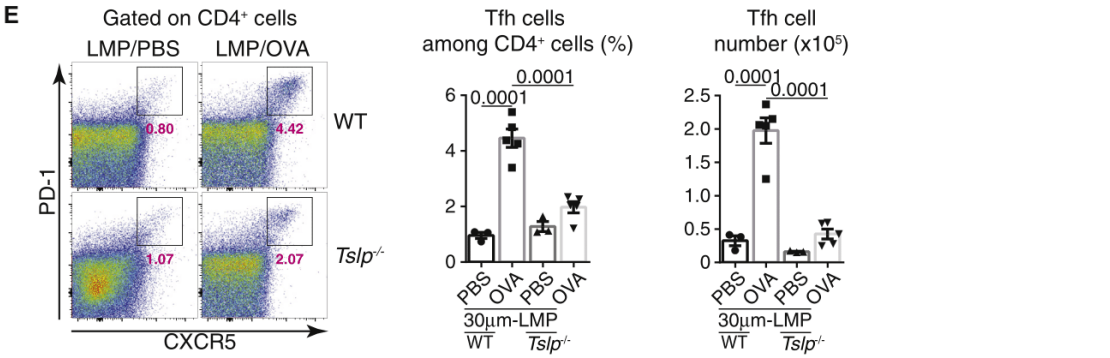
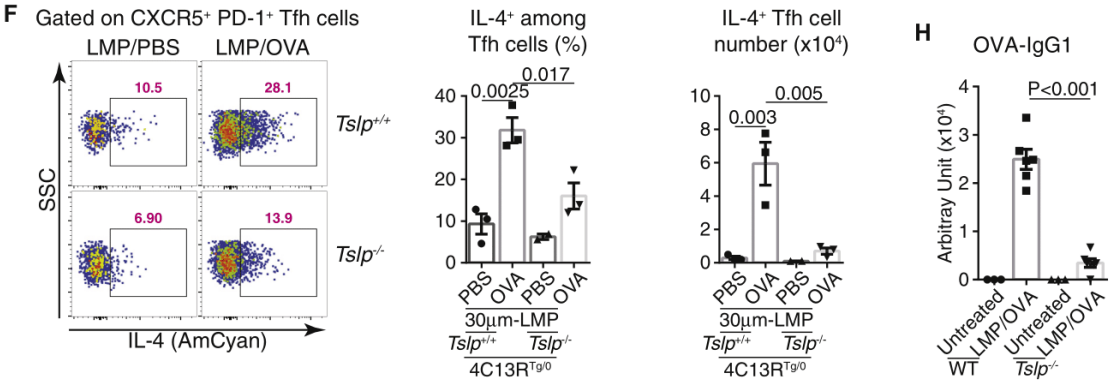


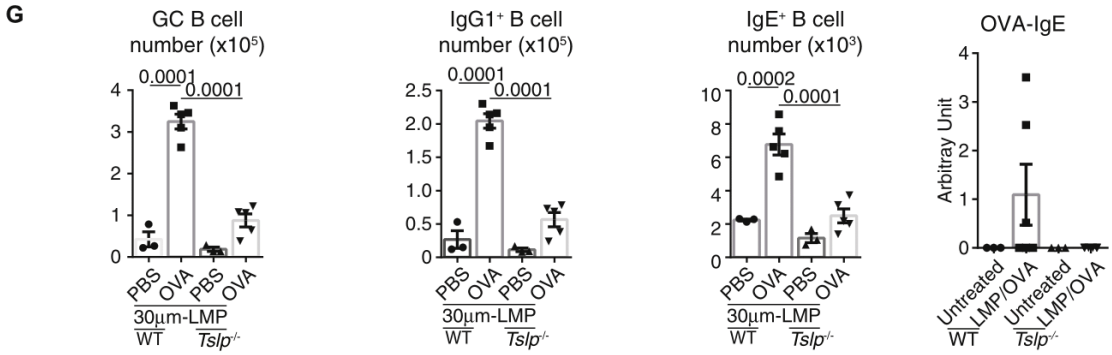
FIG 2. Depletion of Langerin$^+$ cells diminishes the MC903-induced T_{FH}/GC response. **A,** Experimental protocol. Lang^{DTR} mice and littermate CTs were intraperitoneally (*i.p.*) injected with DT at D-2 and D0 and then every 4 days. Mouse ears were topically treated with MC903 or EtOH every other day from D0 to D10 and EDLNs were analyzed at D11. **B,** Frequency and number of T_{FH}s. **C,** IL-4 (AmCyan) expression by T_{FH}s. **D,** Total number of GC, IgG_1^+, and IgE^+ B cells. Values shown are mean ± SEM; 1-way ANOVA with Tukey's multiple comparison *post hoc* test. Data are representative of 3 independent experiments with similar results.


A
Untreated
30µm-LMP
B
48h after treatment
TSLP (pg) / Total proteins (mg)
<10
Untreated
30µm-LMP
30µm-LMP/OVA
EtOH
MC903
C
Untreated
30µm-LMP

D
Epicutaneous treatment with OVA or PBS (vehicle) on LMP ears
D0
D4
D7
D11
Analyses at: D13


E
Gated on CD4+ cells
LMP/PBS
LMP/OVA
PD-1
CXCR5
0.80
4.42
1.07
2.07
WT
Tslp-/-
Tfh cells among CD4+ cells (%)
Tfh cell number (x10^5)
PBS
OVA
30µm-LMP
WT
Tslp-/-


F
Gated on CXCR5+ PD-1+ Tfh cells
LMP/PBS
LMP/OVA
SSC
IL-4 (AmCyan)
10.5
28.1
6.90
13.9
Tslp+/+
Tslp-/-
IL-4+ among Tfh cells (%)
IL-4+ Tfh cell number (x10^4)
4C13R Tg/0
H
OVA-IgG1
P<0.001
Arbitray Unit (x10^4)
Untreated
LMP/OVA


G
GC B cell number (x10^5)
IgG1+ B cell number (x10^5)
IgE+ B cell number (x10^3)
OVA-IgE
Arbitray Unit
PBS
OVA
30µm-LMP
WT
Tslp-/-

and IgE in sera.[33] Here, we developed a novel experimental protocol, in which Precise Laser Epidermal System (P.L.E.A.S.E., Pantec Biosolutions, Ruggell, Lichtenstein)[34] was used to disrupt skin barrier and to generate patterned micropores in mouse skin. This protocol allowed us to deliver allergens to micropores at precise depths of the epidermis, thereby achieving a higher efficiency and reproducibility of allergen sensitization through the skin compared with experiments based on TS. We showed that micropores at a depth of 30 μm (30-μm laser-assisted skin microporation [LMP]) on BALB/c WT mouse ears reached basal layer of ear epidermis (Fig 3, *A*). ELISA analyses indicated that the protein level of TSLP increased at 48 hours after treatment (Fig 3, *B*), in agreement with the previous studies showing that barrier disruption induces TSLP production in mouse[33] and human skin.[35] Notably, such level of TSLP was comparable to our previously reported TSLP level in TS skin,[33] although it was much lower compared with that of MC903-treated skin (Fig 3, *B*; see also Fig E13, *B* in this article's Online Repository at www.jacionline.org). The administration of OVA did not further induce the TSLP level (Fig 3, *B*). *In situ* hybridization showed that TSLP RNA expression was restricted to epidermal keratinocytes in LMP skin (Fig 3, *C*).

As expected, OVA treatment on LMP ears (LMP/OVA) (Fig 3, *D*) induced a T_H2-type skin inflammation in TSLP-dependent manner, showing that OVA sensitization–induced infiltration of eosinophils and basophils (Fig E4, A and B in this article's Online Repository at www.jacionline.org), T_H2 cytokines (IL-4 and IL-13) expression by T cells in the skin (Fig E4, *C*) and by $CXCR5^-CD4^+$ cells in EDLNs (Fig E4, *D*), were all abolished in mice lacking TSLP. Examination of EDLNs revealed that both frequency and number of T_{FH}s were increased in LMP/OVA− compared with in LMP/PBS–treated WT mice, and such increase was largely diminished in $Tslp^{-/-}$ mice (Fig 3, *E*). Note that LMP/PBS was not sufficient to induce T_{FH} differentiation (despite of the induction of TSLP), but LMP plus OVA promoted T_{FH}/GC response that was TSLP-dependent (Fig 3, *E*). Moreover, IL-4 production by T_{FH}s was augmented in LMP/OVA–treated $Tslp^{+/+}$/4C13R$^{Tg/0}$ mice but not $Tslp^{-/-}$/4C13R$^{Tg/0}$ mice (Fig 3, *F*). GC B-cell number analyzed by flow cytometry (Fig 3, *G*) and GC size analyzed by immunofluorescence (Fig E2, *B*) both showed an increase in LMP/OVA–treated WT mice, and this increase was abrogated in the absence of TSLP. IgG_1^+ and IgE^+ B-cell numbers were also increased in LMP/OVA–treated WT mice, and they were much lower in LMP/OVA–treated $Tslp^{-/-}$ mice (Fig 3, *G*). Accordingly, serum levels of OVA-IgG_1 and OVA-IgE were decreased in $Tslp^{-/-}$ mice compared with in WT mice on LMP/OVA treatment (Fig 3, *H*). Together, these results demonstrate that TSLP is crucially required for epicutaneous OVA sensitization–induced T_H2 and T_{FH}/GC responses.

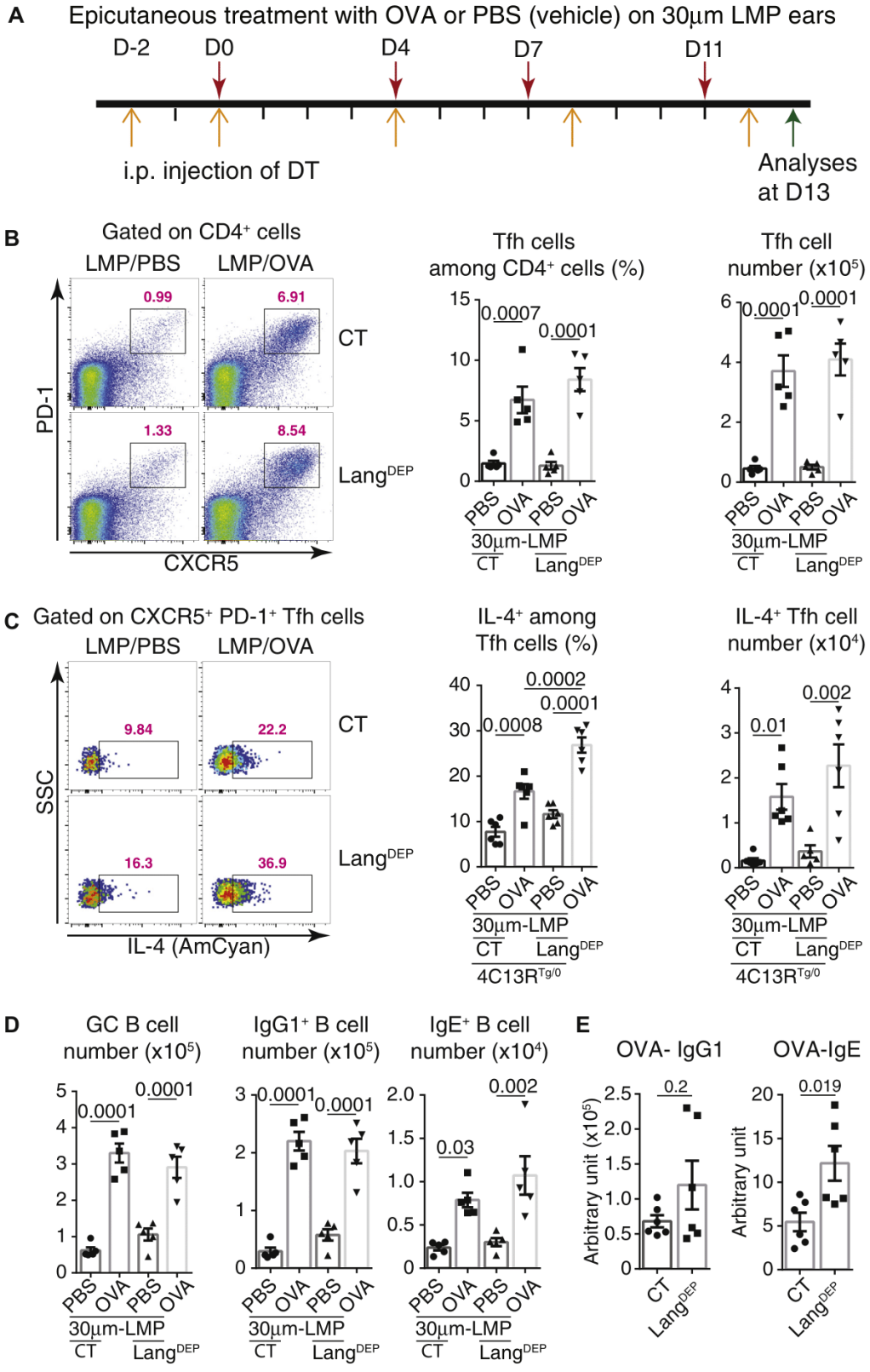
Langerin$^+$ DCs or LCs counteract the T_{FH}/GC response induced by epicutaneous OVA sensitization

Based on the above data from MC903-induced AD, we had expected that Langerin$^+$ DCs would be crucially required for epicutaneous OVA-induced T_{FH}/GC response. To our surprise, when subjected to 30-μm LMP/OVA sensitization (Fig 4, *A*), Lang^{DEP} mice did not exhibit a reduction in frequency and number of $CXCR5^+$ PD-1^+ T_{FH}s, instead they tended to be higher compared with frequency and number in CT mice (Fig 4, *B*). More strikingly, IL-4 expression by T_{FH}s was higher in EDLN from LMP/OVA–treated Lang^{DEP}/4C13R$^{Tg/0}$ mice (Fig 4, *C*). Accordingly, the GC, IgG_1^+, and IgE^+ B-cell numbers were not reduced in LMP/OVA–treated Lang^{DEP} mice (Fig 4, *D*), and serum OVA-specific IgE and OVA-specific IgG_1 were higher or tended to be higher (Fig 4, *E*). Thus, in contrast to our expectation, Langerin$^+$ DCs are not required for the T_{FH}/GC response in LMP/OVA–induced AD model; instead, they appear to play a counteracting role.

Because LCs are located on the suprabasal layer of the epidermis, we suspected that Langerin$^+$ cells would be only required in T_{FH} differentiation when allergens are encountered superficially on the skin. To test this possibility, LMP was performed at the depth of 11 μm, which disrupted only the cornified layer of the epidermis (Fig 5, *A*). We observed that the 11-μm LMP induced also the production of TSLP, even though its level was lower compared with that of 30-μm LMP (Fig 5, *B*). Treatment of CT ears with 11-μm LMP/OVA induced significant increases (although milder than those with 30-μm LMP/OVA) in T_{FH} frequency as well as GC B-cell number, which were all abolished in $Tslp^{-/-}$ mice (Fig 5, *C*), indicating that, despite a low induction of TSLP, the T_{FH}/GC response promoted by 11-μm LMP/OVA is still crucially dependent on TSLP. However, when Lang^{DEP} mice were subjected to 11-μm LMP/OVA treatment, they exhibited a significant increase in the frequency of T_{FH}s, in IL-4 expression by T_{FH}s, as well as in GC, IgG_1^+, and IgE^+ B-cell numbers in EDLNs (Fig 5, *D* and *F*), accompanied by augmented serum levels of OVA-IgG_1 and OVA-IgE (Fig 5, *G*). Similar results were also obtained with huLang^{DEP} mice (Fig 5, *H* and *I*), indicating that LCs significantly counteract the T_{FH}/GC response induced on the 11-μm LMP/OVA sensitization.

Furthermore, we sought to compare antigen-specific T_{FH}s between CT and huLang^{DEP} mice using an activation-induced marker assay.[36] In this assay, the stimulation of LN suspensions with specific antigen drives upregulation of CD154 (CD40L), CD25, and OX40 on T_{FH}s, providing a sensitive method for quantifying antigen-specific T_{FH}s in mice.[36] We showed that *in vitro* stimulation with OVA drove the upregulation of CD154, OX40, and CD25 in EDLN-derived T_{FH}s from

FIG 3. OVA sensitization through LMP skin induces TSLP-dependent T_{FH}/GC response. **A,** Hematoxylin and eosin staining of untreated or 30-μm LMP ears of BALB/c WT mice. The red arrow points to a micropore with the disruption of the epidermis. Bar = 100 μm. **B,** TSLP protein levels in ears of WT mice at 48 hours after the indicated treatment. **C,** RNAscope *in situ* hybridization for TSLP in untreated or 30-μm LMP ears at 48 hours after the microporation. The black arrow points to one of the positive signals. Bar = 50 μm. **D,** Experimental protocol for OVA epicutaneous sensitization through LMP ears. OVA or PBS (vehicle) were topically applied on LMP ears at D0, D4, D7, and D11 and EDLNs were analyzed at D13. **E** and **F,** Frequency and cell number of T_{FH}s **(E)** and IL-4 (AmCyan) producing T_{FH}s **(F)** in EDLNs. **G,** GC, IgG_1^+, and IgE^+ B-cell numbers in EDLNs. **H,** Serum levels of OVA-IgG_1 and OVA-IgE. Values shown are mean ± SEM; 1-way ANOVA with Tukey's multiple comparison *post hoc* test. Data are representative of 3 independent experiments with similar results.


A
Epicutaneous treatment with OVA or PBS (vehicle) on 30µm LMP ears
D-2
D0
D4
D7
D11
i.p. injection of DT
Analyses at D13
B
Gated on CD4+ cells
LMP/PBS
LMP/OVA
0.99
6.91
1.33
8.54
CT
LangDEP
PD-1
CXCR5
Tfh cells among CD4+ cells (%)
0.0007
0.0001
Tfh cell number (x10^5)
0.0001
0.0001
PBS OVA PBS OVA
30µm-LMP
CT
LangDEP
C
Gated on CXCR5+ PD-1+ Tfh cells
LMP/PBS
LMP/OVA
9.84
22.2
16.3
36.9
SSC
IL-4 (AmCyan)
IL-4+ among Tfh cells (%)
0.0002
0.0001
0.0008
IL-4+ Tfh cell number (x10^4)
0.002
0.01
4C13RTg/0
D
GC B cell number (x10^5)
0.0001
0.0001
IgG1+ B cell number (x10^5)
0.0001
0.0001
IgE+ B cell number (x10^4)
0.002
0.03
E
OVA- IgG1
0.2
Arbitrary unit (x10^5)
OVA-IgE
0.019
Arbitrary unit

LMP/OVA–sensitized CT mice, and such upregulation was significantly higher in T_{FH}s from LMP/OVA–sensitized huLang^{DEP} mice (Fig 5, *J*), thus indicating a stronger OVA-specific T_{FH} differentiation in huLang^{DEP} mice on OVA sensitization.

Together, these data indicate that LCs suppress the TSLP-dependent T_{FH}/GC response in epicutaneous OVA-sensitization model.

Langerin$^+$ DCs or LCs limit epicutaneous OVA-induced T_H2 skin inflammation and the subsequent asthma

Having observed the opposite role of Langerin$^+$ DCs or LCs in T_{FH}/GC response in the 2 mouse AD models, we further explored their involvement in the induction of T_H2 response. On MC903 treatment, Lang^{DEP}/4C13R$^{Tg/0}$ mice exhibited a slight decrease in IL-4 and a tendency of decrease in IL-13 production by CXCR5$^-$CD4$^+$ cells in EDLN (see Fig E5, *A* in this article's Online Repository at www.jacionline.org), or by TCRβ^+ cells in dermis (Fig E5, *B*), which suggests a role, even though minor, for Langerin$^+$ DCs in the development of T_H2 response. In contrast, on 30-μm LMP/OVA treatment, Lang^{DEP}/4C13R$^{Tg/0}$ mice exhibited a higher T_H2 response in both skin (Fig 6, *A*) and EDLN (see Fig E6 in this article's Online Repository at www.jacionline.org). This was in accordance with the observation that LMP/OVA–sensitized Lang^{DEP} mice exhibited a stronger skin inflammation (Fig 6, *B*), accompanied with an increase in eosinophils and basophils (Fig 6, *C*). Moreover, when subjected to 11-μm LMP/OVA sensitization, both Lang^{DEP} and huLang^{DEP} mice, compared with CT mice, exhibited an enhanced AD-like skin inflammation (see Fig E7 in this article's Online Repository at www.jacionline.org). Therefore, contrary to their minor role in promoting T_H2 response in MC903-AD, LCs suppress the T_H2 response in OVA-AD.

We further examined whether Langerin$^+$ DCs limit the atopic march. On intranasal OVA challenge following epicutaneous allergen sensitization (Fig 6, *D*), the Lang^{DEP} mice, compared with CT mice, developed a much stronger asthmatic inflammation, exhibiting an increase in the number of eosinophils in BAL (Fig 6, *E*), and in RNA expression of T_H2 cytokines IL-4, IL-5, and IL-13, as well as chemokine receptor CCR3 (eosinophils) and MCPT8 (basophils) by BAL cells (Fig 6, *F*). In addition, hematoxylin and eosin staining of lung sections of OVA-treated Lang^{DEP} mice revealed an increased peribronchial and perivascular infiltration, and periodic acid–Schiff staining showed enhanced goblet cells hyperplasia (Fig 6, *G*). Similar results were obtained with huLang^{DEP} mice (see Fig E8, *A-F* in this article's Online Repository at www.jacionline.org), indicating that LCs counteract the asthma development following epicutaneous allergen sensitization. To exclude the possibility that the enhanced asthmatic inflammation is due to any depletion of lung DCs during the intranasal challenge, we subjected huLang^{DEP} mice to intraperitoneal sensitization with OVA/alum and intranasal OVA challenge, and we observed that these mice developed similar asthmatic inflammation as CT mice did (Fig E8, *G* and *H*). This suggests that the limitation of asthma inflammation by LCs is indeed due to their role in suppressing the epicutaneous allergen sensitization.

Taken together, these studies reveal opposite roles of LCs in 2 AD models: in MC903-AD, LCs play an important role in priming T_{FH}/GC response; they participate but to a lesser extent in promoting T_H2 responses. In OVA-AD, LCs are neither required for T_{FH}/GC nor T_H2 responses, instead, they suppress OVA-induced T_{FH}/GC and T_H2 responses as well as the atopic march.

Langerin$^+$ migratory DCs from MC903-AD but not from OVA-AD mice present profound transcriptomic changes

We next conducted transcriptomic studies to explore molecular insights underlying the opposite roles of Langerin$^+$ DCs in T_{FH} and T_H2 differentiation in MC903-AD and OVA-AD, by taking use of Lang^{GFP} mouse line in which green fluorescent protein (GFP) reports the expression of Langerin.[30] Lang^{GFP} mice were treated with MC903 (at D0, D2, and D4) or LMP/OVA (at D0 and D3), and at D5. Langerin$^+$ (GFP^{pos}) and Langerin$^-$ (GFP^{neg}) migratory DCs (migDCs) were sorted from EDLNs of nontreated (NT), MC903-, or LMP/OVA–treated mice and proceeded to mRNA sequencing (see Fig E9, *A* in this article's Online Repository at www.jacionline.org). The D5 time point was selected to compare gene expression patterns of migDCs at the initiation stage of T_{FH} and T_H2 differentiation.

Principle component analysis for the RNA-sequencing data revealed that the Pos_MC (GFP^{pos} migDCs from MC903-treated Lang^{GFP} mice) was clearly separated from the Pos_NT (GFP^{pos} migDCs from NT Lang^{GFP} mice); however, the Pos_OVA (GFP^{pos} migDCs from LMP/OVA–treated Lang^{GFP} mice) was inseparable from the Pos_NT (Fig 7, *A*). Correspondingly, analyses of differentially expressed genes (DEGs) in Pos_MC versus Pos_NT identified 756 upregulated and 559 downregulated genes (with a fold change >1.5 and adjusted $P < .05$) (Fig 7, *B*); in a sharp contrast, the comparison of Pos_OVA versus Pos_NT revealed only 39 upregulated and 9 downregulated genes (Fig 7, *B*). Therefore, in MC903-AD, Langerin$^+$ migDCs undergo profound transcriptomic changes, but in OVA-AD, they present almost no, or very few, transcriptomic changes.

As to Langerin$^-$ migDCs, principle component analysis showed that Neg_MC (GFP^{neg} migDCs from MC903-treated Lang^{GFP} mice), Neg_OVA (GFP^{neg} migDCs from LMP/OVA-treated Lang^{GFP} mice), and Neg_NT (GFP^{neg} migDCs from NT Lang^{GFP} mice) were all clustered away from each other (Fig 7, *A*). Analyses of DEGs identified 710 upregulated and 698 downregulated genes for Neg_MC versus Neg_NT; and 431 upregulated and 427 downregulated genes for Neg_OVA versus Neg_NT (Fig 7, *B*), suggesting that Langerin$^-$ migDCs present major transcriptomic changes in both MC903-AD and OVA-AD, with considerable numbers of overlapped DEGs (249 upregulated and 215 downregulated).

FIG 4. Depletion of Langerin$^+$ cells does not reduce but rather tends to augment 30-μm LMP/OVA–induced T_{FH}/GC response. **A,** Experimental protocol. Lang^{DTR} mice and littermate CTs were intraperitoneally injected with DT at D-2, D0, and then every 4 days. Mouse ears were treated by 30-μm LMP/OVA or 30-μm LMP/PBS at D0, D4, D7, and D11, and EDLNs were analyzed at D13. **B,** Frequency and number of T_{FH}s. **C,** IL-4 (AmCyan) expression by T_{FH}s. **D,** Number of GC, IgG$_1^+$, and IgE$^+$ B cells. **E,** Serum levels of OVA-specific IgG$_1$ and OVA-specific IgE in 30-μm LMP/OVA–sensitized Lang^{DEP} or CT mice. Data are means± SEM. **B-D,** One-way ANOVA with Tukey's multiple comparison *post hoc* test. **E,** Unpaired *t*-test with Welch's correction. Data are representative of 3 independent experiments with similar results.

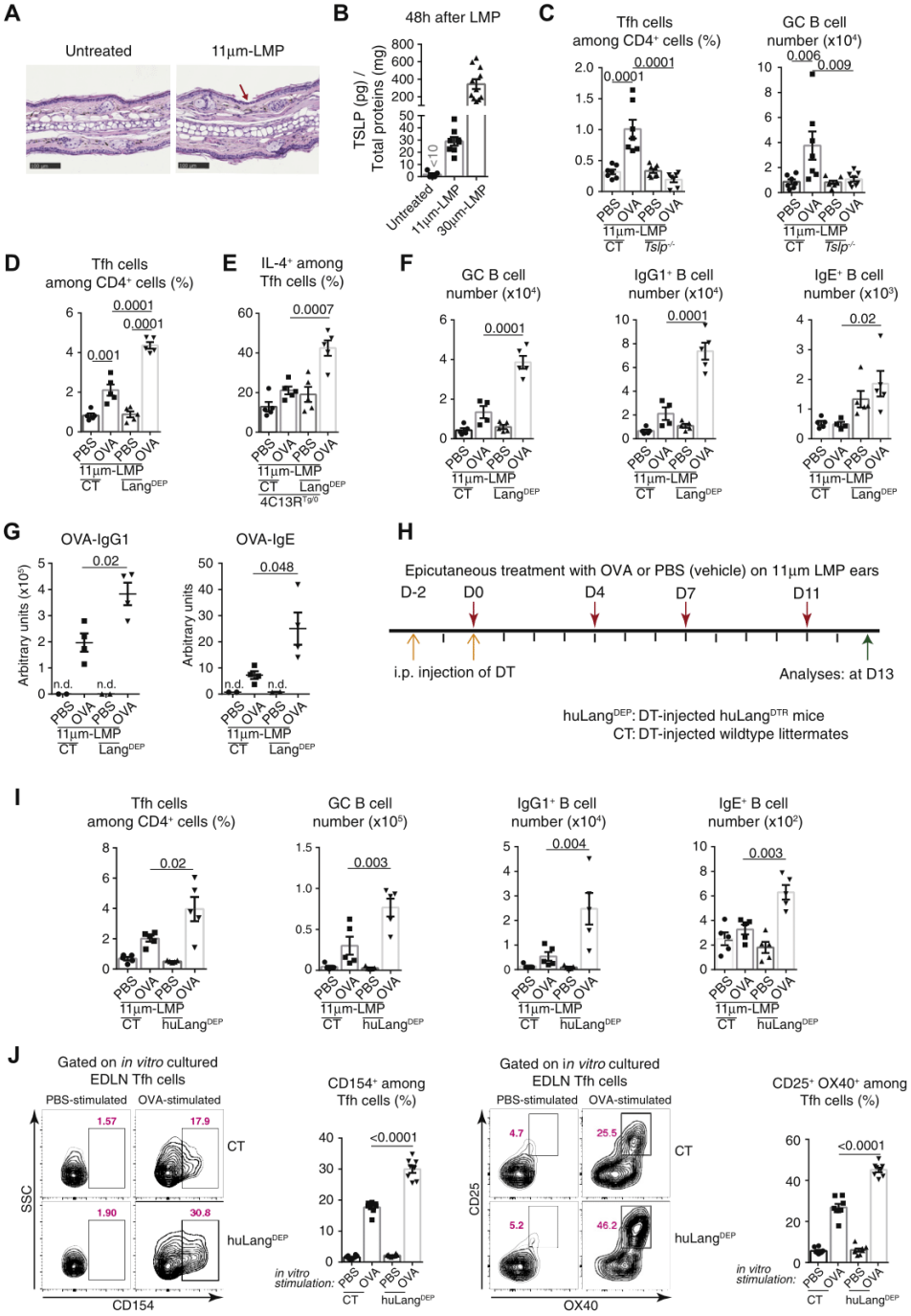


FIG 5. Depletion of Langerin$^+$ cells or LCs enhances 11-µm LMP/OVA–induced TSLP-dependent T_{FH}/GC response. **A,** Hematoxylin and eosin staining of untreated or 11-µm LMP ears of BALB/c WT mice. The red arrow points to a micropore with the impairment of cornified layer. Bar = 100 µm. **B,** TSLP protein levels in ears of WT mice. **C,** Comparison of T_{FH}s and GC B cells in EDLNs from WT or *Tslp*$^{-/-}$ mice. **D-F,** Comparison of T_{FH}s **(D),** IL-4 (AmCyan) expression by T_{FH}s **(E),** and number of GC, IgG$_1^+$, and IgE$^+$ B cells **(F)** in EDLNs from CT or Lang^{DEP} mice. **G,** Serum OVA-IgG$_1$ and OVA-IgE levels. **H,** Experimental protocol. **I,** Comparison of T_{FH}s, GC, IgG$_1^+$, and IgE$^+$ B cells in CT and huLang^{DEP} mice. **J,** Comparison of antigen-specific T_{FH}s between LMP/OVA–sensitized CT and huLang^{DEP} mice. EDLNs were *in vitro* stimulated with OVA or PBS (vehicle control), and activation markers CD154, CD25, and OX40 expressed by EDLN-derived T_{FH}s were examined. Values shown are mean ± SEM; 1-way ANOVA with Tukey's multiple comparison *post hoc* test. Data are representative of 2 independent experiments with similar results.

Gene ontology analyses of DEGs in Langerin$^+$ migDCs from MC903-treated mice

Next, using the upregulated or downregulated DEGs identified in Pos_MC (vs Pos_NT) as input, we performed cluster analyses of all the groups and generated heat maps to visualize trends of expression for genes across the different groups. Results are presented in Fig E9, *B* and Fig E10, *A* in this article's Online Repository at www.jacionline.org. Further, we performed gene ontology analyses of the upregulated genes in Pos_MC (Fig 7, *C*), and examined whether these genes were also significantly upregulated in Neg_MC (vs Neg_NT), and Neg_OVA (vs Neg_NT). We paid particular attention to the upregulated genes shared in all the 3 groups (Pos_MC, Neg_MC, and Neg_OVA), standing here for "commonly upregulated" genes (highlighted in red in Fig 7, *C*), as they could be implicated in TSLP-promoted T_{FH} and/or T_H2, a common feature shared by MC903-AD and OVA-AD. Among them, we found genes related to (1) regulation of cell migration, many of which were reported to facilitate DC migration: *Mmp14,*[37] *Stat5*,[38] *Nrp2*,[39] *Sema7a*[40]; (2) T-cell costimulation: *Cd80* and *Cd86,*[41] *Il2ra,*[42] *Pdcd1lg2*,[43] *Cd274*, *Gpr183*;[44,45] (3) cytokine signal: *Il2ra*, *Tnfrsf11b,* and *Ccl22*; and (4) transcription factors, such as *Ikzf4, Irf4, Stat4,* and *Stat5a*.

We examined the TSLP signaling pathway among the upregulated genes in Pos_MC. Using the reported TSLP-regulated gene set,[46] we identified *Cd84, Cd82, Ccl17, Ccl22,* and *Tnfrsf11b* (in the cluster with higher expression in Pos_MC than Neg_MC), as well as *Cish, Cd86, Cd80, Cd274, Il2ra, Il6, Ccr2,* and *Tgfb1* (in the cluster with higher expression in Neg_MC than Pos_MC) (Fig 7, *D*). In addition, we identified *Irf4*, which has been recently shown to be downstream of TSLP signaling in human migratory LCs.[47] The upregulation of these TSLP-targeting genes by Langerin$^+$ migDCs suggests that these cells could be a direct responder to TSLP signaling, although it remains to be demonstrated that TSLP signals through its receptor on LCs drive their migration/activation. Besides these known TSLP downstream genes, more TSLP pathway genes identified from the group of commonly upregulated genes can be envisaged.

Interestingly, we did not find *Tnfsf4* (encoding OX40L) among the DEGs in Pos_MC, despite that OX40L was reported to be a TSLP-responsive gene and mediated TSLP-promoted T_H2[48] and T_{FH}[49] differentiation. Actually, OX40L expression by GFP^{pos} cells was barely detected in Pos_NT, Pos_MC, or Pos_OVA (Fig 7, *E*). On the other hand, OX40L was expressed in Neg_NT, and its expression was further upregulated in Neg_MC and Neg_OVA. Therefore, it is unlikely that OX40L would be responsible for the T_{FH}-promoting function of Langerin$^+$ DCs, while its precise function as a potential TSLP downstream factor in Langerin$^-$ DCs remains to be defined (Fig 7, *E*).

Among the above-mentioned TSLP-regulated genes, IL-6 has been shown to be a critical cytokine for T_{FH} differentiation.[50,51] We thus tested whether IL-6 neutralization decreases T_{FH}/GC response in MC903-AD. Results showed that IL-6 was not required for the initiation of T_{FH} differentiation and the overall GC reaction (see Fig E11 in this article's Online Repository at www.jacionline.org), although it is possible that its function in T_{FH} response is redundant with other signals as suggested by Eto et al.[52] Besides IL-6, several other T_{FH}-promoting factors derived from DCs have been recently reported, including IRF4,[53] IL-2Ra,[42,54] and EBI2 (*Gpr183*),[44,45] whose expression was all commonly upregulated in Pos_MC, Neg_MC, and Neg_OVA (Fig 7, *D*). The role of these potential candidates in TSLP-promoting T_{FH} differentiation remains to be examined.

Finally, among the downregulated genes (Fig E10, *B*), less knowledge was available, but we could see *Il12b* (IL-23/IL-12p40), whose expression in DCs was previously reported to be suppressed by TSLP.[55] Other commonly downregulated ones included genes related to the following: T-cell costimulation: *Havcr2*, *Lgals8*; regulation of cell migration: *Adam15* and *Ptk2* (negative regulators for cell migration); and regulation of transcription: *Foxc2, Thrb, Tcf7l2, Ehf,* and *Lmo2*.

DISCUSSION

In this study, we analyzed how T_{FH}s were generated in 2 experimental AD mouse models, triggered by $TSLP^{over}$ by topical application of MC903, or induced by epicutaneous OVA sensitization. We demonstrated a crucial role for TSLP in promoting T_{FH}s and GC response in MC903-AD as well as OVA-AD. Intriguingly, we revealed a dual function of LCs in TSLP-promoted T_{FH}/T_H2 differentiation: while they promoted T_{FH} differentiation in MC903-AD, they inhibited T_{FH}/GC response and suppressed T_H2 skin inflammation and the atopic march in OVA-AD.

TSLP: Critical player for T_H2 and T_{FH} response in AD

It has been recognized that TSLP is overproduced in AD lesional skin;[56] however, its expression varies from high to low, which could be related with the cause (eg, genetic mutation of *Spink5,* which induces a high level of TSLP,[57] vs skin barrier impairment, which induces a low level of TSLP[35]), age (eg, TSLP serum level in AD children is high at early stage and decreases with age[58]), or the nature of disease (eg, intrinsic or extrinsic AD). Our study demonstrates that in AD models associated with either high or low TSLP expression, TSLP is crucial for promoting T_{FH}/GC response in AD. Recently, the link between TSLP and T_{FH} differentiation was suggested by the study with human blood DC–T-cell coculture system.[49] Thus, the T_{FH}-promoting function of TSLP appears to be conserved between mouse and human, which suggests that it is relevant and valuable to employ AD mouse models to elucidate mechanisms underlying the TSLP (skin)–T_{FH} (draining LN) axis, particularly as the access of tissue and lymphoid organs is rather limited in human study.

Our data add new evidence that neutralization of TSLP or blocking the TSLP downstream pathway will be helpful for reducing T_H2 and T_{FH} responses in AD. Notably, TSLP is crucial for driving the downstream IL-4/IL-13 expression by T_H2s, as well as IL-4 expression by T_{FH}s. Indeed, blocking antibody against IL-4/-13R (dupilumab), which may actually target both T_H2 and T_{FH} responses, has been shown to achieve significant therapeutic effect on AD.[59] Intriguingly, the neutralization TSLP antibody tezepelumab has been demonstrated to significantly reduce annual asthma exacerbation rate in patients with uncontrolled asthma.[60] A recent study with tezepelumab showed numeric improvements in patients with moderate to severe AD, despite that there were certain limitations in that study, including patient selection, use of topical corticosteroids,

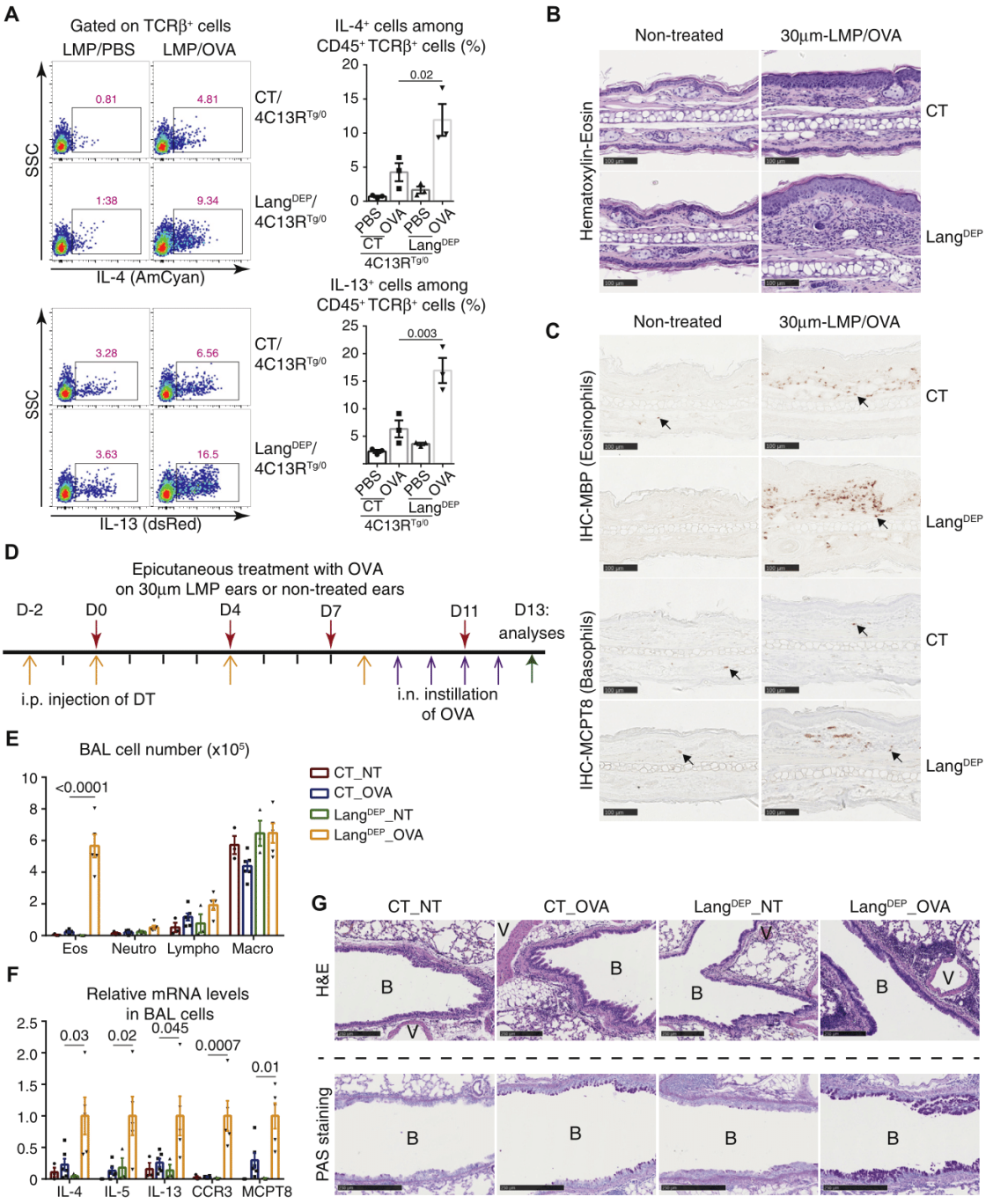


FIG 6. Langerin^{+} cells counteract LMP/OVA sensitization–induced skin T_H2 inflammation and the subsequent asthmatic phenotype. **A,** IL-4 (AmCyan) and IL-13 (dsRed) expression in TCRβ^{+} dermal cells. **B,** Hematoxylin and eosin (*H&E*) staining of mouse ears. **C,** Immunohistochemistry staining of mouse ears with anti-MBP (for eosinophils) or anti-MCPT8 (for basophils). Arrows point to positive signals. **D,** Experimental protocol for OVA epicutaneous sensitization and airway challenge. Mice were intraperitoneally injected with DT at D-2, D0, and then every 4 days. Mice were either treated with OVA on LMP ears at D0, D4, D7, and D11 or were NT. All mice were subjected to intranasal instillation with OVA from D9 to D12 and analyzed at D13. **E,** Differential cell counting for eosinophils (*Eos*), neutrophils (*Neutro*), lymphocytes (*Lympho*), and macrophages (*Macro*) in BAL. **F,** RNA levels of indicated genes in BAL cells by quantitative RT-PCR. **G,** Lung sections were stained with H&E for histology or periodic acid–Schiff (*PAS*) for goblet cell hyperplasia analyses. Bar = 100 μm. Values shown are mean ± SEM; 1-way ANOVA with Tukey's multiple comparison *post hoc* test. Data are representative of 2 independent experiments with similar results. *B,* Bronchiole; *MBP,* major basic protein; *V,* blood vessel.

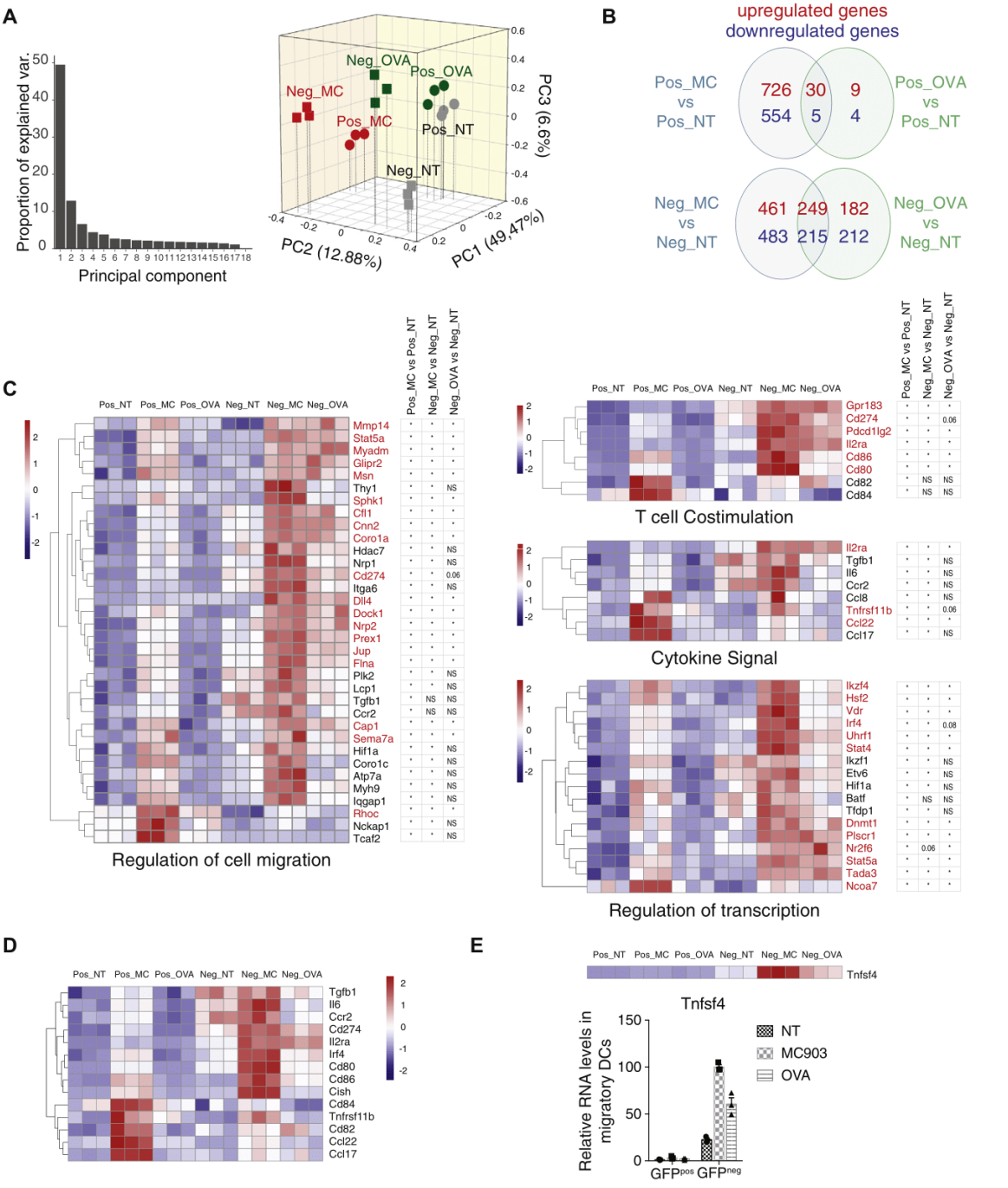


FIG 7. Transcriptomic analyses of migDCs in EDLNs of Lang^{GFP} mice on MC903 treatment or epicutaneous OVA sensitization. Lang^{GFP} mice were treated with MC903 at D0, D2, and D4 or 30-μm LMP/OVA on D0 and D3; EDLNs were collected at D5 for cell sorting and RNA-sequencing analyses. **A,** Left, percentage of variability explained by each principal component (*PC*). Right, principal component analyses showing PC1, PC2, and PC3. **B,** Venn diagram showing the number of upregulated and downregulated genes (fold change > 1.5; $P < .05$; raw read > 200 in at least 1 sample of all groups), and the number of commonly upregulated or downregulated genes between the comparisons, as indicated. **C,** Selected genes corresponding to gene ontology terms. *$P < .05$. **D,** Heat maps of the reported TSLP pathway genes, which are significantly upregulated in Pos_MC versus Pos_NT. **E,** Heat map of Tnfsf4 (encoding OX40L) from RNA-sequencing data and quantitative RT-PCR analyses. *NS,* Nonsignificant.

duration of treatment, and uncertain inhibition of TSLP with the dose used.[61] Given the preclinical evidence for the role of TSLP in AD, more clinical studies are required to evaluate TSLP as a therapeutic target in AD.

It should be also noted that recent studies have recognized the importance of T_{FH}s in AD,[19-21] but the *in vivo* function of T_{FH}s remains to be further delineated using AD mouse models. This is challenged by the lack of appropriate tools to deplete T_{FH}s. We are under the way to generate mouse line in which DTR can be selectively expressed in T_{FH}s, thus allowing the DT-induced depletion of T_{FH}s.

LCs: Function as migDCs to promote T_{FH} differentiation

LCs represent one of the most studied but controversial DC subtypes. Our study shows that LCs are importantly engaged in the initiation T_{FH} differentiation and GC response triggered by $TSLP^{over}$ in MC903-AD. This provides new evidence on the T_{FH}-promoting function of LCs in AD, in addition to several studies reporting the requirement of LCs for humoral responses in other contexts.[62-64] In MC903-AD, we observed that LCs play a dominant role in T_{FH} differentiation, although dermal $Langerin^-$ DCs may also contribute. On the other hand, $Langerin^-$ DCs (cDC2) appear to be the major player for the $TSLP^{over}$-induced T_H2, while LCs have a somewhat but minor contribution. Nevertheless, to provide direct evidence for the contribution of cDC2 in TSLP-driven T_{FH} and T_H2 responses in AD, further studies could be performed using DC-specific knockout of IRF4 or Dock8 mice, which have impaired development and migration of $CD11b^+$ cDC2,[65] or CD301b-DTR mice in which $CD301b^+$ cDC2 can be transiently depleted.[66]

There have been long debates on the migration, antigen uptake, and T-cell differentiation of LCs in different contexts; but transcriptomic study on migratory LCs in skin-draining LNs under inflammatory pathological contexts was lacking. Our transcriptomic data are therefore of value; however, a drawback is that migratory LCs and cDC1 were not separated in $Langerin^+$ (GFP^{pos}) migDCs, thus the gene expression data still need to be cautiously interpreted concerning LCs. Nevertheless, we have shown that $Langerin^+$ migDCs in EDLN of MC903-induced $TSLP^{over}$ mice presented substantial transcriptional changes, suggesting that the activation and migration of $Langerin^+$ DC to the draining LNs underlie its function to prime T_{FH} differentiation in MC903-AD. Indeed, when comparing numbers of GFP^{pos} and GFP^{neg} migDCs in EDLN of MC903-treated $Lang^{GFP}$ mice at D5, we observed that both were increased (see Fig E12 in this article's Online Repository at www.jacionline.org), supporting that both $Langerin^+$ DCs and $Langerin^-$ DCs migrate to draining LNs in MC903-AD.

Do LCs function as nonmigratory cells in the skin to suppress T_{FH}/T_H2 response?

Our study revealed a suppressive role of LCs for epicutaneous OVA-induced T_{FH} and T_H2 differentiation. This is in contrast with 2 previous studies that reported a role of LCs in provoking AD inflammation by using a TS/OVA-sensitization model.[67,68] To examine whether the discrepancy is due to the different effects of LMP compared with TS, we performed TS/OVA sensitization on mouse ears. Results showed that, similar to LMP/OVA, TS/OVA–sensitized $Lang^{DEP}$ mouse EDLNs exhibited increased frequency and number of T_{FH}s, increased IL-4 expression by T_{FH}s, higher numbers of GC, IgG_1^+, and IgE^+ B cells, with elevated OVA-IgG_1 and OVA-IgE in sera (Fig E13, *A-G*). Moreover, when intranasally challenged with OVA, $Lang^{DEP}$ mice developed a stronger asthmatic inflammation (Fig E13, *H* and *I*). Therefore, the discrepancy with the previous reports[67,68] is not explained by the difference of LMP versus TS technique in epicutaneous OVA sensitization; rather, it could be due to other factors yet to be determined, such as the allergen application method: topical OVA versus long exposure (2 days) of OVA placed on patch-test tape; the difference of mouse background: BALB/c versus C57BL/6; or the site of allergen application: ear versus back.

Why are LCs not implicated in the promotion of T_{FH}/T_H2 differentiation in EDLN in this context? Transcriptomic analyses showed that in sharp contrast to MC903-AD, $Langerin^+$ migDCs in OVA-AD presented almost the same transcriptomic program as in untreated mice, suggesting an absence of migration/activation of these cells. Indeed, $Langerin^+$ migDC numbers in EDLNs from LMP/OVA–treated or TS/OVA–treated mice at D5 were nearly unchanged, whereas $Langerin^-$ migDC number was increased (Fig E12). This is in agreement with previous studies showing that when skin was treated with fluorescence-conjugated OVA,[66] house dust mites,[69] or dextran,[70] antigen uptake and transport to draining LNs were mainly exerted by $Langerin^-$ DCs. Of note, it was recently shown that LCs can transfer antigen to cDC2 in the context of Langerin mAb-mediated targeting.[71] It will be interesting to see whether this occurs in AD models, and whether efficiency of LC antigen transfer could be altered in the 2 models, as another possible explication of different implication of LCs in T_{FH} differentiation.

Then how do LCs exert their anti-T_{FH}/T_H2 role in OVA-AD? A recent study showed that LCs played an immunosuppressive role when OVA was applied on the intact skin, in company with the induction of IL-10 in LCs in skin-draining LNs.[72] However, this does not seem to be our case, because $Langerin^+$ migDCs in EDLN did not exhibit any transcriptional change of regulatory T cell–inducing signals including IL-10 and TGF-β, or ALDH1A2. More likely, the anti-T_{FH}/T_H2 effect of LCs is related to their immune suppression function *in situ* in the skin, in keeping with LC ontogeny not only as DCs but also as nonmigratory macrophages.[73,74] It should be further studied how LCs exert such functionality, for example, by limiting the antigen-uptake by cDC2 in the skin, or by promoting local regulatory T cells in OVA-sensitized skin.[75,76] Transcriptomic analysis of LCs isolated from the OVA-treated skin site may provide further molecular insights.

Which signals switch the function of LCs?

One intriguing question is what microenvironment cues and molecular signals switch the function of LC between anti-T_{FH}/T_H2 to pro-T_{FH}/T_H2 in AD contexts. Notably, MC903-AD and OVA-AD exhibit a similar AD phenotype that is TSLP-dependent, but the quantity of TSLP and the nature of antigen are different in these 2 models. In MC903-AD, MC903 induced a high production of TSLP,[7] which was sufficient to induce T_{FH} and T_H2 differentiation. As there was no administration of experimental allergen, the nature of antigen

implicated in T-cell differentiation in the MC903 model may involve endogenous antigens or microbiota coexisting in the skin. On the other hand, in OVA-AD, the disruption of skin barrier with LMP induced TSLP expression, however, to a much lower extent. It is possible that LCs sense the quantity of TSLP. Indeed, as a danger signal, TSLP may convert the function of LCs when its level is above a certain threshold. *In vitro* studies have shown that TSLP triggers DC migration[77] or promotes the survival, maturation, and migration of human LCs, and allogenic naïve $CD4^+$ T cells cocultured with TSLP-conditioned LCs produced cytokines IL-4 and IL-13,[78] but quantitative study on TSLP signaling has never been performed. It will be interesting to explore whether and how quantitative TSLP signaling determines the role of LCs, by conducting *in vivo* or *ex vivo* dose-dependent experiments. In addition, the nature and quantity of antigens can be also involved in the functional switch of LCs. To unravel such complexity, the emerging mathematic modeling[79,80] may eventually help to integrate multiple parameters for a better understanding of functional switch of LCs.

It will be interesting to further explore in AD patients whether and how TSLP levels are correlated with the states and function of LCs. A better understanding of what molecular switch determines the function of LCs either as pro-T_{FH}/T_H2 or as anti-T_{FH}/T_H2, and of how LCs exert such functions, will allow us to shape LCs to act in suppressing the skin inflammation, limiting the allergen sensitization through AD skin, thus preventing the progression from AD to asthma. On the other hand, the potential of LCs to induce T_{FH} differentiation and GC response and the subsequent induction of antigen-specific antibodies has been of interest for transcutaneous vaccination.[63,81] Therefore, the knowledge we obtain from this study should also be insightful for LC-based skin vaccination, including the use of TSLP at an appropriate level as an effective adjuvant for promoting T_{FH} differentiation and humoral response.

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Key messages

- **TSLP is critically involved in mounting T_{FH}/GC response in mouse AD driven by MC903 or OVA sensitization.**
- **LCs promote T_{FH}/GC response in MC903-induced AD.**
- **LCs suppress T_{FH}/GC response and T_H2 skin inflammation in OVA sensitization–induced AD.**

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Supplementary information

Methods

Experimental mice. Balb/c mice were purchased from Charles River Laboratory. T*slp*$^{-/-}$ [1], 4C13R$^{Tg/0}$ [2], Langerin^{DTR} [3] and huLangerin^{DTR} [4] were as described and were all backcrossed to >99.9 % Balb/c genetic background. Lang^{GFP} reporter mice [3] were in C57BL/6J background. Breeding and maintenance were performed under institutional guidelines, and all of the animal experiments were approved by the animal care and ethics committee of animal experimentation of the IGBMC.

MC903 topical application. MC903 (Calcipotriol, Sigma) was dissolved in 100% ethanol and topically applied on mouse ears (2 nmol in 25 µl per ear) as previously described [5].

Epicutaneous OVA sensitization and airway challenge. Laser-assisted skin microporation (LMP) was performed using P.L.E.A.S.E.® research system (Pantec Biosolutions) on the dorsal side of mouse ears. For the depth of 30µm (30µm_LMP): 2 pulses per pore, with fluence of 7.5 J/cm2, pulse length of 75 µs, RepRate of 500 Hz and power of 1.0 W; for the depth of 11µm (11µm_LMP): 1 pulse per pore with fluence of 1,8 J/cm2, pulse length of 50 µs, RepRate of 500 Hz and power of 0.7 W. In all cases, the pore array size was set 14 mm and the pore density was set 15%. To induce epicutaneous OVA sensitization, 10 µl of sterile PBS solution containing 200 µg of OVA (Sigma-aldrich) were applied immediately on LMP ear skin at the time points indicated in experimental schemes in the Figures. In case of airway challenge, 25 µL of saline solution containing 50 µg of OVA was intranasally instilled.

Depletion of Langerin$^+$ DCs or LCs in mice. Lang^{DTR} or huLang^{DTR} mice were intraperitoneally injected with diphtheria toxin (DT; Sigma-Aldrich) (1 μg per 25 g body weight) at the time points indicated in the experimental schemes in the Figures. The DT-injected wild-type littermate mice were used as controls.

Cell preparation for flow cytometry analyses. For cell preparation from ear-draining lymph nodes (EDLN) for Tfh/GC staining, EDLNs were dissociated with piston, passed through a 70μm strainer (Falcon) and resuspended in PBS containing 0.5% BSA and 2mM EDTA. Cells were then centrifuged and resuspended in FACS buffer (PBS containing 1% FCS and 2mM EDTA), counted and used for FACS staining. In case of preparation of EDLN cells for DC staining, EDLNs were cut in small pieces and incubated 30 minutes at 37°C in 2mg/mL collagenase D (Roche), 0.25mg/mL DNase I (Sigma) and 2.5% foetal calf serum (Thermofisher) in PBS prior passing through the strainer.

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

Surface staining for flow cytometry analyses. Two million cells were used for antibody staining. Cells were first incubated with anti-CD16/CD32 (clone 93, eBioscience) to block unspecific binding, followed by surface staining with the following antibody panels : CD11c biotin (clone HL3), IgE biotin (clone R35-72), CD95 PE-Cy7 (clone Jo2), CD19 FITC (clone 1D3), CXCR5 biotin (clone 2G8), CD4 Alexa Fluor 700 (clone RM-5), CD4 BV421 (clone GK1.5), streptavidin BV605 were from BD Biosciences; CD8a PerCP-Cy5.5 (clone 53-6.7), B220 APC (clone RA3-6B2), GL-7 PE (clone GL-7), I-

A/I-E PE (clone M5/114.15.2) and streptavidin APC were from eBioscience. PD-1 PE-Cy7 (clone RMP1-30), IgG1 PerCP-Cy5.5 (clone RMG1-1) were from Biolegend. Viability staining was performed by adding propidium iodide to a final concentration of 4 µg/mL prior to cell passing with the cytometer. Stained cells were analysed on a Fortessa or LSRII flow cytometer (BD Biosciences). Results were analysed using FlowJo (Treestar).

LN cell culture and antigen stimulation. To identify OVA-specific Tfh cells by activation-induced marker assay[6], one million of freshly isolated EDLN single cell suspensions were cultured in 96-well U-bottom plate in 200µl of medium (RPMI 1640 supplemented with 10% FCS, HEPES, 0.05mM 2-mercaptoethanol, 100U/ml penicillin, 100U/ml streptomycin), stimulated with 500µg/ml of OVA or PBS (vehicle) for 18h. Anti-CD154 BV650 antibody (clone MR1, BD Biosciences) was added to all culture conditions. After the culture, cells were incubated with anti-CD16/CD32 (Clone 93, eBioscience) to block unspecific binding, and stained with viability dye 506 (eBioscience) and antibody panels: B220 FITC (clone RA3-6B2, Biolegend), CD4 BV421 (clone GK1.5, BD Biosciences), CXCR5 biotin (Clone 2G8, BD Biosciences), Streptavidin PE (eBioscience), PD-1 PE-Cy7 (clone RMP1-30, Biolegend), OX40 APC (clone OX86, eBiosience) and CD25 PerCP-Cy5.5 (clone PC61, BD Biosciences).

RNA sequencing. Migratory DCs from EDLNs were FACS-sorted with ARIA II (BD) (see Fig 8A for sorting strategies). RNA was extracted using RNeasy Micro Kit (Qiagen). RNA-seq was performed in IGBMC high-throughput mRNA sequencing facility. Full length cDNAs were generated from 1ng of total RNA using Clontech SMART-Seq v4 Ultra Low Input RNA kit for Sequencing (Takara Bio Europe, Saint Germain en Laye, France) according to manufacturer's instructions with 12 cycles of PCR for cDNA amplification by Seq-Amp polymerase. Six hundred pg of pre-amplified cDNA were

then used as input for Tn5 transposon tagmentation by the Nextera XT DNA Library Preparation Kit (96 samples) (Illumina, San Diego, CA) followed by 12 cycles of library amplification. Following purification with Agencourt AMPure XP beads (Beckman-Coulter, Villepinte, France), the size and concentration of libraries were assessed by capillary electrophoreris. Libraries were sequenced as 50bp single-end reads on an Illumina HiSeq 4000 sequencer.

Reads were preprocessed in order to remove adapter, polyA and low-quality sequences (Phred quality score below 20). After this preprocessing, reads shorter than 40 bases were discarded for further analysis. These preprocessing steps were performed using cutadapt version 1.10. Reads were mapped onto the mm10 assembly of mouse genome using STAR version 2.5.3a. Read counts have been normalized across samples with the median-of-ratios method proposed by Anders and Huber [7], to make these counts comparable between samples. Comparisons of interest were performed using the method proposed by Love et al. [8] and implemented in the DESeq2 Bioconductor library version 1.16.1. P-values were adjusted for multiple testing using the Benjamini and Hochberg method. Gene expression quantification was performed from uniquely aligned reads using htseq-count version 0.6.1p1, with annotations from Ensembl version 96 and "union" mode. The RNA-Seq data have been deposited in the NCBI's Gene Expression Omnibus (GEO) and are accessible as GSE149039.

Bronchoalveolar lavage (BAL) cell analyses. BAL was taken in anaesthetized mice by instilling and withdrawing 0.5 ml of saline solution (0.9% NaCl, 2.6mM EDTA) in the trachea. After six times lavages, BAL fluid was centrifuged, and BAL cells were counted using a Neubauer hemocytometer. $5x10^4$ BAL cells were cytospined and stained with Hemacolor kit (Merck) to identify macrophages, lymphocytes, neutrophils and eosinophils. After differential counting to obtain their frequencies, the number of each cell type was calculated according to the total BAL cell number and the

frequency. For RT-qPCR analyses, RNA was extracted from BAL cells using NucleoSpin RNA XS kit (Macherey-Nagel), reverse transcribed by using random oligonucleotide hexamers and SuperScript IV Reverse Transcriptase (Invitrogen) and amplified by means of quantitative PCR with LightCycler 480 SYBR Green kit (Roche), according to the manufacturer's instructions. Relative RNA levels were calculated with hypoxanthine phosphoribosyl- transferase (HPRT) as an internal control. For analyses of each set of gene expression, an arbitrary unit of 1 was given to the samples with the highest level, and the remaining samples were plotted relative to this value. Sequences of qPCR primers are: Hprt (TGGATACAGGCCAGACTTTG; GATTCAACTTGCGCTCATCTTA; 161 bp); IL4 (GGCATTTTGAACGAGGTCAC; AAATATGCGAAGCACCTTGG; 132 bp); IL5 (AGCACAGTGGTGAAAGAGACCTT; TCCAATGCATAGCTGGTGATTT; 117 bp); Il13 (GGAGCTGAGCAACATCACACA; GGTCCTGTAGATGGCATTGCA; 142 bp); Ccr3 (TAAAGGACTTAGCAAAATTCACCA; TGACCCCAGCTCTTTGATTC; 150 bp); Mcpt8 (GTGGGAAATCCCAGTGAGAA; TCCGAATCCAAGGCATAAAG; 160 bp).

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

To measure OVA-specific IgG1 and IgE in sera, microtiter plates were coated with OVA and then blocked with BSA. Serum samples were incubated in the coated plates overnight at 4°C followed by incubation with a biotinylated rat anti-mouse IgE (BD Biosciences; clone R35-118) or IgG1 (BD Biosciences; clone A85-1). Extravidin horseradish peroxidase (Sigma) and TMB

(tetramethylbenzidine) Substrate Reagent Set (BD Biosciences) were used for detection. Levels of OVA-specific IgG1 and OVA-specific IgE were calculated relevant to a pre-prepared serum pool from OVA-sensitized and challenged mice and expressed as arbitrary units.

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

Immunohistochemistry (IHC). For IHC staining of major basic protein (MBP) and mast cell protease 8 (MCPT8), 5µm paraffin sections were treated with 0.6% H_2O_2 to block endogenous peroxidase activity before antigen retrieval with either Pepsin (for IHC of MBP; Life technologies) or citric buffer (10 mmol/L citric acid, pH 6; for IHC of MCPT8). Slides were then blocked with normal rabbit serum (Vector Laboratories) and incubated overnight with primary antibody (Rat anti-mouse MBP antibody (Mayo Clinic, Rochester); Rat anti-mouse TUG8 (Biolegend)). Slides were then incubated with biotinylated rabbit anti-rat IgG (dilution: 1/300) and treated with AB complex (Vector Laboratories). Staining was finally visualized with AEC high-sensitivity substrate chromogen solution (Dako) and counterstained with hematoxylin.

RNA in situ hybridization. Mouse ears were fixed in formalin and embedded in paraffin. RNA in situ hybridization was performed on freshly 5µm sections using RNAscope® 2.5 HD Reagent Kit-RED (Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer's instructions. Probe Mm-Tslp was used for detection of TSLP (Cat 432741).

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

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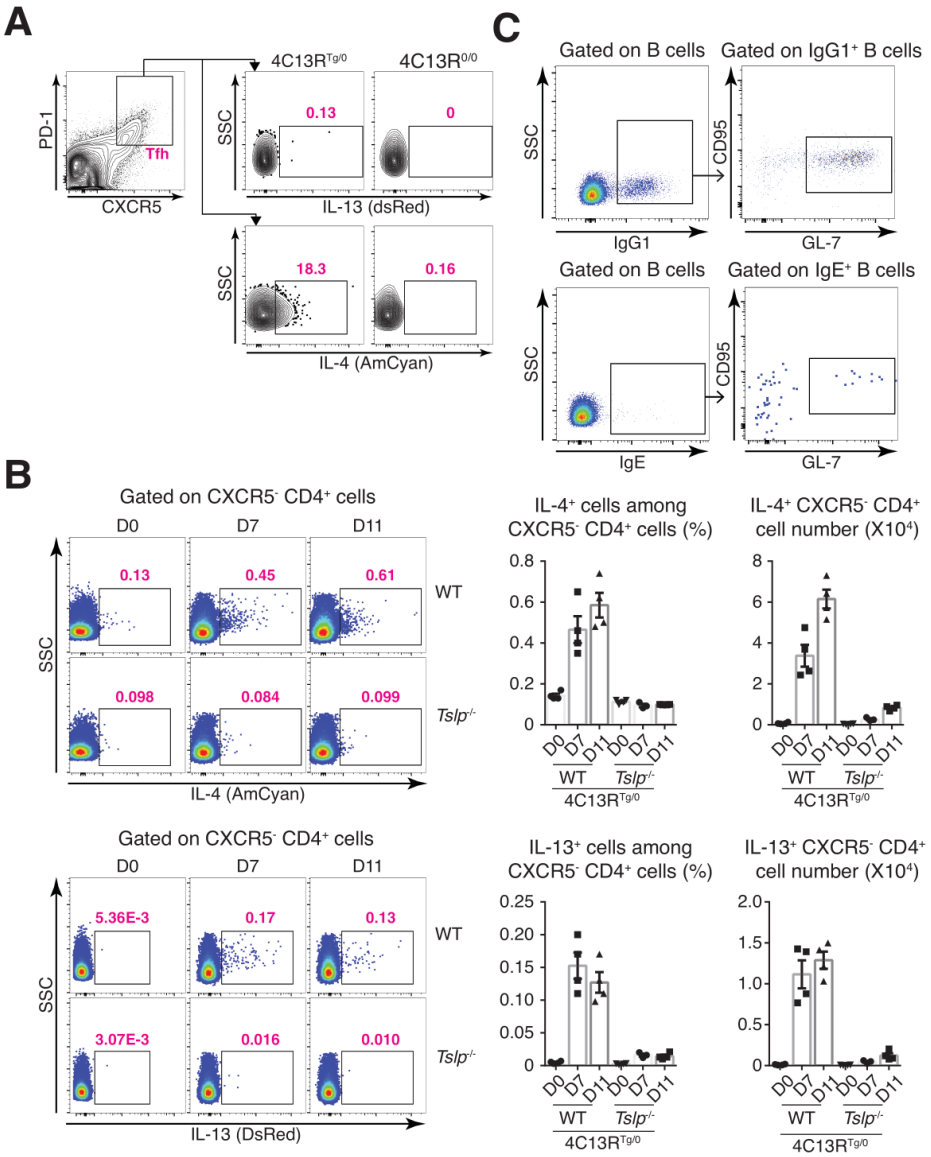

FIG E1. (A) CXCR5$^+$ PD-1$^+$ Tfh cells produce IL-4 (AmCyan) but not IL-13 (dsRed) in EDLNs of MC903-treated 4C13R$^{Tg/0}$ mice at D11. 4C13R$^{0/0}$ EDLNs were used as gating control. **(B)** Frequency and number of CXCR5$^-$ CD4$^+$ (non-Tfh) cells producing IL-4 (AmCyan) or IL-13 (dsRed), representing Th2 cells, in EDLNs from Balb/c wildype (WT) and Tslp$^{-/-}$ mice in the background of 4C13R$^{Tg/0}$, treated with MC903 or ethanol, and analyzed at D0, D7 and D11. **(C)** The majority of IgG1$^+$ but not IgE$^+$ B cells in EDLNs from MC903-treated wildtype Balb/c mice are GL-7$^+$ CD95$^+$.

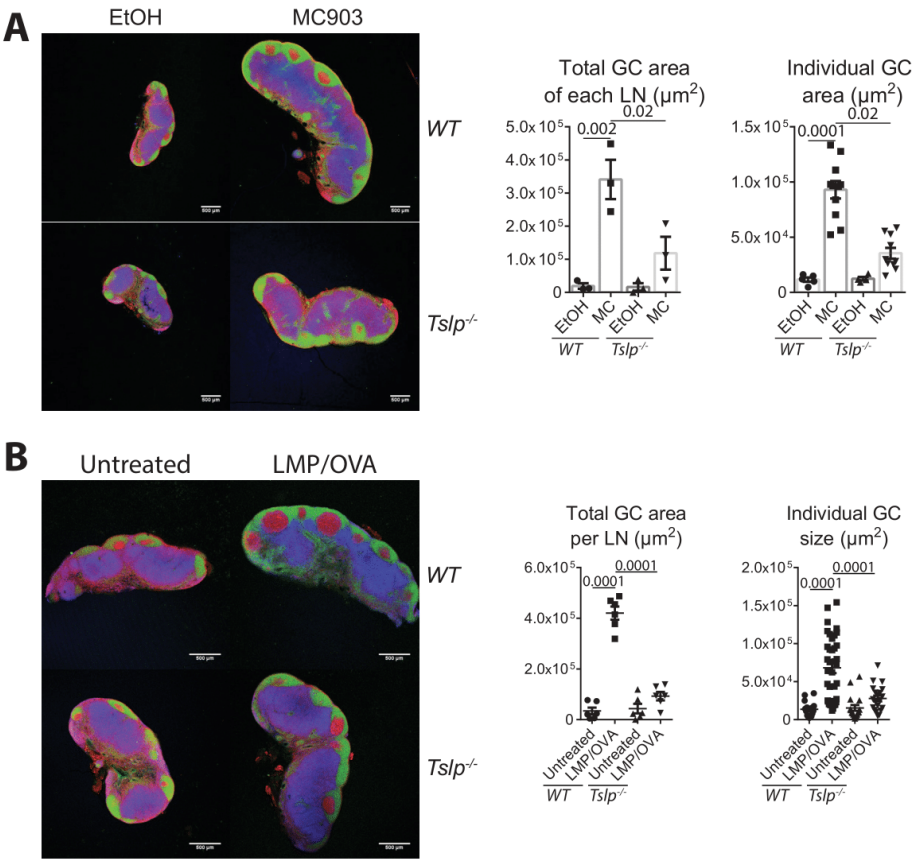

FIG E2. Germinal center staining. Wildtype *(WT)* and *Tslp*$^{-/-}$ mice were treated with MC903 (**A**) or subjected to OVA-sensitization (**B**) as shown in figure 1A and 4D respectively. EDLN were collected and fixed overnight with 4% PFA at 4°C. After 2 times 30 minutes of wash in PBS at room temperature (RT), samples were included in 4% low melting point agarose in PBS. Vibratome sections of 100μm were blocked with 5% normal donkey serum (NDS), 0.1% Triton X-100 in PBS and then stained overnight at 4°C with anti CD4-AlexaFluor 647 (RM4-5, biolegend, d=1/100; shown in blue), anti IgD-FITC (11-26c.2a, BD Biosciences, d=1/50; shown in green) and biotinylated PNA (Vectorlabs, d=1/250; shown in red) diluted in 5% NDS, 0.1% Triton X-100 in PBS. Sections were subsequently incubated 1h at RT with Neutravidin-Dylight550 (ref 84606, Thermofisher, d=1/200) diluted in PBS. After 2 washing of 30 minutes with PBS at RT, sections were kept at 4°C in PBS containing Hoechst 33342 (Sigma Aldrich) and images were acquired using Leica LSI confocal macroscope. Measurements were performed with ImageJ software. Data are means ± SEM; one-way ANOVA with Tukey's multiple comparison post-hoc test.

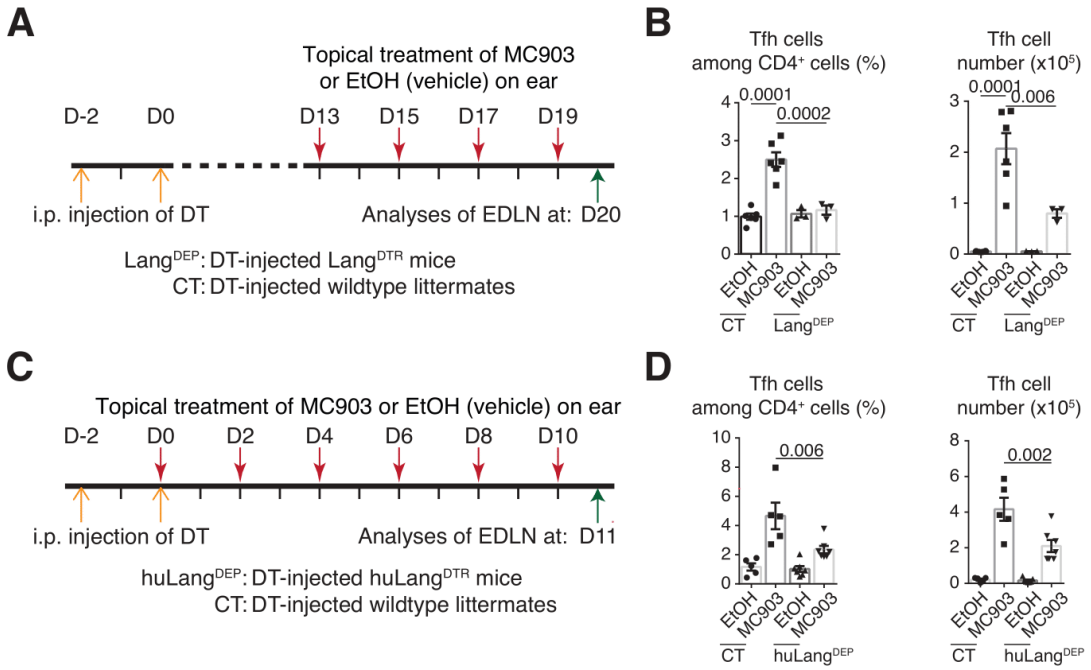

FIG E3. Selective depletion of LCs leads to a diminished Tfh cell differentiation in MC903 model. (**A**) Experimental protocol. Lang^{DTR} mice and wildtype littermate controls were intraperitoneally (i.p.) injected with diphteria toxin (DT) at D-2 and D0. Mouse were then topically treated with MC903 or EtOH every other day from D13 to D19 and ear draining lymph nodes (EDLN) were analyzed at D20. (**B**) Frequency and number of CXCR5^{+} PD-1^{+} Tfh cells in Lang^{DEP} mice and CT at D20. (**C**) Experimental protocol. HuLang^{DTR} mice and wildtype littermate controls were intraperitoneally i.p. injected with DT at D-2 and D0. Mouse were then topically treated with MC903 or EtOH every other day from D0 to D10 and EDLN were analyzed at D11. (**D**) Frequency and number of CXCR5^{+} PD-1^{+} Tfh cells in huLang^{DEP} mice and CT at D11. Values shown are means ± SEMs; one way ANOVA with Tukey's multiple comparison post-hoc test. Data are representative of 2 independent experiments with similar results.

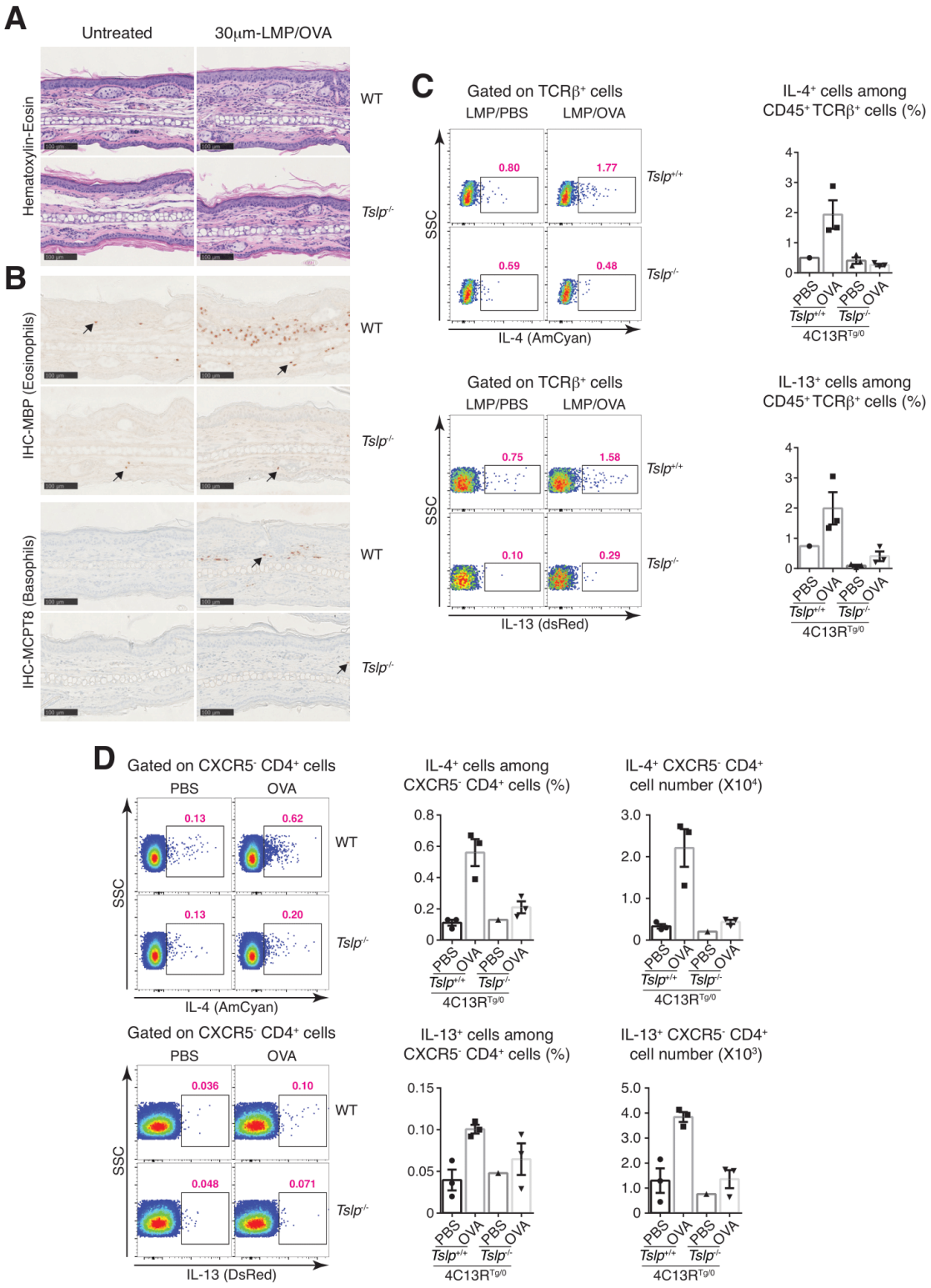

FIG E4. TSLP is crucially required for 30μm-LMP/OVA-induced skin Th2 inflammation. **(A)** Hematoxylin and eosin (HE) staining of mouse ears. **(B)** Immunohistochemistry staining of mouse ears with anti-MBP antibody (for eosinophils) or anti-MCPT8 antibody (for basophils). Arrow points to one of the positive cells. Scale bar, 100μm. **(C-D)** IL-4 (AmCyan) and IL-13 (dsRed) expression in TCRβ$^+$ dermal cells **(C)** or CXCR5$^-$ CD4$^+$ (non-Tfh) cells **(D)**.

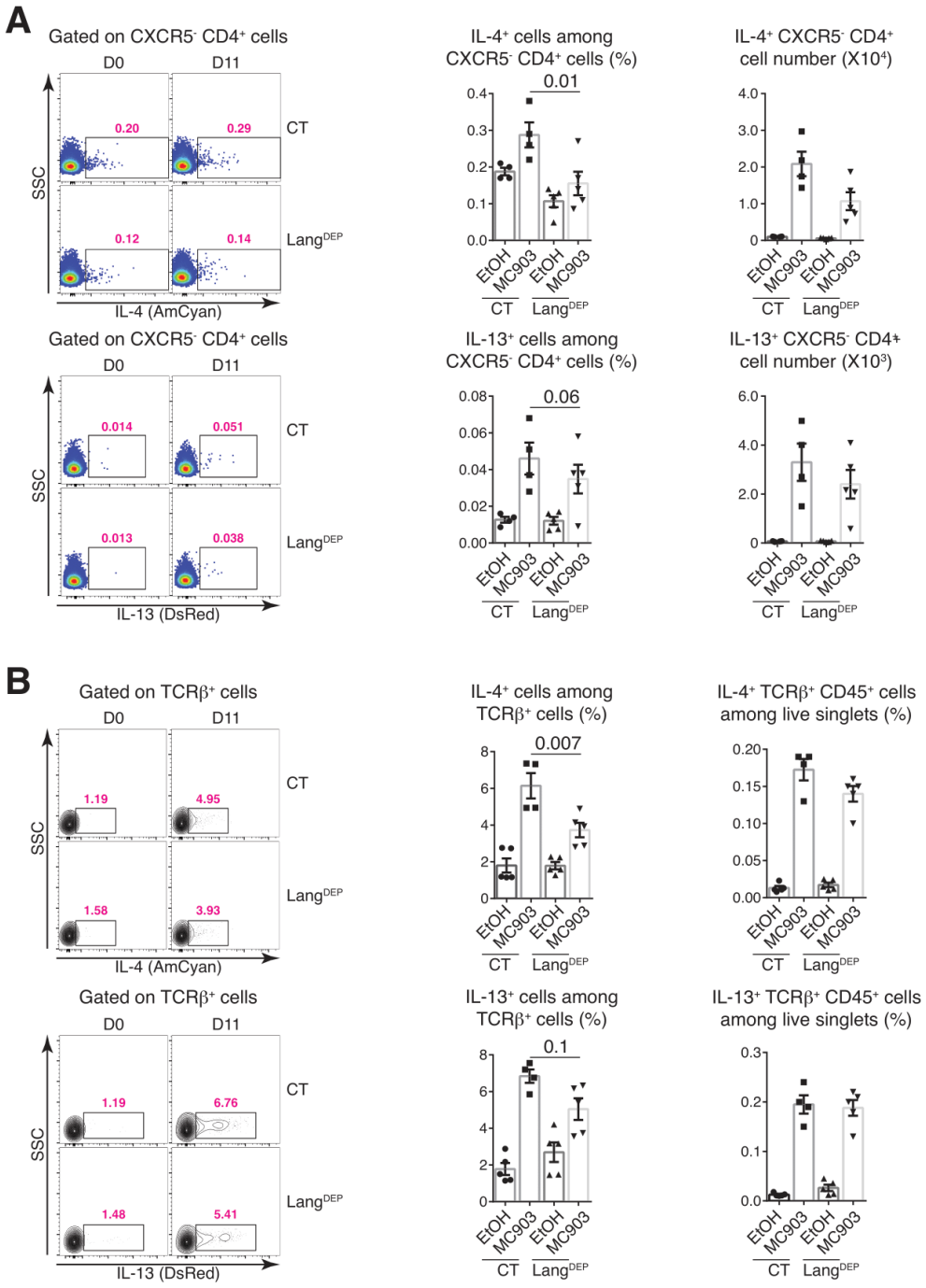

FIG E5. Depletion of Langerin+ cells slightly diminishes the MC903- induced Th2 cell response. Comparison of IL-4 and IL-13 expression among CXCR5-CD4+ (non-Tfh) cell in the EDLN **(A)**, or among TCRβ+ cells in the dermis **(B)** of EtOH- or MC903-treated control (CT) or Lang^{DEP} mice, all in the background of 4C13R$^{Tg/0}$.

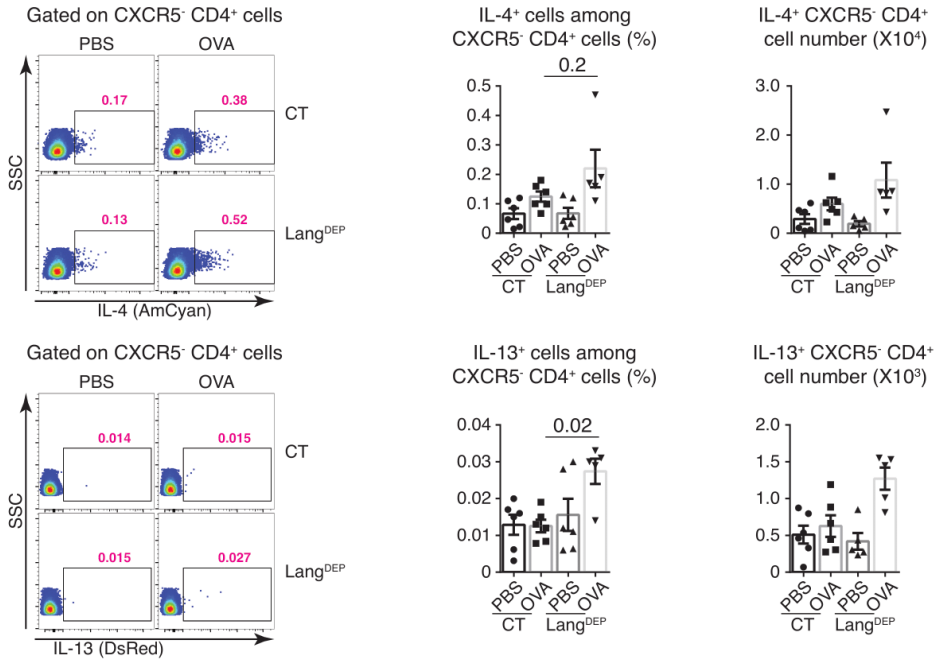

FIG E6. Depletion of Langerin⁺ cells increases the LMP/OVA-induced Th2 cell response in EDLNs. Comparison of IL-4 and IL-13 expression among CXCR5⁻CD4⁺ (non-Tfh) cell in EDLNs from LMP/OVA-treated control (CT) or Lang^{DEP} in the background of 4C13R$^{Tg/0}$ mice.

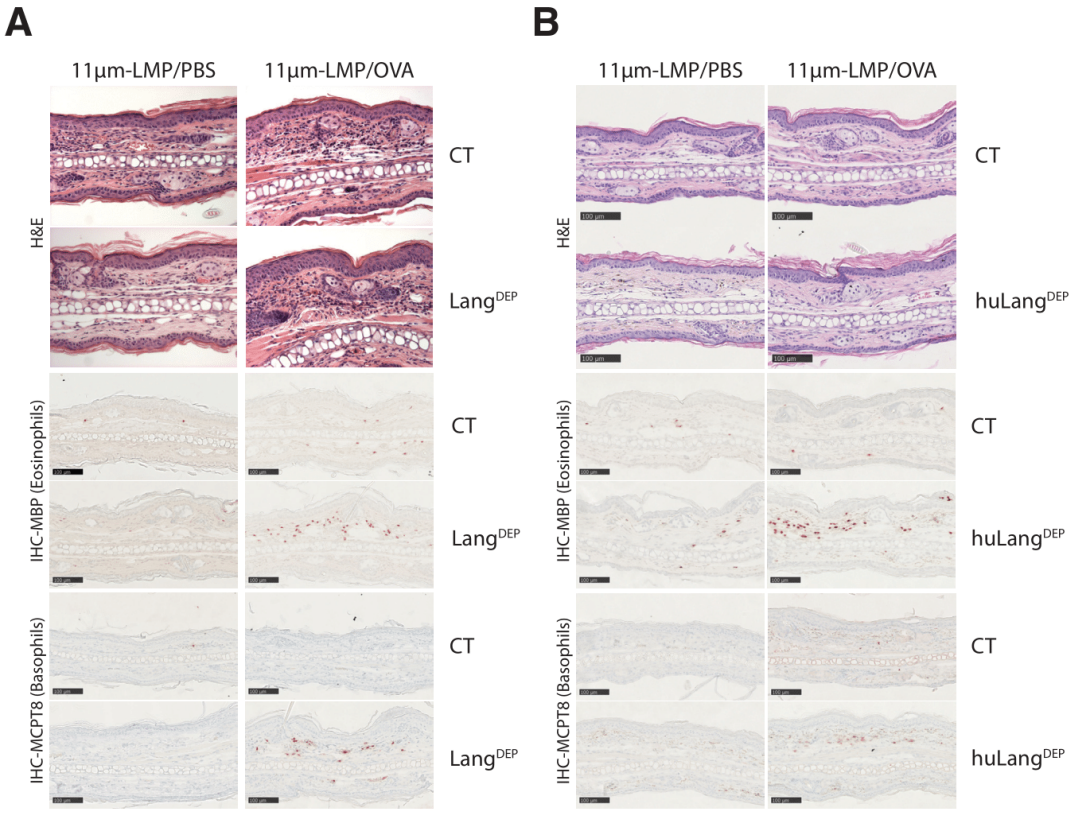

FIG E7. 11μm-LMP/OVA-induced skin inflammation is enhanced in mice with the depletion of Langerin$^+$ DCs or LCs. Hematoxylin and eosin staining of ears from Lang^{DEP} (**A**, top) and huLang^{DEP} (**B**, top) mice after 11μm-LMP/OVA sensitization. Immunohistochimstry for MBP (eosinophils) and MCPT8 (basophils) of ears from Lang^{DEP} (**A**, bottom) and huLang^{DEP} (**B**, bottom) mice after 11μm-LMP/OVA treatment. Scale bar, 100μm.

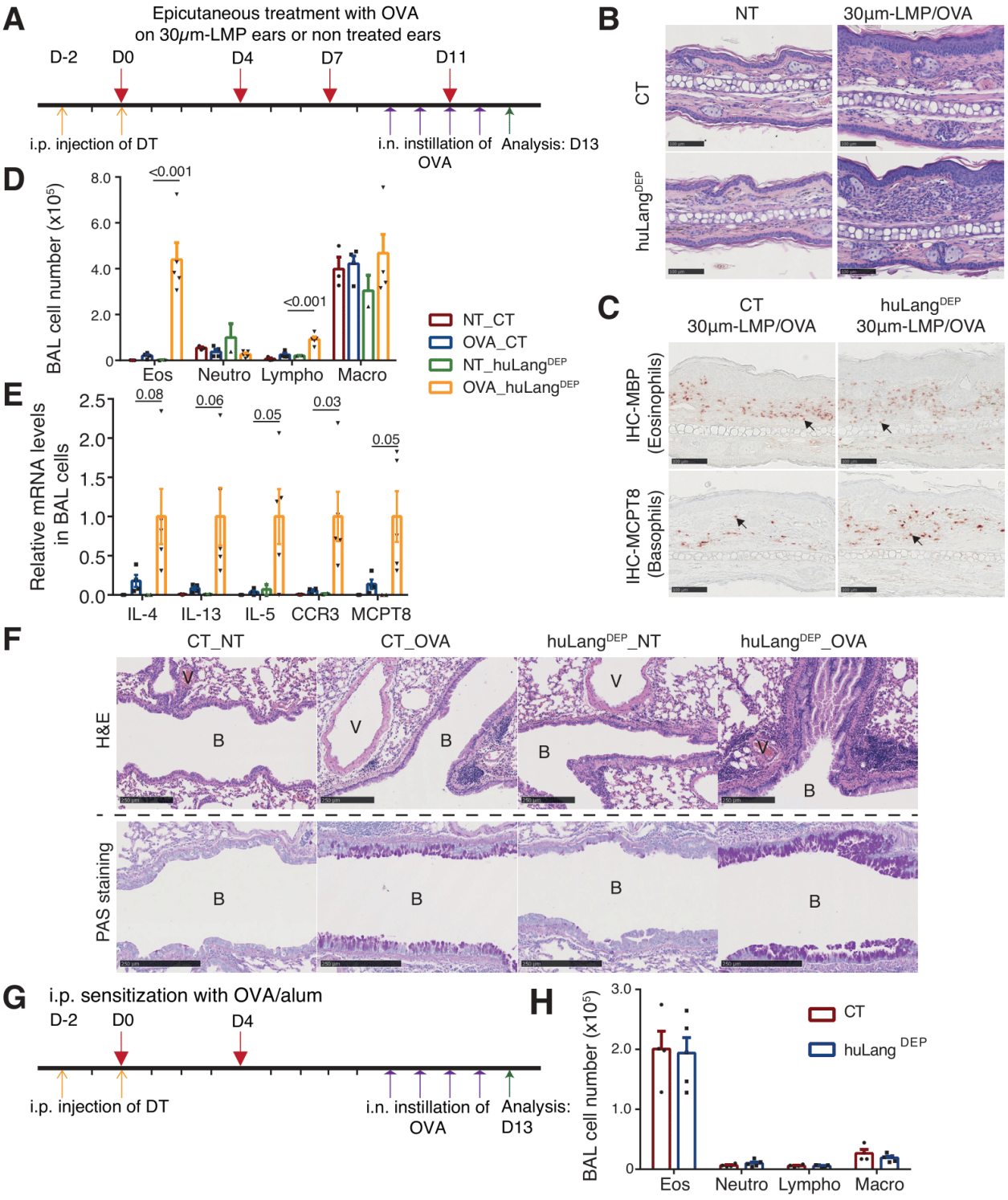

FIG E8. LCs counteract LMP/OVA sensitization-induced skin inflammation and the subsequent asthmatic response. **(A)** Experimental protocol for OVA epicutaneous sensitization and airway challenge. Mice were intraperitoneally injected with DT at D-2, D0. Mice were either treated with OVA on LMP ears at D0, D4, D7 and D11 or ears were non treated (NT). All mice were subjected to intranasal (i.n.) instillation with OVA from D9 to D12. Ears and lungs were analyzed at D13. **(B)** H&E staining of mouse ears. Scale bar, 100 um. **(C)** IHC staining of mouse 30μm-LMP/OVA ears with anti-MBP (for eosinophils) or anti-MCPT8 (for basophils). **(D)** Differential counting of eosinophils (Eos), neutrophils (Neutro), lymphocytes (Lympho) and macrophages (Macro) in BAL. **(E)** RNA levels of indicated genes in BAL cells by RT-qPCR. **(F)** Lung sections were stained with H&E for histological analyses or PAS for goblet cell hyperplasia analyses. B: bronchiole; V: blood vessel. Scale bar, 250 um. **(G)** Experimental protocol for OVA i.p. sensitization and airway challenge. Mice were i.p. injected with DT at D-2 and D0. Mice were treated with OVA/alum i.p.at D0 and D4. Mice were subjected to intranasal instillation with OVA from D9 to D12. Lungs were analyzed at D13. **(H)** Differential cell counting in BAL. Data are means ± SEM; unpaired two-tailed t-test.

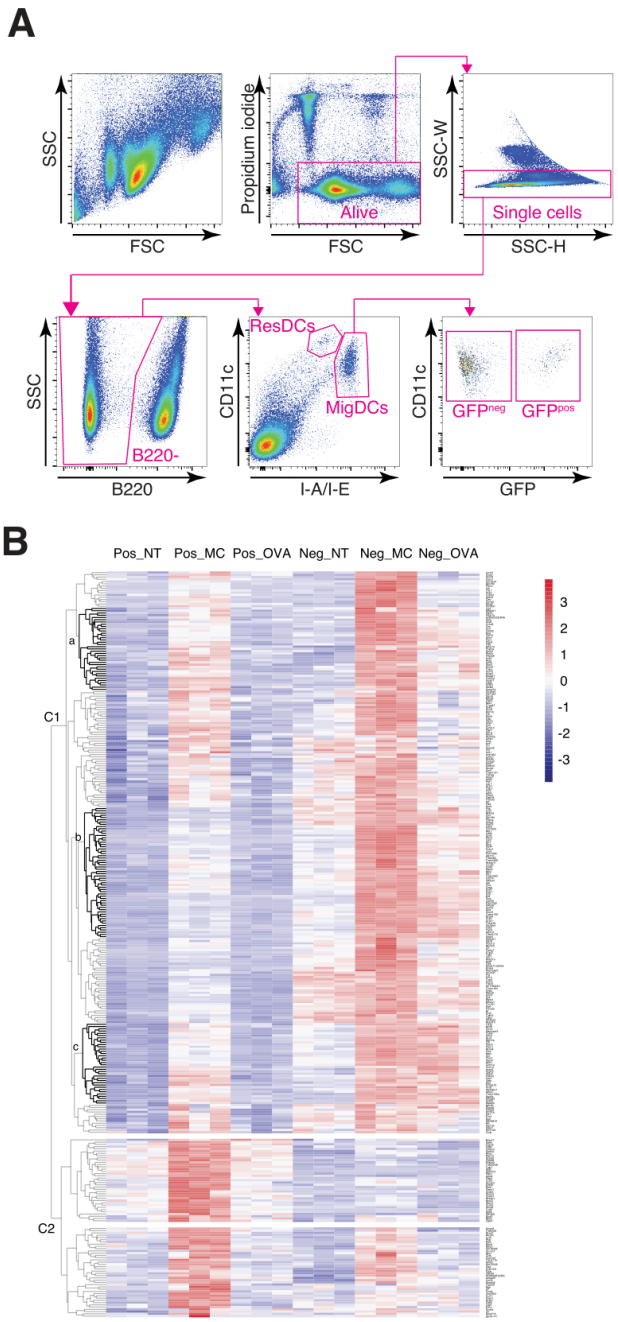

FIG E9. Transcriptomic analyses of migratory DCs in EDLNs of Lang^{GFP} mice upon MC903 treatment or epicutaneous OVA sensitization. Lang^{GFP} mice were treated with MC903 at D0, D2 and D4 or 30μm-LMP/OVA on D0 and D3; EDLNs were collected at D5 for cell sorting and RNAseq analyses. (**A**) Gating strategy used to sort resident (res) and migratory (mig) GFP^{pos} and GFP^{neg} DCs. (**B**) Heatmap generated with the input of upregulated genes identified in Pos_MC compared with Pos_NT(FC > 1.5; p < 0.05; raw read > 200 in at least one sample of the Pos groups), to visually assess the results of clustering on the data to observe trends of expression for genes across all groups. Z score of the expression level is used to generate heatmap. Pos_NT, Pos_MC, Pos_OVA: GFPPos migDCs from non-treated, MC903-treated or LMP/OVA-treated LangGFP mice; Neg_NT, Neg_MC, Neg_OVA: GFPneg migDCs from non-treated, MC903-treated or LMP/OVA-treated LangGFP mice.

Two clusters C1 and C2 were revealed. The cluster C1 genes exhibited the expression trends: 1) in non-treated groups, they had a lower expression in GFP^{pos} cells than in GFP^{neg} cells (Pos_NT < Neg_NT); 2) in MC903-treated groups, their expression in GFP^{Pos} cells increased, reaching a similar or higher expression than non-treated GFP^{neg} cells (Pos_MC = or > Neg_NT), and their expression in GFP^{neg} cells was also increased (Neg_MC > Neg_NT), with a higher level than Pos_MC cells; 3) in OVA-treated groups, the expression of some genes was also increased in GFP^{neg} cells (Neg_OVA versus Neg_NT) (subcluters of C1: a, b and c) while others remained not changed. Together, expression features of the cluster C1 suggest that in the MC903-AD, Langerin^{+} migDCs acquire many gene expression of Langerin^{-} migDCs, and share the upregulation of these genes with Langerin^{-} migDCs; and moreover, the upregulation of some (although less) of these genes also occurs in Langerin^{-} migDCs (but not Langerin^{+} migDCs) in OVA-AD.

Different from the cluster C1, the cluster C2 genes were highly upregulated in Pos_MC; some of them were also upregulated in Neg_MC (but reaching a lower level) and very few of them were upregulated in Neg_OVA, suggesting that this cluster represents the upregulated genes rather specific for Langerin^{+} migDCs under MC903 treatment.

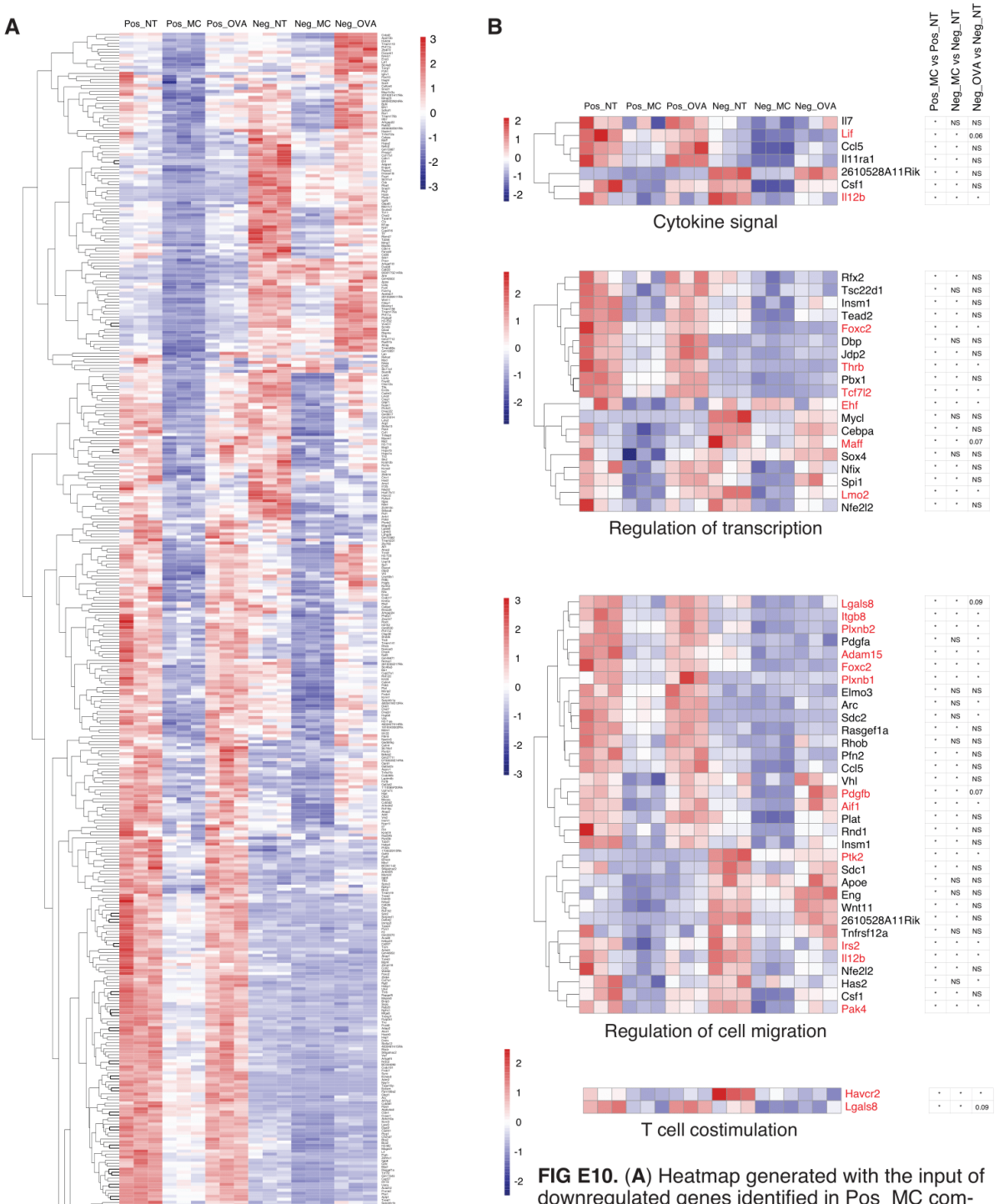

FIG E10. (**A**) Heatmap generated with the input of downregulated genes identified in Pos_MC compared with Pos_NT (FC > 1.5; $p < 0.05$; raw read > 200 in at least one sample of the Pos groups), to visually assess the results of clustering on the data to observe trends of expression for genes across all groups. Z score of the expression level was used to generate heatmap. (**B**) Selected genes corresponding to gene ontology terms for Cytokine activity, Regulation of transcription, Regulation of cell migration, or T cell costimulation. *, adjusted $p<0.05$; NS, non significant.

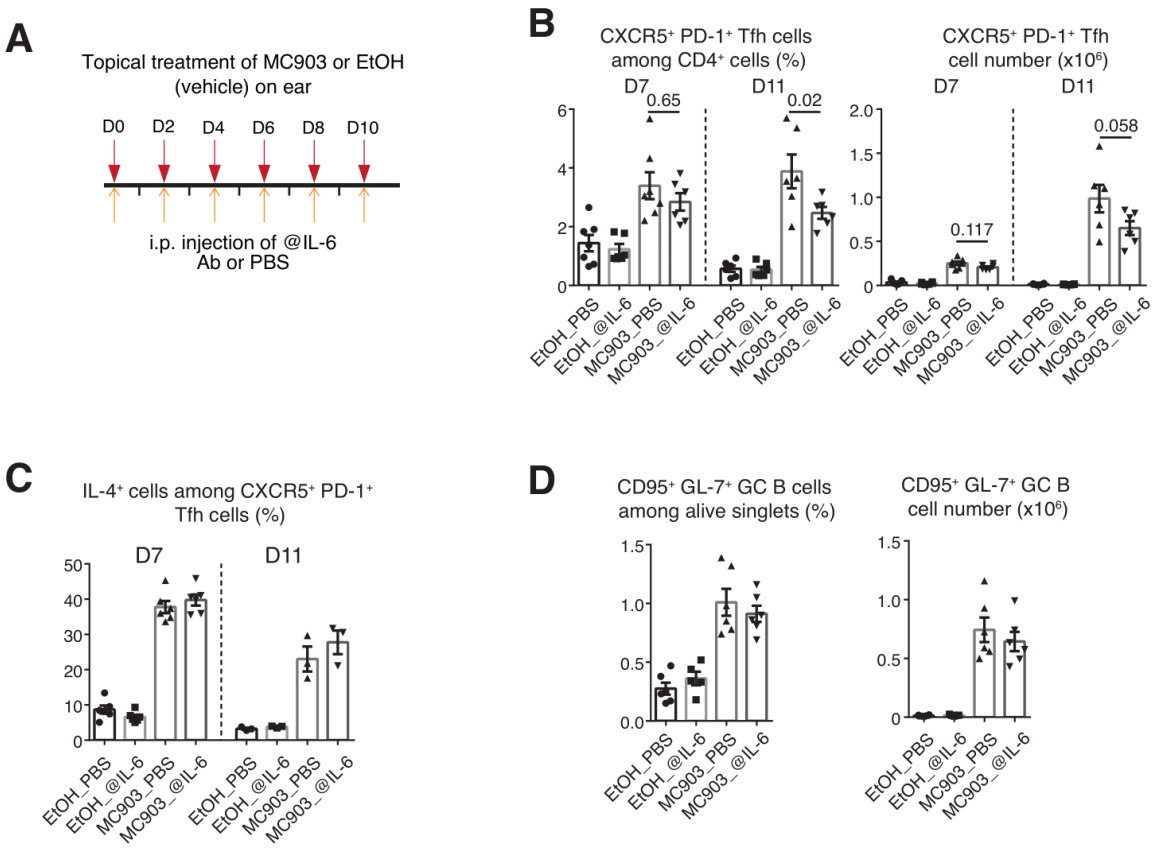

FIG E11. IL-6 neutralization does not significantly diminish Tfh cell differentiation and GC B cell numbers. (**A**) experimental scheme. 4C13R$^{Tg/0}$ mice were i.p. injected with 200 μg anti-IL-6 neutralizing antibody (@IL-6; Clone MP5-20F3, BioXcell) every other day from D0 to D10, and mouse ears were topically treated with MC903 or EtOH every other day from D0 to D10. EDLNs were analyzed at D7 or D11. (**B**) Frequency and number of CXCR5$^+$ PD-1$^+$ Tfh cells. (**C**) IL-4 (AmCyan) expression by Tfh cells. (**D**) Frequency and number of CD95$^+$ GL-7$^+$ GC B cells at D11. Data are means ± SEM, one-way ANOVA with Tukey's multiple comparison post-hoc test.

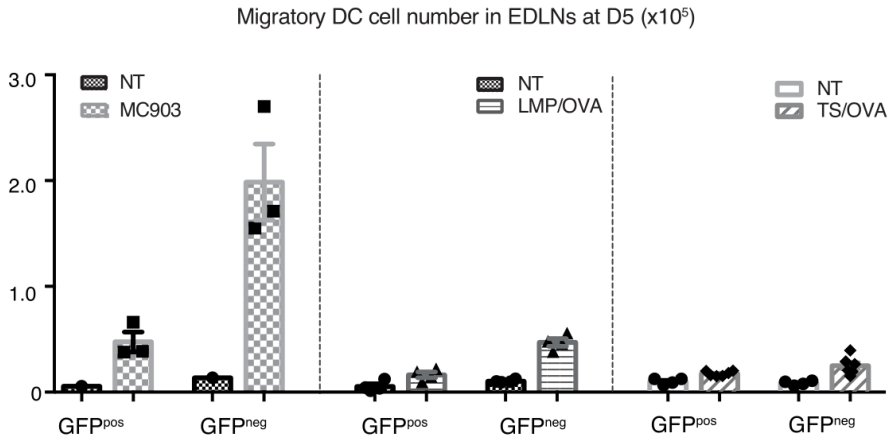

FIG E12. MC903 treatment leads to increased numbers of both langerin-GFP^{pos} and langerin-GFP^{neg} migratory DCs in EDLNs at D5. Lang^{GFP} mice were treated with MC903 at D0, D2 and D4, or 30μm-LMP/OVA at D0 and D3, or tape-stripping (TS)/OVA at D0 and D3, and EDLNs were collected at D5 for flow cytometry analyses. Absolute numbers of GFP-positive (GFP^{pos}) and -negative (GFP^{neg}) migratory DCs in EDLN, compared with non-treated (NT), are shown.

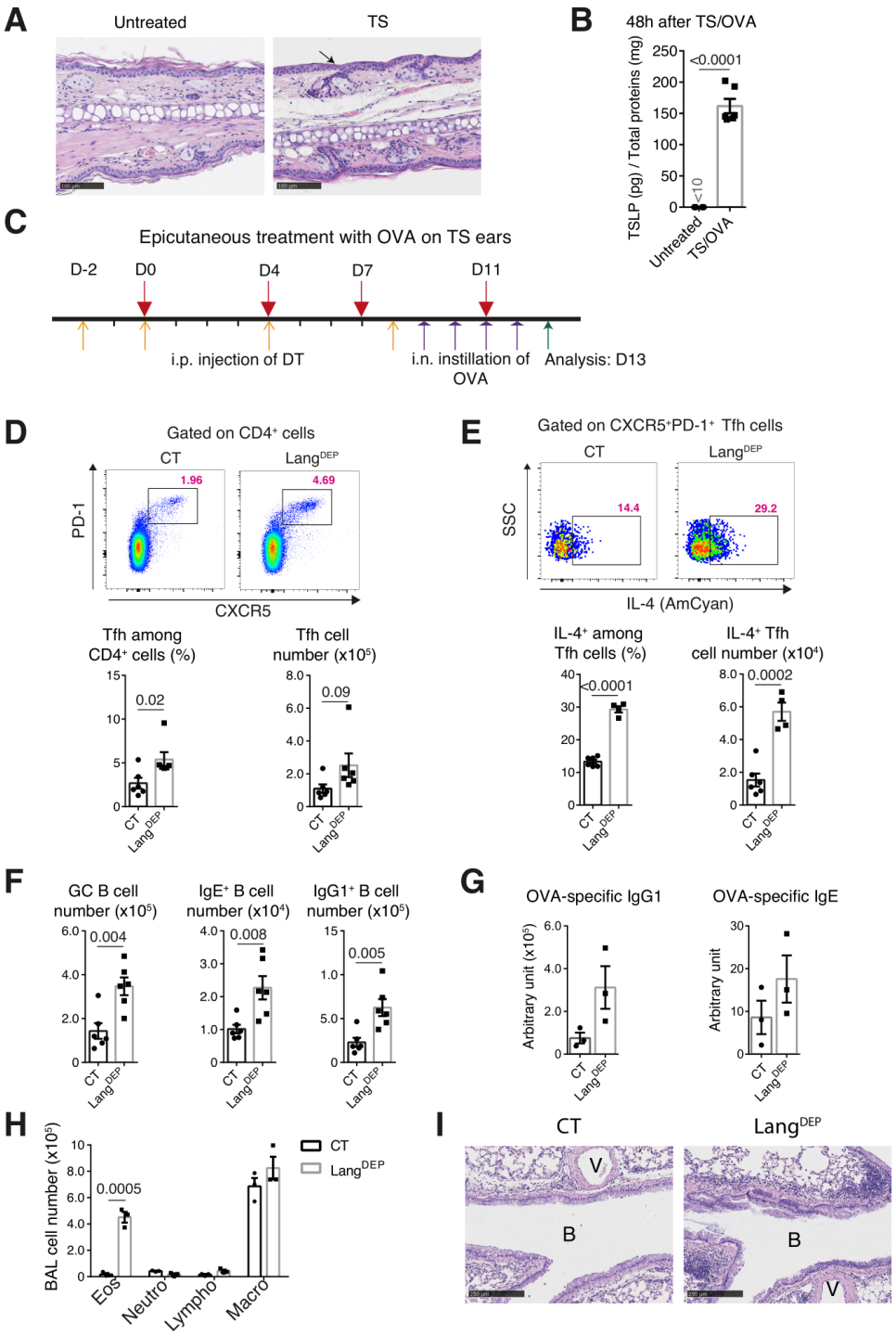

FIG E13. Depletion of Langerin+ cells enhances the TS/OVA-induced Tfh/GC response and the subsequent asthmatic phenotype. **(A)** H&E staining of untreated or tape-stripped (TS) Balb/c wildtype mice. Arrow points to the absence of stratum corneum in TS-ear. Scale bar, 100μm. **(B)** Dorsal side of ears of WT mice were tape-stripped 10 times and topical treated with 200μg of OVA in 10μl PBS. TSLP protein levels in ears were measured by ELISA at 48h after treatment. **(C)** Experimental protocol. Lang-^{DTR} mice and wildtype littermate controls (CT), in the background of 4C13R$^{Tg/0}$, were i.p. injected with DT at D-2, D0 and then every 4 days. OVA (200μg) were topically applied on TS-ears at D0, D4, D7 and D11. All mice were subjected to intranasal (i.n.) instillation with 50μg of OVA from D9 to D12 and analyzed at D13. **(D-F)** Frequency and number of Tfh cells (**D**), IL-4 (AmCyan) expression by Tfh cells (**E**) and numbers of CD95+ GL-7+ GC B cells, IgG1+ and IgE+ B cells in EDLNs (**F**). (**G**) Serum levels of OVA-IgG1 and OVA-IgE. (**H**) Differential cell counting for eosinophils (Eos), neutrophils (Neutro), lymphocytes (Lympho) and macrophages (Macro) in BAL. (**I**) H&E staining of lung sections. B: bronchiole; V: blood vessel. Scale bar, 250μm. Data are means ± SEM; unpaired two-tailed t-test.

Ongoing studies and perspectives

1. Is TSLPR on DCs required for OVA-induced Tfh cell differentiation?

Introduction

I previously showed that TSLP induces Tfh cell differentiation in allergic skin sensitization, however the underlying mechanisms remain to be elucidated. It is commonly accepted that DCs induce naive T cell differentiation. Indeed, I showed that Lang^{+} DCs, and more specifically LCs, play an inhibiting role in Tfh cell differentiation during TSLP-promoted allergic sensitization, suggesting a promoting role for Lang^{-} DCs, as reported by different studies[174,216]. TSLP signals through its receptor comprising two sub-units, IL-7Rα and TSLPR, which is expressed by different cell populations, including, but not only, DCs. TSLP could therefore act on other cell populations, which in turn would activate DCs. For example, it has been shown that ILCs express TSLP receptor and that TSLP-TSLPR signalling is important for ILC recruitment to the skin[76]. Therefore, I investigated 1) whether TSLP signals directly through TSLPR expressed on DCs to induce Tfh cell differentiation in allergic skin sensitization using *Crlf2*$^{CD11c-/-}$ mouse line, in which TSLPR is ablated specifically in DCs. 2) whether TSLPR expressed by LCs is required for Tfh / GC B cell response in allergic skin sensitization, using *Crlf2*$^{iLC-/-}$ in which TSLPR is specifically ablated in LCs upon 4-hydroxytamoxifen (OH-Tam) treatment.

Methods

Mice

Crlf2$^{fl/fl}$ mice (on a C57BL/6J genetic background) were generated by my team. HuLang-Cre$^{ERT2(Tg/0)}$ mice were described previously[311]. CD11c-Cre$^{Tg/0}$ (on a C57BL/6J genetic background) were purchased from Jax. All mice were backcrossed to Balb/c background for more than 10

generations. Twelve of seventeen weeks old female mice were used in experiments. Breeding and maintenance of mice were performed under institutional guidelines, and all experimental protocols were approved by the ethics committee of animal experimentation of the IGBMC, and by the Ministère de l'enseignement supérieur, de la recherche et de l'innovation.

Epicutaneous ovalbumin sensitization

Laser-assisted skin microporation was performed on the dorsal side of mouse ears using P.L.E.A.S.E system (Pantec Biosolutions). The parameters for the depth of 30µm were as follow: 2 pulses per pore, with fluence 7.5J/cm², pulse length of 75µs, RepRate of 500Hz and power of 1 W. To induce OVA epicutaneous sensitization, 10µl of PBS solution containing 200µg of OVA (Sigma-Aldrich, Cat No. A5503) were applied on ears immediately after LMP at D0, D4, D7 and D11.

4-hydroxytamoxifen treatment

4-hydroxytamoxifen (Sigma-Aldrich, Cat No. H6278) was dissolved in 100% ethanol and was topically applied (8µg in 10µl per ear) on HuLang-Cre$^{ERT2(Tg/0)}$ *Crlf2*$^{fl/fl}$ mouse ears from D-3 to D-1 to induce *Crlf2* excision in Langerhans cells.

ELISA

To measure OVA-specific immunoglobulins, microtiter plates were coated with OVA and blocked with BSA (Sigma-Aldrich, Cat no. A7030). Serum samples were incubated overnight at 4°C followed by incubation with biotinylated rat anti-mouse IgG1 (BD Biosciences, Cat No. 553441, clone A85-1) or IgE (BD Biosciences, Cat No. 553419, clone R35-118). Extravidin horseradish peroxidase (Sigma-Aldrich, Cat No. E2886) and TMB Substrate Reagent (BD Biosciences, Cat No. 555214) set were used for detection. Serum levels of OVA-specific IgE and OVA-specific IgG1 were calculated relevant to a pre-prepared serum pool from OVA-sensitized and challenged mice and expressed as arbitrary units.

Cell preparation and flow cytometry analyses

For cell preparation of ear-draining LNs, LNs were dissociated with a piston, passed through a 70µm cell strainer and resuspend in FACS buffer (1% FCS, 2mM EDTA in PBS), counted, and used for surface staining. $3x10^6$ LN cells were incubated with anti-CD16/CD32 (clone 93, eBioscience) to block unspecific binding, followed by surface staining with the following antibodies: GL7 (clone GL-7, eBioscience), CD8 (clone, 53-6.7, eBioscience), B220 (clone RA3-6B2, eBioscience), streptavidin (eBioscience), CXCR5 (clone 2G8, BD Biosciences) CD95 (clone Jo2, BD Biosciences),

IgE (clone R35-72, BD Biosciences), CD19 (clone 1D3, BD Biosciences), PD-1 (clone RMP1-30, Biolegend), IgG1 (clone RMG1-1, Biolegend), CD4 (clone GK1.5, Biolegend). Cells were stained with propidium iodide prior to passing to eliminate dead cells. Samples were passed on LSRFortessa x20 and data were analysed using FlowJo.

LN cell culture and antigen stimulation

OVA-specific Tfh cells were identified by activation-induced marker assay. Briefly, $1x10^6$ LN cells were stimulated with 500µg/ml OVA or PBS (vehicule control) in 200µl of medium (RPMI 1640 supplemented with 10% FCS, HEPES, 0.05mM 2-mercaptoethanol, 100U/ml penicillin, 100U/ml streptomycin) for 18h. Anti-CD154 BV650 antibody (clone MR1, BD Biosciences) was added to all culture conditions. After culture, the cells were incubated with CD16/32 (Clone 93, eBioscience), and stained with FVD506 (eBioscience) and the following antibodies: B220 FITC (clone RA3-6B2, Biolegend), CD4 BV421 (clone GK1.5, BD Biosciences), CXCR5 biotin (Clone 2G8, BD Biosciences), Streptavidin PE (eBioscience), PD-1 PE-Cy7 (clone RMP1-30, Biolegend), OX40 APC (clone OX86, eBiosience) and CD25 PerCP-Cy5.5 (clone PC61, BD Biosciences).

Fluorescence-assisted cell sorting

For preparation of epidermal cells, ears were split into ventral and dorsal halves and incubated 1h at 37°C with 2.5mg/ml of Dispase (Gibco). Epidermis was separated from dermis and incubated 30min at 37°C with 1mg/ml collagenase D (Roche), 0.25mg/ml DNAse I (Sigma-Aldrich) and 2.5% of FCS in PBS. Cells were passed through a 70µm cell strainer, resuspend in FACS buffer (1% FCS, 2mM EDTA in PBS) and used for surface staining. Cells were first incubated with anti-CD16/CD32 to block unspecific binding and then stained with the following antibodies: MHCII (clone M5/114.15.2, eBioscience), CD3 (clone 145-2C11, eBioscience), CD45 (clone 30-F11, eBioscience). Viability staining was performed with DAPI. Cells were sorted using BD FACSAria II. Keratinoyctes were identified as $CD45^-$. LCs were identified as $CD45^+$ $CD3^-$ $MHCII^+$.

RT-qPCR

Total RNA was extracted from sorted cells using NucleoSpin RNA XS kit (Macherey-Nagel) according to manufacturer's instructions. RNA was reverse transcribed by using random oligonucleotide hexamers and amplified by quantitative PCR with the LightCycler 480 SYBR Green kit (Roche) on a LightCycler 480 (Roche Diagnostics). Relative RNA levels were calculated with hypoxantine phosphoribosyl-transferase (HPRT) as an internal control. Sequences of qPCR primers are: HPRT (TGGATACAGGCCAGACTTTG, GATTCAACTTGCGCTCATCTTA), IL-7Rα (AAAGCATGATGTGGCCTACC, GGATCCCATCCTCCTTGATT),

TSLPR (GGCCATGGTGTTTAAGGCTA, CCCACTGTCAAGTCACAGCA).

Results

TSLPR expression on DCs is crucial for OVA-induced Tfh cell differentiation

To investigate whether TSLP acts directly on DCs to induce Tfh cell differentiation, *Crlf2*$^{fl/fl}$ mice were crossed with CD11c-Cre$^{Tg/0}$ mice to obtain CD11c-Cre$^{Tg/0}$ *Crlf2*$^{fl/fl}$ mice (named *Crlf2*$^{CD11c-/-}$) in which TSLPR is ablated specifically in DCs. *Crlf2*$^{CD11c-/-}$ and their littermate controls (CT) were then subjected to LMP/OVA treatment (Fig. 1A). Results showed that frequency and number of Tfh cells were drastically reduced in LNs from *Crlf2*$^{CD11c-/-}$ mice compared to CT (Fig. 1B). Next, using an activation-induced marker assay[312], in which OVA stimulation leads to the upregulation of CD25, CD154 and OX40 in allergen-specific Tfh cells, data showed that allergen-specific Tfh cells were also diminished in *Crlf2*$^{CD11c-/-}$ mice (Fig. 1C). In addition, GC B cell and IgG1^{+} B cell numbers, as well as OVA-IgG1 and OVA-IgE levels were reduced in *Crlf2*$^{CD11c-/-}$ mice compared to CT mice (Fig. 1D, E).

Together, these results indicate that TSLPR on DCs is crucially required for TSLP-induced Tfh cell differentiation during allergic skin sensitization.

TSLPR expression on LCs is not required for OVA-induced Tfh cell differentiation

Next, to investigate whether TSLP-TSLPR axis in LCs is implicated in Tfh / GC B cell response during allergic skin sensitization, *Crlf2*$^{fl/fl}$ mice were crossed with HuLang-Cre$^{ERT2(Tg/0)}$ mice to knock-out TSLPR specifically in LCs upon OH-Tam treatment (named *Crlf2*$^{iLC-/-}$).

First, results showed that topical OH-Tam treatment on ears abolished RNA levels of TSLPR in sorted LCs from epidermis of *Crlf2*$^{iLC-/-}$ compared to CT mice, but not in sorted keratinocytes (Fig. 2A). As control, IL-7Rα expression was not changed between *Crlf2*$^{iLC-/-}$ and CT mice. These results indicate that OH-Tam treatment induces *Crlf2* excision specifically in Langerhans cells from *Crlf2*$^{iLC-/-}$ mice.

Next, OH-Tam-treated CT and *Crlf2*$^{iLC-/-}$ mice were subjected to LMP/OVA treatment (Fig. 2B) and showed that the frequency and number of Tfh cells were similar in CT and *Crlf2*$^{iLC-/-}$ mice (Fig. 2C). Moreover, GC B cell, IgG1^{+} cell and IgE^{+} cell numbers were not changed (Fig. 2D). These data suggest that TSLPR in LCs is not implicated in Tfh/GC B cell induction, nor in their tolerogenic role.

Conclusion and perspectives

To conclude, my study suggests that TSLP acts through TSLPR on DCs to induce Tfh cell differentiation in allergic skin sensitization. Different DC populations have been reported to induce Tfh cell response in vivo. For example, cDC2 are required for Tfh cell differentiation in OVA-induced airway inflammation[174]. mo-DCs have also been reported to enhance Tfh cell response in antigen+adjuvant immunized mice[225]. Which DC subpopulation mediates Tfh cell differentiation in allergic skin sensitization remains to be determined. It would be interesting to deliver fluorochrome-coupled OVA to WT mice to identify which DC subpopulation uptake the allergens and could be involved in Tfh response. To get a definitive answer, further studies should be performed using *Dock8*$^{-/-}$, *Irf4*$^{-/-}$ or *Ccr2*$^{-/-}$ mice, in which different DC populations are altered.

DC induction of Tfh cell differentiation requires different signals, of which are costimulatory molecules. TSLP-activated DCs have been shown to promote Tfh cell differentiation through OX40L in vitro[78]. To investigate OX40L role in Tfh / GC B cell response during allergic skin sensitization, we generated *Ox40l*$^{-/-}$ mice. Moreover, as both DCs and B cells express OX40L, we generated CD11c-Cre$^{Tg/0}$ *Ox40l*$^{fl/fl}$ and MB1-Cre$^{Tg/0}$ *Ox40l*$^{fl/fl}$ mice with specific ablation in DCs and B cells respectively, to identify whether OX40L expression is required on DCs or B cells.

My results also show that TSLPR expression on LCs is not required for Tfh cell differentiation. Moreover, we showed that LCs exhibit a tolerogenic function and present an inactivated state in LNs suggesting that their inhibitory role might not be happening in the LNs, but directly in the skin instead[68]. One hypothesis is that LCs could prevent the uptake and processing of allergens by other DC populations, limiting the subsequent T and B cell responses. To test this hypothesis, it would be interesting to deliver fluorochrome-coupled OVA to LC^{DEP} and CT mice and analyse OVA uptake by DC subpopulations

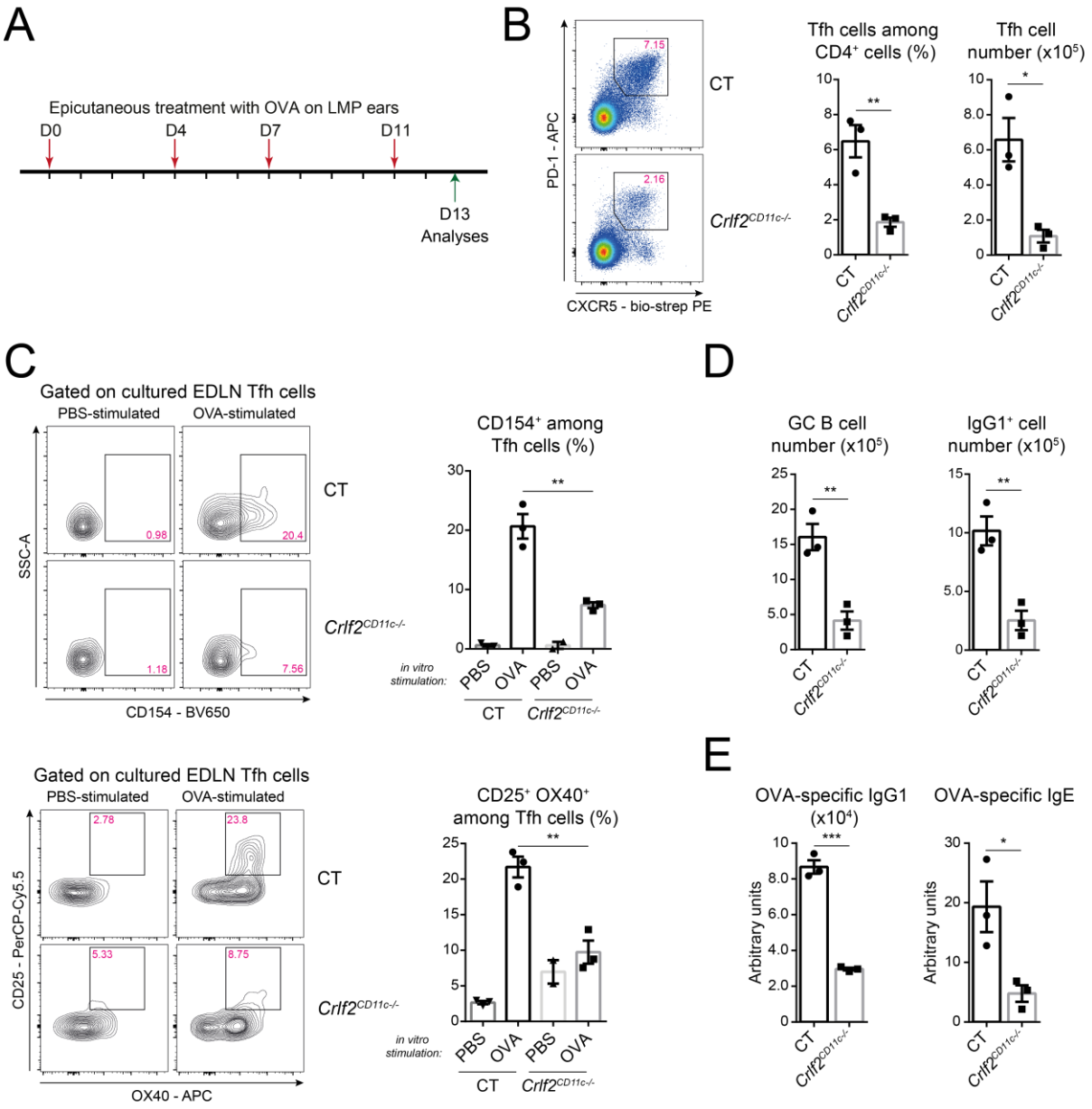

FIG 1. TSLPR expression on DCs is required for OVA-induced Tfh/GC response. **A,** Experimental protocol for OVA epicutaneous sensitization through LMP ears. CD11c-Cre$^{Tg/0}$ *Crlf2*$^{fl/fl}$ mice (named *Crlf2*$^{CD11c-/-}$) and CD11c-Cre$^{0/0}$ *Crlf2*$^{fl/fl}$ littermate controls (named CT) were topically sensitized with OVA on LMP ears at D0, D4, D7 and D11 and EDLNs were analyzed at D13. **B,** Frequency and cell number of Tfh cells in EDLNs. **C,** Frequency of antigen-specific Tfh cells. EDLN cells were in vitro stimulated with OVA or PBS (vehicle control) and activation markers CD154, CD25 and OX40 were examined in Tfh cells. **D,** Cell number of GC B cells and IgG1$^+$ B cells in EDLNs. **E,** Serum levels of OVA-IgG1 and OVA-IgE. Values shown are mean ± SEM. Unpaired two-tailed t-test.

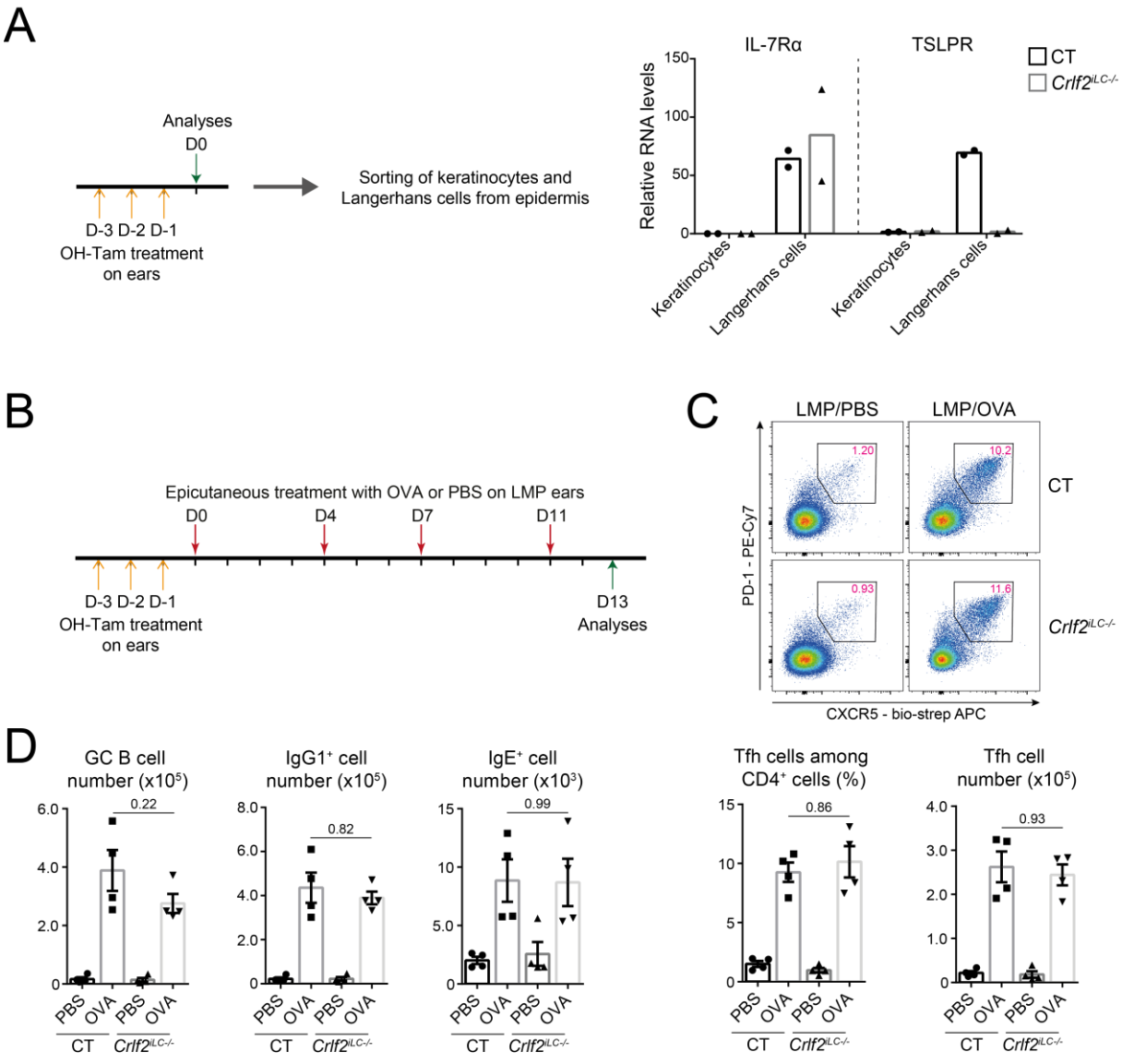

FIG 2. TSLPR expression on LCs is not required for OVA-induced Tfh/GC response. **A,** Experimental protocol. HuLang-Cre$^{ERT2(Tg/0)}$ *Crlf2*$^{fl/fl}$ mice and HuLang-Cre$^{ERT2(0/0)}$ *Crlf2*$^{fl/fl}$ littermate controls were topically treated with 8µg of 4-hydroxytamoxifen (OH-Tam) on ears from D-3 to D-1 to generate *Crlf2*$^{iLC-/-}$ mice and control (CT) mice, respectively. Ears were analyzed at D0. After 1h incubation with 2.5mg/ml dispase, dermis and epidermis were separated and keratinocytes (CD45$^-$) and Langerhans cells (CD45$^+$ CD3$^-$ MHCII$^+$) were sorted from the epidermal cell suspension. Relative RNA levels of IL-7Rα and TSLPR in sorted keratinocytes and Langerhans cells. **B,** Experimental protocol for OVA epicutaneous sensitization through LMP ears. 8µg of OH-Tam was topically applied on ears from D-3 to D-1 to generate *Crlf2*$^{iLC-/-}$ mice and CT mice. OVA or PBS (vehicle) were topically applied on LMP ears at D0, D4, D7 and D11 and EDLNs were analyzed at D13. **C,** Frequency and cell number of Tfh cells in EDLNs. **D,** Cell number of GC B cells, IgG1$^+$ B cells and IgE$^+$ B cells in EDLNs. Values shown are mean ± SEM. One-way ANOVA test.

2. Characterization of a new mouse line to deplete Tfh cells

Introduction

Study of the role Tfh cells in vivo requires mouse tools to deplete these cells. To this day, several mouse models are used to study Tfh cells, including adoptive transfer models or CD4-Cre$^{Tg/0}$ *Bcl6*$^{fl/fl}$ mice. Transfer models are widely used to investigate the role of a cell population, including Tfh cells as reported by Dolence et al.[303] who sorted CD4^{+} ST2^{-} CXCR5^{+} cells to transfer in *Tcrb*$^{-/-}$ mice in a model of peanut allergy. However, adoptive transfer models present several limitations such as the difficulty of the technique, the number of cells to transfer, cell viability and activation status. In addition, CD4-Cre$^{Tg/0}$ *Bcl6*$^{fl/fl}$ mouse line is also commonly used as these mice exhibit an absence of Tfh cells. However, Kobayashi et al.[302] reported that Th2 cytokine production is increased in CD4-Cre$^{Tg/0}$ *Bcl6*$^{fl/fl}$ mice at steady state, in agreement with the inhibitory role of Bcl6 on GATA3 expression[313,314]. Therefore, to bypass the bias towards a Th2 phenotype of this mouse model, and to avoid the difficulty of adoptive transfer, we aimed at generating an inducible genetic tool based on DTR expression to deplete Tfh cells in order to study their role in allergic skin sensitization.

Methods

Mice

Bcl6$^{HR/+}$ mice were generated (on a C57BL/6J genetic background) by my team. CD4-Cre$^{Tg/0}$ (in C57BL/6J background) mice were purchased from Jax. Ten to sixteen weeks old mice were used in experiments. Breeding and maintenance were performed under institutional guidelines, and all the experiments were approved by the animal care and ethics committee of animal experimentation of the IGBMC, and by the Ministère de l'enseignement supérieur, de la recherche et de l'innovation.

Alum/OVA/LPS immunization

Mice were immunized by intraperitoneal (i.p.) injection of 100µg OVA + 10µg LPS (Sigma-Aldrich, Cat No. L4524) in 2mg alum (ThermoFisher, Cat No. 77161) at day 0 (D0) and D4. Spleen analyses were performed at D10.

CFA/OVA immunization

To induce immunization, mice were subcutaneously (s.c.) injected with 50µg OVA in CFA (Sigma-Aldrich, Cat No. F5881) in the lower back, in direction of the left inguinal LN at D0. The procedure was repeated in the direction of the right inguinal LN. Inguinal LN analyses were performed at D10. In case of Tfh cell depletion, mice were i.p. injected with diphtheria toxin (1µg per 25g of body weight) at D8 and D9.

Flow cytometry

For cell preparation of inguinal LNs and spleen, LNs and spleen were dissociated with a piston and passed through a 70µm cell strainer and resuspend in FACS buffer (1% FCS, 2mM EDTA in PBS), counted, and used for surface staining. $3x10^6$ LN or spleen cells were incubated with anti-CD16/CD32 (clone 93, eBioscience) to block unspecific binding, followed by surface staining with the following antibodies: streptavidin (eBioscience), GL7 (clone GL-7, eBioscience), CD8 (clone, 53-6.7, eBioscience), CXCR5 (clone 2G8, BD Biosciences) CD95 (clone Jo2, BD Biosciences), CD4 (clone RM4-5, BD Biosciences), CD19 (clone 1D3, BD Biosciences), PD-1 (clone RMP1-30, Biolegend). Cells were stained with propidium iodide prior to passing to eliminate dead cells. Samples were passed on LSRFortessa x20 and data were analysed using FlowJo.

Statistics

Data were analysed using GraphPad Prism 6. Comparison of two samples was performed by Student's unpaired two-tailed t-test.

Results

To investigate the role of Tfh cells, we generated a new mouse line allowing the depletion of Tfh cells upon diphtheria toxin injection. The two LoxP sites were placed to flank the blue fluorescent protein (BFP), a STOP codon and the neomycin resistance (NeoR) gene. This cassette and the human diphtheria toxin receptor (hDTR) were inserted just before the stop codon of *Bcl6* gene to prevent any alteration of Bcl6 expression (Fig. 1A). The allele containing the targeting construct

was named HR (homologous recombination). *Bcl6*$^{HR/+}$ mice were generated and analysed for BFP (Bcl6) expression. Next, *Bcl6*$^{HR/+}$ mice were crossed with CD4-Cre$^{Tg/0}$ mice to obtain CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mice, allowing the excision of BFP-STOP-NeoR sequence in CD4$^+$ and CD8$^+$ cells. This strategy allows hDTR expression specifically in CD4$^+$ Bcl6$^+$ cells, corresponding to Tfh cells, as CD8$^+$ cells do not express Bcl6.

First, *Bcl6*$^{HR/+}$ mice were characterized for BFP expression. *Bcl6*$^{+/+}$ and *Bcl6*$^{HR/+}$ mice were immunized with OVA/LPS diluted in alum to induce Tfh cells in the spleen (Fig. 1B), and BFP expression was analysed in B cells, CD8$^+$ T cells, Tfh cells and CD4$^+$ CXCR5$^-$ PD-1$^-$ non-Tfh cells by flow cytometry. Results showed that B cells and Tfh cells from non-treated *Bcl6*$^{HR/+}$ mice expressed BFP compared to *Bcl6*$^{+/+}$ mice, which was further enhanced in OVA/LPS/alum-treated *Bcl6*$^{HR/+}$ mice (Fig. 1C). BFP was not detected in CD8$^+$ T cells and CD4$^+$ CXCR5$^-$ PD-1$^-$ non-Tfh cells. *Bcl6*$^{+/+}$ and *Bcl6*$^{HR/+}$ mice were then subcutaneously (s.c.) immunized with CFA/OVA in the lower back to induce Tfh / GC B cells in inguinal LNs at D10 (Fig. 1D). As expected, B cells and Tfh cells from *Bcl6*$^{HR/+}$ mice expressed BFP but no signal was detected in CD8$^+$ T cells and CD4$^+$ CXCR5$^-$ PD-1$^-$ non-Tfh cells (Fig. 1E). These results validate the correct insertion of the construct in the *Bcl6* gene as the expression of BFP is restricted to B and Tfh cells, in adequation with their expression of Bcl6.

Next, *Bcl6*$^{HR/+}$ mice were crossed with CD4-Cre$^{Tg/0}$ mice to generate CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mice. First, BFP expression was analysed in B cells, CD8$^+$ T cells, Tfh cells and CD4$^+$ CXCR5$^-$ PD-1$^-$ non-Tfh cells by flow cytometry. Results showed that similar level of BFP were observed in B cells from CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ and CD4-Cre$^{0/0}$ *Bcl6*$^{HR/+}$ mice (Fig. 2A). In contrast, BFP expression in Tfh cells from CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mice was abolished compared to CD4-Cre$^{0/0}$ *Bcl6*$^{HR/+}$ mice, indicating that BFP was excised by the recombinase Cre in Tfh cells, but not in B cells. Second, to examine the Tfh cell depletion in CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mice, these mice and their littermate controls were subcutaneously immunized with CFA/OVA and i.p. injected with diphtheria toxin (DT) at D8 and D9 (Fig. 2B). Inguinal LNs were analysed at D10. Results showed a decrease in total LN cell number in CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mice compared to CT (Fig. 2C). Both CD4$^+$ T cell and Tfh cell frequencies were decreased in CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mice (Fig. 2E, F, see Fig. 2D for gating strategy). On the other hand, CD8$^+$ T cells, CD4$^+$ CXCR5$^-$ PD-1$^-$ non-Tfh cell as well as B cell frequencies were not reduced in CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mice (Fig. 2E-G). In addition, a reduction in frequency of GC B cells was observed (Fig. 2H), which could be the consequence of the depletion of Tfh cells. Together, these results confirm the depletion of Tfh cells in CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mice (named Tfh^{DEP}) upon DT injection.

Conclusion and perspectives

To conclude, characterization of the mouse line *Bcl6*$^{HR/+}$ indicates that it can be used as a reporter line for Bcl6^{+} cells. Moreover, crossing CD4-Cre$^{Tg/0}$ with *Bcl6*$^{HR/+}$ restricts hDTR expression to Bcl6^{+} CD4^{+} T cells corresponding to Tfh cells. Results show that DT injection results in Tfh cell depletion in the LNs, validating CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mouse line as a new tool to study Tfh cells.

Overall, this mouse line could be useful for different studies, including allergy, autoimmunity, cancer, infection or vaccination. My study provides a new tool to investigate the function of Tfh cells on Th2 and humoral responses during allergic skin sensitization and the atopic march. Indeed, eosinophils and basophils can crosslink IgE resulting in degranulation which contributes to inflammation. Moreover, Tfh cells have been suggested to give rise to pathogenic Th2 cells in asthma[305]. Therefore, it would be interesting to subject Tfh-depleted CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mice to LMP/OVA, followed by intranasal OVA exposure to induce asthma, to investigate the role of Tfh cells not only in GC-B formation and IgE/IgG1 production, but also on the subsequent skin and airway phenotypes.

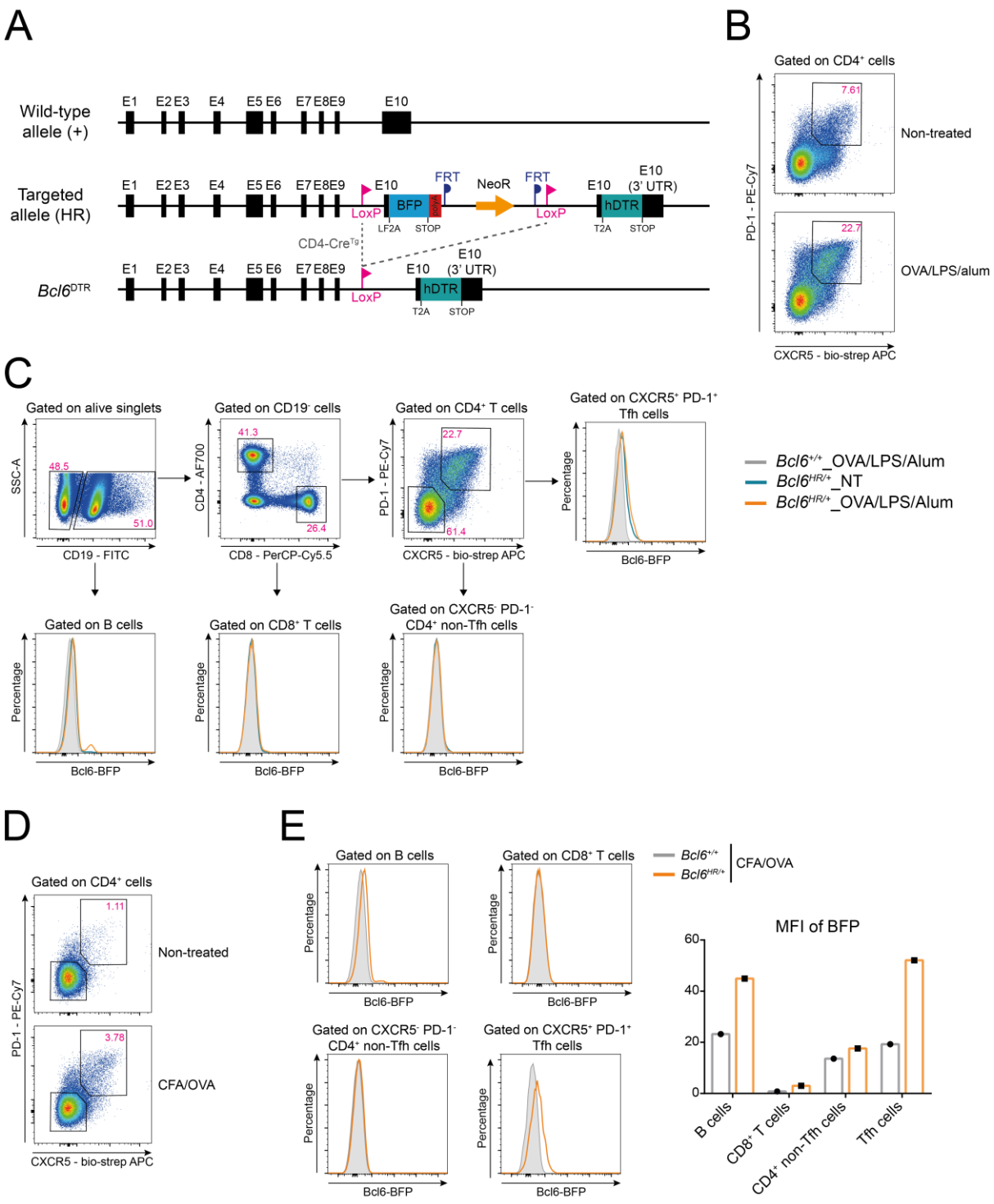

FIG 1. BFP expression is restricted to B cells and Tfh cells in *Bcl6*$^{HR/+}$ mice. **A,** Schematic representation of the wild-type allele (+), the targeted allele (HR) containing the BFP sequence and the FRT-flanked neomycin resistance gene, and the *Bcl6*^{DTR} allele after Cre-mediated excision of BFP-STOP-NeoR. E: exon. **B, C,** Mice were intraperitoneally immunized with OVA/LPS/alum at D0 and D4. Spleen was analyzed at D10. **B,** Plots of CXCR5$^+$ PD-1$^+$ CD4$^+$ Tfh cells in spleen of non-treated or immunized mice. **C,** Histograms and Mean Fluorescence Intensity (MFI) of BFP (reporting Bcl6) in B cells, CD8$^+$ T cells, CXCR5$^-$ PD-1$^-$ CD4$^+$ non-Tfh cells and Tfh cells in spleen of non-treated or immunized *Bcl6*$^{HR/+}$ and *Bcl6*$^{+/+}$ mice. **D, E,** Mice were subcutaneously immunized with CFA/OVA at D0. Inguinal lymph nodes (LNs) were analyzed at D10. **D,** Representative plots of CXCR5$^+$ PD-1$^+$ CD4$^+$ Tfh cells in inguinal LNs of CFA/OVA or non-treated mice. **E,** Histograms and Mean Fluorescence Intensity (MFI) of BFP (reporting Bcl6) in B cells, CD8$^+$ T cells, CXCR5$^-$ PD-1$^-$ CD4$^+$ non-Tfh cells and Tfh cells in inguinal LNs of CFA/OVA-treated *Bcl6*$^{HR/+}$ and *Bcl6*$^{+/+}$ mice.

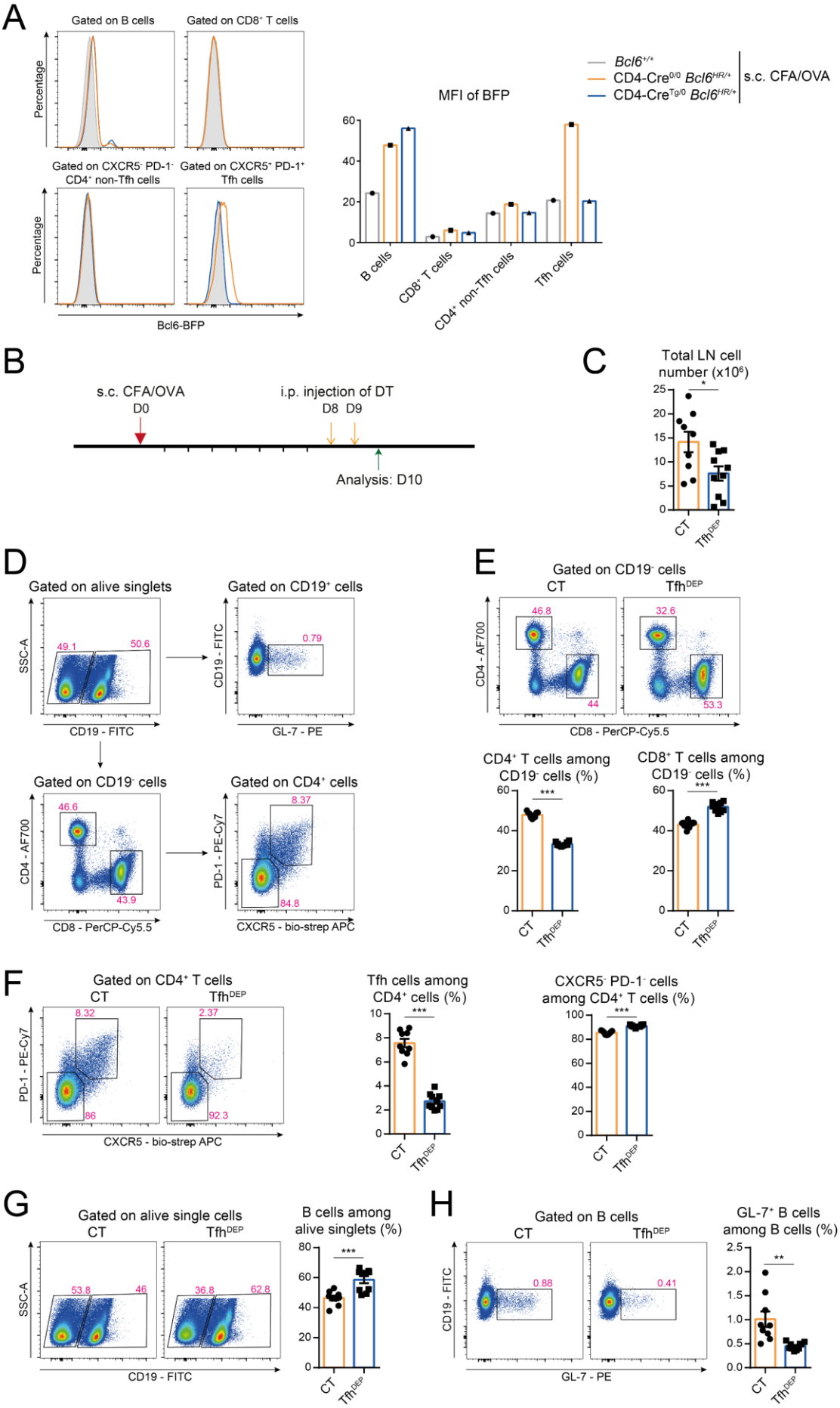
A
Gated on B cells
Gated on CD8⁺ T cells
Gated on CXCR5⁻ PD-1⁻ CD4⁺ non-Tfh cells
Gated on CXCR5⁺ PD-1⁺ Tfh cells
Percentage
Bcl6-BFP
MFI of BFP
Bcl6+/+
CD4-Cre0/0 Bcl6HR/+
CD4-CreTg/0 Bcl6HR/+
s.c. CFA/OVA
B cells
CD8⁺ T cells
CD4⁺ non-Tfh cells
Tfh cells
B
s.c. CFA/OVA
D0
i.p. injection of DT
D8 D9
Analysis: D10
C
Total LN cell number (x10⁶)
CT
TfhDEP
D
Gated on alive singlets
SSC-A
CD19 - FITC
Gated on CD19⁺ cells
GL-7 - PE
Gated on CD19⁻ cells
CD4 - AF700
CD8 - PerCP-Cy5.5
Gated on CD4⁺ cells
PD-1 - PE-Cy7
CXCR5 - bio-strep APC
E
Gated on CD19⁻ cells
CT
TfhDEP
CD4⁺ T cells among CD19⁻ cells (%)
CD8⁺ T cells among CD19⁻ cells (%)
F
Gated on CD4⁺ T cells
Tfh cells among CD4⁺ cells (%)
CXCR5⁻ PD-1⁻ cells among CD4⁺ T cells (%)
G
Gated on alive single cells
B cells among alive singlets (%)
H
Gated on B cells
GL-7⁺ B cells among B cells (%)

FIG 2. DT induces Tfh cell depletion in CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mice. **A,** *Bcl6*$^{+/+}$, CD4-Cre$^{0/0}$ *Bcl6*$^{HR/+}$, CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ mice were subcutaneously (s.c.) injected with CFA/OVA at D0. Histograms and MFI of BFP (reporting Bcl6) in B cells, CD8^{+} T cells, CXCR5^{-} PD-1^{-} CD4^{+} non-Tfh cells and Tfh cells in inguinal LNs at D10. **B,** Experimental protocol. Control CD4-Cre$^{0/0}$ *Bcl6*$^{HR/+}$ (named CT) mice and CD4-Cre$^{Tg/0}$ *Bcl6*$^{HR/+}$ (named Tfh^{DEP}) mice were s.c. injected with CFA/OVA at D0, and were intraperitoneally injected with DT (1µg) at D8 and D9 to induce DTR-expressing cell depletion. Inguinal LNs were analysed at D10. **C,** Total inguinal LN cell number. **D,** Gating strategy. **E-H** Frequency of CD4^{+} T cells, CD8^{+} T cells **(E)**, Tfh cells, CXCR5^{-} PD-1^{-} CD4^{+} non-Tfh cells **(F)**, B cells **(G)** and GC B cells **(H)** in inguinal LNs. Values shown are mean ± SEM. Unpaired two-tailed t-test.

PART 2:
Context-dependent function of TSLP and IL-1β in skin allergic sensitization and atopic march

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Context-dependent function of TSLP and IL-1β in skin allergic sensitization and atopic march

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Abstract

Atopic diseases, including atopic dermatitis (AD) and asthma, affect a large proportion of the population, with increasing prevalence worldwide. AD often precedes the development of asthma, known as the atopic march. Allergen sensitization developed through the barrier-defective skin of AD has been recognized to be a critical step leading to asthma, in which thymic stromal lymphopoietin (TSLP) was previously shown to be a key player. In this study, using an innovative laser-assistant microporation system to disrupt targeted skin layers for generating micropores at a precise anatomic depth of mouse skin, we modeled allergen exposure superficially or deeply in the skin, leading to epicutaneous sensitization or dermacutaneous sensitization that is associated with different cytokine microenvironment. Our work reveals a differential requirement for TSLP in these two contexts, and identifies an important role for IL-1β, which is independent of TSLP, in promoting allergen sensitization and the subsequent allergic asthma.

Introduction

Atopic diseases, including AD and asthma, affect a large proportion of the population, with increasing prevalence worldwide. These diseases result in considerable morbidity, and are in some circumstances life-threatening, raising these diseases as major health problems. AD is a chronic inflammatory skin disease characterized by defective skin barrier, cutaneous inflammation with infiltration of Th2 cells, eosinophils as well as expression of Th2 cytokines and allergen-specific immunoglobulin (Ig) E production[1, 2]. It usually starts in early infancy and precedes other atopic diseases such as asthma. More than 50% of moderate to severe AD children develop asthma and/or allergic rhinitis at a later stage, called the atopic march [3, 4, 5, 6]. In addition to the efforts in the development of medical therapy for asthma, it is critically important to develop strategies to prevent and block the atopic march.

It has been found that AD children are prone to develop allergen sensitization, indicated by T cell and B cell memories to allergens. Cutaneous exposure to allergens, such as aeroallergen house dust mites (HDM) is recognised as a critical route for sensitization in AD patients [7]. It is thus assumed, which has been supported by studies from mouse models [8, 9], that following the phase of skin sensitization, allergen challenge upon re-encountering of the allergen in the airway at a later stage results in the development of asthmatic symptoms. Recently, a human cohort study showed that AD with allergen sensitization has a higher risk of asthma, whereas AD without concomitant allergic sensitization is not associated with an increased risk of asthma[10], providing further evidence for the key role of allergic sensitization occurring during AD in the process of the atopic march. The understanding of how allergic sensitization occurs and how it is regulated in AD context is therefore crucially required for developing strategies to prevent and stop the atopic march.

It is known that AD skin not only bears defective skin barrier allowing allergen penetration but also provides an inflammatory cytokine microenvironment conducive to the development of the sensitization to allergens[2]. We and others have previously reported that thymic stromal lymphopoietin (TSLP), a cytokine produced by skin keratinocytes, is induced by skin barrier disruption in mouse[8] or human[11], and promotes ovalbumin (OVA)-induced Th2-type sensitization through the tape-stripped skin and the subsequent asthma in mice[8]. More recently, we also provided evidence that skin TSLP promotes epicutaneous OVA-induced follicular helper T (Tfh)

cells, which provide critical B cell help in the germinal center (GC) of lymphoid organs[12] for the generation of allergen-specific IgE[13].

Despite of these pieces of evidence suggesting TSLP as an important target for AD therapy and for preventing the atopic march, it had not yet been explored the role of TSLP in allergen sensitization occurring in AD skin with different severity. Indeed, AD is well recognized for its heterogeneity, bearing varied skin barrier defects[14] due to various genetic or/and environmental causes, or at different stages of the diseases. Consequently, allergen exposure could occur at lesioned skin at the different anatomic depth, and be associated with different cytokine microenvironment. We thus aimed to investigate the role of TSLP in the allergen cutaneous sensitization occurring at different anatomic depth of mouse skin and the subsequent development of allergic asthma. In this work, using an innovative laser-assistant microporation (LMP) system to disrupt the targeted skin layers for generating micropores at a precise anatomic depth of mouse skin, we model allergen exposure superficially or deeply in the skin, leading to an epicutaneous sensitization or a dermacutaneous sensitization that is associated with different cytokine microenvironment. Our study reveals a differential requirement for TSLP in these two contexts, and identifies an important role for IL-1β, which is independent of TSLP, in promoting allergen sensitization and the subsequent allergic asthma.

Results

TSLP is differentially required for e.c. or d.c. HDM-induced Th2/Tfh responses

To mimic the cutaneous allergen sensitization occurring at different anatomic depth of mouse skin, we used the Precise Laser Epidermal System (P.L.E.A.S.E., Pantec Biosolutions) to fractionally ablate the targeted skin layers and generate patterned micropores, which allows us to deliver allergens to micropores at a precise depth of the skin, as recently reported[13]. As shown in Fig. 1a, the laser microporation (LMP) at the depth of 30µm (LMP_30µm) ablates the stratum corneum and the suprabasal layer of the epidermis of mouse ears, while the LMP at the depth of 91µm (LMP_91µm) ablates the epidermis and reaches the dermis. ELISA analyses showed that TSLP production was similarly induced in LMP_91µm and LMP_30µm ears (Fig. 1b), and RNA in situ hybridization identified that in both cases TSLP expression was restricted to the epidermis, with no signal detected in the dermis (Fig. 1c), in agreement with previous reports showing that keratinocyte-derived TSLP is induced by barrier disruption in mouse[8, 13] or human skin[11]. We next set out to investigate allergen sensitization occurring at different anatomic depth of the skin and subsequently the development of asthma, by establishing an experimental protocol in which house dust mite (HDM) is applied on LMP_30µm skin (to achieve epicutaneous e.c. sensitization*)* or on LMP_91µm (to achieve dermacutaneous d.c. sensitization), followed by intranasal (i.n.) challenge with HDM to induce allergic asthma (Fig. 1d).

We first analysed the Th2-type skin inflammation induced by e.c. or d.c. HDM sensitization, and compared the requirement for TSLP in these two contexts. As shown in Fig 1e, the e.c. HDM treatment induced an inflammatory cell infiltration in the dermis of Balb/c wildtype (WT) mice, including eosinophils and basophils, two characteristic cells in Th2-type inflammation in allergic AD, which was totally abolished in *Tslp*$^{-/-}$ mice (Fig. 1e). In the case of the d.c. HDM treatment, more eosinophils and basophils were observed to infiltrate into the dermis of WT mice compared to e.c. HDM treatment (Fig. 1e). However, unlike the e.c. HDM, the d.c. HDM-induced infiltrate of eosinophils and basophils was not abolished in *Tslp*$^{-/-}$ mice, despite of a partial reduction (Fig. 1e; see Supplementary Fig. 1a for cell counts comparison per microscopic field). We next examined the Th2 cells in the sensitized ears, which are central for allergen sensitization-induced T cell responses. To analyse whether the expression of Th2 cytokines IL-4 and IL-13 is dependent on TSLP, the Il4/Il13 dual-reporter transgenic mice (4C13R^{Tg}), in which AmCyan-coding sequence is under IL-4 regulatory elements and DsRed-coding sequence is under IL-13 regulatory elements[15]

were bred with *Tslp*$^{-/-}$ mice to generate *Tslp*$^{-/-}$ 4C13R^{Tg} mice. As shown in Fig. 1f **(**see Supplementary Fig. 2a for FACS gating strategy), upon the e.c. HDM treatment, expression of both Amcyan (IL-4) and DsRed (IL-13) was increased in TCR-β^{+} cells in WT / 4C13R^{Tg} ears when compared to e.c. PBS treatment, and such increase was completely abolished in e.c. HDM-treated *Tslp*$^{-/-}$ / 4C13R^{Tg} skin (Fig. 1f). Upon d.c. HDM treatment, we observed a higher IL-4 and IL-13 expression by Th2 cells in the skin compared to e.c. HDM (Fig. 1f), and similar to what was observed for eosinophils and basophils, the IL-4 and IL-13 expression by skin Th2 cells was partially reduced but not abolished in *Tslp*$^{-/-}$ mice (Fig. 1f).

We also examined the Tfh/GC responses in ear-draining lymph nodes (EDLN), which are known to be crucial for allergen sensitization-induced B cell responses and IgE and IgG1 production. In e.c. HDM-treated WT mice, the total LN cell number, Tfh cell (identified as CXCR5^{+}PD1^{+}) frequency in CD4^{+} T cells and its cell number, as well as IL-4 frequency in Tfh cells and IL-4^{+} Tfh cell number, were all increased, which were dramatically reduced in e.c. HDM-treated *Tslp*$^{-/-}$ mice (Fig. 1g-h, see Supplementary Fig. 2b for FACS gating strategy). Correspondingly, the numbers of GC B cells (identified as Fas^{+}GL7^{+}), as well as of IgE^{+} B cells and IgG1^{+}B cells (see Supplementary Fig. 2c for FACS gating strategy), were increased in e.c. HDM-treated compared to e.c. PBS-treated WT mice, which were all diminished in *Tslp*$^{-/-}$ mice (Fig. 1i). This was in agreement with the observation that serum levels of HDM-specific IgE and IgG1 in e.c. HDM-treated *Tslp*$^{-/-}$ mice were significantly reduced or tended to reduce, respectively (Fig. 1j). In contrast to e.c. HDM, d.c. HDM treatment induced higher numbers for Tfh cells with IL-4 expression and GC B cells in EDLNs of WT mice (Fig. 1g-i). Again, these increases were not abolished in *Tslp*$^{-/-}$ mice, despite of certain reduction (Fig. 1g-i). Particularly, IgE^{+} and IgG1^{+} B cell numbers in EDLNs (Fig. 1i), as well as serum levels of HDM-specific IgE and IgG1 (Fig. 1j), were not reduced in d.c. HDM-treated *Tslp*$^{-/-}$ mice.

Together, these data indicate that e.c. HDM and d.c. HDM sensitization both induce Th2/Tfh cell responses, however TSLP is either crucially or only partially implicated in these two contexts.

TSLP is differentially implicated for e.c. or d.c. HDM sensitization-triggered asthma

We next examined the asthmatic inflammation developed in mice at D13 following i.n. HDM challenge (Fig. 1d). Note that in this experiment, all the mice were i.n. challenged with HDM. We observed first that the e.c. and d.c. PBS treatments (i.e. the vehicle control PBS was applied on

LMP_30µm or LMP_91µm skin) did not result in any change in bronchoalveolar lavage (BAL) cells (Fig. 2a, b), Th2 cytokine expression (Fig. 2c), eosinophil and basophil infiltration in the lung, or goblet cell hyperplasia (Fig. 2d). Thus, without allergen HDM, skin LMP (at the depth of 30µm or 91µm) on its own does not drive any lung inflammation, indicating that the asthmatic phenotype developed in this experimental protocol is triggered by allergen sensitization through the LMP skin.

Second, BAL cell analyses showed that the number of eosinophils was increased in e.c. HDM-sensitized WT mice compared to e.c. PBS-treated WT mice (Fig. 2b), which was accompanied by an increase of the expression of Th2 cytokines IL-4, IL-5, IL-13 as well as CCR3 (an indicator for eosinophils) and MCPT8 (an indicator for basophils) by BAL cells (Fig. 2c). All these increases were abolished in *Tslp*$^{-/-}$ mice (Fig. 2b-c). Moreover, e.c. HDM sensitized WT mice exhibited perivascular and peribronchiolar cell infiltrations including eosinophils and basophils in the lung (Fig. 2d, hematoxylin & eosin (H&E) and immunohistochemistry (IHC) analyses), mucus-producing goblet cell hyperplasia (Fig. 2d, periodic acid schiff (PAS) staining), as well as enhanced airway responsiveness to methacholine (Fig. 2e, shown by lung resistance R_L), which were again all abolished in *Tslp*$^{-/-}$ mice. Together, these data indicate that TSLP is crucially required for e.c. HDM-induced allergic asthma.

In contrast to e.c. HDM sensitization, d.c. HDM sensitization appeared to lead to a stronger asthmatic phenotype in WT mice, including a higher number of total cells, including that of eosinophils as well as neutrophils in the BAL (Fig. 2a, b), accompanied by a higher (or a tendency to be higher) RNA level of IL-4, IL-5, IL-13, CCR3 and MCPT8 in BAL cells (Fig. 2c), a stronger infiltration of eosinophils and basophils in the lung (Fig. 2d; see Supplementary Fig. 1b for cell counts comparison), as well as an enhanced goblet cell hyperplasia (Fig. 2d) and airway hyperresponsiveness (AHR) (Fig. 2e). In *Tslp*$^{-/-}$ mice, we observed that some of these d.c. HDM-induced asthmatic phenotypes, including BAL eosinophils, lung infiltration of eosinophils and basophils and goblet cell hyperplasia,were partially reduced however not abolished (Fig. 2a-d), while the AHR was comparable between *Tslp*$^{-/-}$ and WT mice (Fig. 2e).

These results thus indicate that e.c. HDM sensitization promotes a typical Th2 asthmatic inflammation, which is abolished in *Tslp*$^{-/-}$ mice; in contrast, d.c. HDM sensitization-triggered allergic asthma is only partially reduced in *Tslp*$^{-/-}$ mice. Therefore, in addition to TSLP, there should be other factor(s) derived from the skin, which is (are) implicated in d.c. HDM sensitization and thereby impact(s) the subsequent development of allergic asthma.

IL-1β enhances the e.c. HDM sensitization in a TSLP-independent manner

Searching for what could be the factors in addition to TSLP contributing to the d.c. HDM-induced skin sensitization and the subsequent asthma, we found with interest that IL-1β protein level in the skin was much higher in LMP_91µm ears compared to LMP_30µm ears (Fig. 3b). RNA in situ hybridization analyses showed that while IL-1β-expressing cells were barely detected in non-treated WT skin, some and many more were detected in LMP_30µm and LMP_91µm ears, respectively; and different from TSLP, most of IL-1β-expressing cells appeared to be infiltrated immune cells located in the dermis (Fig. 3a; see below for cellular characterization).

To examine whether IL-1β may be functionally responsible for the difference between e.c. HDM- and d.c. HDM-induced sensitization and the subsequent asthma, we designed an experimental protocol in which the recombinant mouse IL-1β was supplemented to e.c. HDM treatment on LMP_30µm ears, followed by HDM i.n. challenge (Fig. 3c). Note that the administration of IL-1β did not result in an increase of TSLP production (by ELISA, Fig. 3d; and by RNAscope analyses, Supplementary Fig.3). Histology and IHC analyses of ears showed that the co-administration of IL-1β with HDM (e.c. HDM + IL-1β) in WT mice exacerbated the dermal cell infiltration compared to e.c. HDM, including abundant eosinophils and basophils (Fig. 3e; see Supplementary Fig. 1c for cell counts comparison). Although a small increase of eosinophils and basophils was noted in the dermis by IL-1β alone (without HDM treatment), which may suggest an effect of IL-1β in skin inflammation, this was very mild compared to e.c. HDM or e.c. HDM + IL-1β skin (Fig. 3e). Interestingly, we found that in *Tslp*$^{-/-}$ mice, e.c. HDM + IL-1β also induced the infiltration of eosinophils and basophils (Fig. 3e; see Supplementary Fig. 1c for cell counts comparison), as well as Th2 cytokine (particularly IL-13) expression in TCR-β^{+} cells in the dermis (Supplementary Fig. 4a), indicating that IL-1β is able to promote e.c. HDM-induce skin Th2 inflammation without the need of TSLP.

Analyses of Tfh/GC response in EDLNs showed that e.c. HDM+ IL-1β treatment led to an increase in the number of Tfh cells, IL-4-expressing Tfh cells, GC B cells, IgE^{+} and IgG1^{+} B cells compared to e.c. HDM treatment, in both WT and *Tslp*$^{-/-}$ mice (Fig. 3f and Supplementary Fig. 4b). Correspondingly, measurement of HDM-specific IgE and IgG1 in sera showed that the co-administration of IL-1β with e.c. HDM enhanced the production of HDM-specific IgE and IgG1 in WT and particularly in *Tslp*$^{-/-}$ mice (Fig. 3g).

We further examined asthmatic phenotypes after the i.n. HDM challenge. We observed first that the e.c. IL-1β alone (without HDM) treatment did not result in any change in BAL cells (Fig. 4a, b), Th2 cytokine expression (Fig. 4c), eosinophil and basophil infiltration in the lung (Fig. 4d, see Supplementary Fig. 1d for cell counts comparison), or goblet cell hyperplasia (Fig. 4d), indicating that without HDM, IL-1β administration in the skin does not promote any asthmatic inflammation on its own, even though e.c. IL-1β slightly enhances the skin inflammation. Second, co-administration of IL-1β in e.c. HDM-treated WT mice led to a strong increase in the number of total BAL cells (Fig. 4a), including that of eosinophils, lymphocytes and neutrophils (Fig. 4b), accompanied by a higher (or a tendency to be higher) expression of IL-4, IL-5, IL-13, CCR3 and MCPT8 in BAL cells (Fig. 4c), a stronger infiltration of eosinophils and basophils, and an increased goblet cell hyperplasia (Fig. 4d). Notably, the exacerbation of all these allergic asthma phenotypes by IL-1β was dependent of T and B cell responses, as no change of lung inflammation was observed in *Rag1*$^{-/-}$ mice (lacking T and B cells) treated with e.c. HDM + IL-1β compared with e.c. HDM alone (Supplementary Fig. 5). Again, the exacerbation of these allergic asthma phenotypes by IL-1β was observed not only in WT mice but also in *Tslp*$^{-/-}$ mice (Fig. 4a-d).

Altogether, these results suggest that co-administration of IL-1β with e.c. HDM sensitization enhances allergen-triggered Th2 and Tfh/GC responses, as well as the subsequent asthmatic inflammation, in a TSLP-independent manner.

Infiltrated neutrophils and monocytes / macrophages express IL-1β

We further characterized IL-1β-expressing cells in d.c. HDM-sensitized skin. First, flow cytometry analyses (Supplementary Fig. 6, for gating strategy) showed that in d.c. HDM-treated skin, there was a significant infiltration of CD45^{hi} Siglec-F^{-} CD49b^{-} Gr-1^{hi} and Gr-1^{int} cells (Fig. 5a), which corresponded to Ly-6G^{+} Ly-6C^{-} neutrophils and Ly-6G^{-} Ly-6C^{+} monocytes/macrophages, respectively (Supplementary Fig. 6). The frequency of eosinophils (CD45^{+} Siglec-F^{+} SSC-A^{hi}), basophils (CD45^{int} Siglec-F^{-} CD49b^{+}) or TCR-β^{+} T cells (CD45^{+} Siglec-F^{-} TCR-β^{+}) cells was not significantly increased (Fig. 5a). In contrast, the e.c. HDM treatment induced only a mild infiltration of Gr-1^{int} and Gr-1^{hi} cells (Fig. 5a). Second, intracellular staining of IL-1β showed that the Gr-1^{int} monocytes / macrophages and the Gr-1^{hi} neutrophils recruited to d.c. HDM-treated skin exhibited the highest expression level of IL-1β among the cell populations examined (Fig. 5b). Calculation of frequency of the gated IL-1β^{+} cells showed that the majority of IL-1β-expressing cells comprised

Gr-1^{hi} neutrophils and Gr-1^{int} monocytes / macrophages (Fig. 5c).

We then sought to test whether the depletion of Gr-1^{hi} and/or Gr-1^{int} cells would reduce the IL-1β level in the d.c. HDM-treated skin, by administrating anti-Gr-1 antibody (Ab) (clone NIMP-R14) which depletes both Gr-1^{hi} and Gr-1^{int} cells[16] or anti-Ly6G Ab which was reported to selectively deplete Gr-1^{hi} cells in Balb/c (but not C57BL/6) WT mice[17, 18] (Fig. 5d). As expected, at D1, NIMP-R14 Ab efficiently depleted Gr-1^{hi} neutrophils and Gr-1^{int} monocytes/macrophages, whereas anti-Ly6G Ab depleted only Gr-1^{hi} neutrophils but not Gr-1^{int} monocytes/macrophages, without impacting other cells like T cells or eosinophils (Fig. 5e and f). This was accompanied by a strong reduction of IL-1β level in the d.c. HDM-sensitized skin from the NIMP-R14-injected mice, and to a lesser extent from the anti-Ly6G-injected mice (Fig. 5g). In contrast, TSLP levels remained unchanged (Fig. 5g). These results thus indicate that both Gr-1^{hi} neutrophils and Gr-1^{int} monocytes / macrophages are cellular sources for IL-1β in d.c. HDM-sensitized skin, and that the depletion of these cells reduces IL-1β but does not impact TSLP production.

We also examined whether the infiltration of IL-1β-expressing neutrophils and monocytes / macrophages in the skin requires TSLP. Upon e.c. HDM or d.c. HDM treatment, WT and *Tslp*$^{-/-}$ mice exhibited similar levels for IL-1β (Fig. 5h) and similar frequencies for Gr-1^{hi} and Gr-1^{int} cells in the skin (Fig. 5i). In addition, administration of recombinant TSLP did not induce IL-1β level in e.c. HDM-treated skin (Fig. 5j). These results thus indicate that the infiltration of IL-1β-expressing neutrophils and monocytes / macrophages is an event independent of TSLP signaling.

Moreover, we observed that the increased infiltration of neutrophils and monocytes / macrophages in d.c. HDM compared to e.c. HDM treatment was associated with the higher induction of neutrophil-chemoattractant factors[19, 20] including CXCL2, CXCL3, CXCL5, CCL3, S100A7, S100A8 and S100A9 (but not CXCL1, CCL2 or IL-17C) in the skin (Supplementary Fig. 7a). Notably, their expression in the epidermis exhibited a higher level in d.c. HDM-treated compared to e.c. HDM-treated mice (Supplementary Fig. 7b), suggesting that these chemoattractant factors, which are possibly derived (or at least partially) from the epidermis, could be implicated in mediating the infiltration of IL-1β-expressing neutrophils and monocyte/macrophages.

NIMP-R14 antibody treatment during d.c. HDM sensitization reduces the subsequent asthma

We then wondered whether the depletion of the IL-1β-producing cells during the d.c. HDM-sensitization phase (but not during the i.n. challenge) led to the reduction of the subsequent allergic asthma. To test that, mice were i.p. injected at D-1 and D2 with NIMP-R14 or anti-Ly6G Ab, and d.c. sensitized with HDM at D0 and D3, followed by i.n. challenge with HDM at D10-13 (Fig. 6a). This experimental protocol was designed based on the previous report that Gr-1^{hi} and Gr-1^{int} cells could be efficiently depleted 1 day after the i.p. injection of NIMP-R14 Ab, but started to recover 4 days after[21]. We confirmed that a repeated Ab injection at D2 maintained the cell depletion during the d.c. sensitization phase, while Gr-1^{hi} and Gr-1^{int} cells were recovered before the i.n. HDM challenge (at D9, Supplementary Fig. 8), therefore allowing us to investigate the role of these cells in sensitization phase.

Analyses of BAL at D14 following i.n. HDM challenge showed that total cell number was decreased in both NIMP-R14- and anti-Ly6G-injected mice (Fig. 6b). Cell differential counting showed that eosinophil numbers were decreased in both NIMP-R14- and anti-Ly6G-injected mice compared to PBS-treated ones, whereas lymphocyte cell number was significantly decreased in NIMP-R14- but not in Ly6G-injected mice (Fig. 6b). In contrast, neutrophil number was comparable between PBS-, NIMP-R14- and anti-Ly6G-injected mice (Fig. 6b). RT-qPCR analyses of BAL cells showed a decrease in RNA levels of IL-13, IL-5, IL-4, CCR3 and MCPT8 in both NIMP-R14- and anti-Ly6G-injected mice, although it appeared more prominent with NIMP-R14 than anti-Ly6G (Fig. 6c). In keeping with these data, lung histological analyses showed that NIMP-R14 or anti-Ly6G-injected mice exhibited a reduced peribronchiolar and perivascular infiltration (H&E, Fig. 6d), as well as a reduced hyperplasia of mucus-secreting goblet cells (PAS staining, Fig. 6d). Finally, ELISA analyses showed that HDM-specific IgE was significantly reduced in both NIMP-R14 and anti-Ly6G-treated mice (Fig. 6e).

Together, these results indicate that the depletion of Gr-1^{hi} and Gr-1^{int} cells, or the depletion of Gr-1^{hi} cells alone, during the d.c. HDM sensitization, reduces the subsequent lung inflammation, suggesting that these IL-1β-expressing cells, particularly neutrophils, are crucial for d.c. HDM sensitization-triggered allergic asthma.

Skin IL-1β restores asthmatic inflammation in NIMP-R14-treated mice

We further asked whether the role of Gr-1^{hi} and Gr-1^{int} cells in d.c. HDM sensitization-triggered allergic asthma is mediated through IL-1β. To answer this question, Balb/c WT mice were i.p.

injected with NIMP-R14 Ab at D-1 and D2, and d.c. sensitized with HDM supplemented with IL-1β at D0 and D3, followed by i.n. HDM challenge at D9-12 (Fig. 7a). Analyses at D13 showed that the co-administration of IL-1β largely restored the allergic asthmatic phenotypes in NIMP-R14-injected mice, from the number of total BAL cells including that of eosinophils and lymphocytes (Fig. 7b), the expression of IL-5, IL-13, CCR3, MCPT8 (and to a lesser extent, IL-4) by BAL cells (Fig. 7c), to the perivascular and peribronchiolar cell infiltration and goblet cell hyperplasia (Fig. 7d). In addition, measurement of lung resistance showed that the d.c. HDM-induced AHR was diminished in NIMP-R14-injected mice, which was restored by the co-administration of IL-1β (Fig. 7e). Finally, serum HDM-specific IgE and IgG1 levels also tended to be restored by IL-1β in the NIMP-R14-injected mice (Fig. 7f). Together, these results suggest that IL-1β mediates the role of Gr-1^{hi} and/or Gr-1^{int} cells for d.c. HDM sensitization-triggered allergic asthma.

Moreover, we tested whether a direct blockade of IL-1β signalling, by administrating anti-IL-1β Ab or Anakinra (a recombinant IL-1 receptor antagonist) during the d.c. HDM sensitization, could reduce the subsequent asthmatic phenotype. Although the effects observed were less striking compared to the depletion with NIMP-R14 Ab, the results showed that mice with i.p. injection with anti-IL-1β during d.c. HDM sensitization developed a weaker asthmatic inflammation, including a decreased number of eosinophils in BAL cells, a decrease tendency for RNA levels of IL-13, IL-5, CCR3 and MCPT8 (but not IL-4), a milder and more patchy hyperplasia of goblet cells (Supplementary Fig. 9 a-d). We noted that the H&E staining did not show a striking reduction for inflammatory cell infiltration in the lung (Supplementary Fig. 9d). Injection of Anakinra did not reach a better reduction for lung inflammation either (Supplementary Fig. 9e-f), suggesting that unlike the depletion of IL-1β-expressing neutrophils and monocytes/macrophages, the blockade of IL-1β or IL-1 signaling using the available anti-IL-1β Ab or anakinra only reaches a mild effect in reducing the d.c. HDM sensitization-triggered allergic asthma.

Discussion

In this study, we have modeled in mice the allergen sensitization occurring in the skin with barrier disruption at different anatomic depth, either superficially (named epicutaneous e.c. sensitization) or deeply (named dermacutaneous d.c. sensitization). We report that compared with e.c. HDM sensitization, d.c. HDM sensitization leads to stronger Th2 and Tfh/GC cell responses accompanied by type 2 skin inflammation, and consequently, it triggers a stronger allergic lung inflammation as well as AHR upon the i.n. challenge of HDM. We provide experimental evidence that in these two contexts, epidermal keratinocyte-derived TSLP plays either a major or only a partial role for HDM skin sensitization and the subsequent allergic asthma. We further identify that IL-1β, whose level is induced by a deeper barrier disruption and whose expression is detected in neutrophils and monocytes / macrophages infiltrated to the skin, promotes the d.c. HDM sensitization and the atopic march in a TSLP-independent manner. Together, our study suggests context-dependent roles for TSLP and IL-1β in skin allergic sensitization and atopic march (Fig. 8): for e.c. sensitization occurring more superficially in the skin, which may correspond to the context of milder AD, TSLP is dominantly crucial, while for d.c. sensitization occurring more deeply in the skin, which may correspond to the context of more severe AD, IL-1β is another important player, in addition to TSLP, to generate the allergen sensitization and develop the atopic march.

It has been reported that TSLP overexpressed by epidermal keratinocytes in AD lesions[22] drives AD pathogenesis[23, 24, 25, 26] and the atopic march[8, 27, 28, 29], with its role recognized in promoting Th2 and Tfh cell differentiation in mouse and human[13, 30, 31, 32]. The current study provides novel evidence on the context-dependent contribution of TSLP in allergen sensitization which occurs at different depth of the skin mimicking the heterogeneous situations in AD lesion. Note that first, TSLP is similarly induced by superficial or deep disruption of the barrier skin, and second, its functional contribution to allergic sensitization and atopic march decreases once skin sensitization goes deeper. This finding may thus provide insight for the recent clinical trial results in AD obtained from TSLP neutralizing antibody Tezepelumab, showing that moderate to severe AD adults treated with Tezepelumab presented only a numeric but not significant improvement compared to placebo-treated group[33]. Very recently, a phase 2b clinical trial (NCT03809663) was terminated / withdrawn because Tezepelumab as a monotherapy in moderate to severe AD patients did not reach the efficacy required. As these clinical trials with limited/negative results were performed in moderate to severe AD patients, the lack of efficacy of Tezepelumab could be at least partially explained by the context-dependent implication of TSLP in allergic skin inflammation and

sensitization in these patients.

We show in this study that IL-1β level is elevated following a deeper disruption of the skin, but unlike TSLP produced by epidermal keratinocytes, infiltrated Gr-1^{hi} (Ly-$6G^{+}$) neutrophils and Gr-1^{int} (Ly-$6C^{+}$) monocytes/macrophages are the major cellular sources for skin IL-1β. Actually, elevated IL-1β level has been reported not only in the skin of AD-like mouse models with genetic mutations of *Flg*[34], *Cdsn*[35], or *Spink5*[36], but also in human AD lesional skin[37, 38]. Moreover, serum IL-1β levels were shown to be significantly increased in AD patients[39], to correlate with the severity of AD[40], and following improvement to decrease in a significant manner[40]. Our study provides experimental evidence for the role of IL-1β in skin sensitization and the atopic march: first, the supplement of IL-1β exacerbates e.c. HDM-sensitization (where endogenous IL-1β level is low) and the subsequent asthma, thus somehow mimicking the effects of d.c. HDM sensitization; second, the depletion of IL-1β-expressing Gr-1^{hi} or Gr-1^{hi+int} cells reduces the d.c. HDM-triggered asthma, which is restored by the skin administration of IL-1β. One limitation of our study is that the blockade with anti-IL-1β or Anakinra (an IL-1 receptor antagonist, IL-1RA) appears to reach only limited reduction in asthmatic phenotypes. We suspect that this could be due to the inefficiency of the blockade. For example, Anakinra blockade efficiency in mice seems to be influenced by genetic background[41], and its treatment has led to both positive and negative results in different human diseases suggesting complex role of IL-1 receptor antagonist and its usage difficulties from tissues to tissues. The ongoing efforts for developing more potent treatments than Anakinra[42] may hopefully provide better reagents for targeting IL-1 signaling.

Importantly, we conclude from our study that IL-1β and TSLP are two independent factors promoting allergic sensitization and atopic march. In one hand, we show that first, the depletion of IL-1β-expressing neutrophils and monocytes/macrophages does not impact TSLP production; second, the supplement of IL-1β does not induce TSLP expression, which is in contrast to a previous report showing that in vitro IL-1β induces TSLP in reconstructed human epidermis culture[43]; and third, the supplement of IL-1β enhances the e.c. HDM sensitization in *Tslp*$^{-/-}$ mice similarly as in WT mice, suggesting that the effect of IL-1β in promoting skin allergic sensitization is TSLP-independent. On the other hand, we show that the supplement of TSLP does not increase IL-1β level in the skin, and that TSLP is not required for d.c. HDM-induced IL-1β or infiltration of neutrophils and monocytes/macrophages, in keeping with a recent paper reporting TSLP-TSLPR signaling is not implicated in skin recruitment of neutrophils [44]. Taken together, these data emerge IL-1β as a potential target which is independent of TSLP for allergic sensitization in AD patients,

particularly those moderate to severe cases.

As a plurifunctional cytokine, IL-1β is known to be implicated in innate and adaptive immunity. Here we report an adjuvant function of IL-1β in promoting allergen-triggered skin sensitization and asthma, rather than an immune regulation function on its own. Also, IL-1β appears to act rather locally than systemically, as an increase in IL-1β protein was detected in the skin, but not in a distant organ like lung or in circulation at any time points examined upon d.c. HDM treatment (Supplementary Fig. 10). In particular, we show that skin IL-1β promotes allergen-induced Th2 and Tfh cell differentiation in the skin-draining LNs, both of which are critically implicated in T- and B-cell mediated memory to allergen during the atopic march process. In agreement with our data, the role of IL-1β on Th2 response has been also reported in several studies of allergy mouse models, where lung inflammation and / or Th2 cytokines are decreased in *Il1b*$^{-/-}$ mice sensitized by i.p. OVA/alum and i.n. challenged by OVA[45], or exacerbated in mice co-treated with IL-1β with i.n. OVA[46, 47]. Besides, it has been also shown that IL-1β promotes the differentiation of Tfh cells, which express IL-4 and promote GC B cells and antigen-specific IgE and IgG1, upon i.p. immunization with OVA/Alum[48] or i.n. OVA or peanut exposure[47, 49]. Despite all these studies, cellular and molecular mechanisms underlying the role of IL-1β for Th2/Tfh cell differentiation remain still to be investigated. It is possible that IL-1β activates dendritic cells (DCs) as previously shown in mouse and human[50, 51], or directly acts on T cells[52]. For example, Ritvo et al. showed that Tfh cells expressed IL-1β receptor IL-1R1, and in vitro stimulation of Tfh cells with IL-1β induces their production of IL-4[48]. Recently, IL-1β was also reported to act on CD4^{+} T cells to induce Bcl6, CXCR5 and ICOS expression thus promoting Tfh cells in response to live vaccines[53].

In addition to its prominent effect on enhancing eosinophils and basophils in lung inflammation developed following the i.n. HDM challenge, we note that skin IL-1β has also an impact, although less striking, in the elevation of neutrophils in BAL. First, compared to e.c. HDM sensitization, d.c. HDM sensitization triggers a small but significant increase in neutrophils in BAL (Fig. 2b). Second, we show that co-delivery of IL-1β during e.c. HDM sensitization leads to a higher neutrophil number in BAL (Fig. 4b). Third, the administration of IL-1β appears to restore the BAL neutrophil number in d.c. HDM sensitized WT mice depleted of Gr-1 cells (Fig. 7b). In contrast to IL-1β, TSLP does not seem to have any role in BAL neutrophils, as their number remains unchanged between d.c. HDM-sensitized WT and *Tslp*$^{-/-}$ mice (Fig. 2b). Given the recognized role of airway neutrophils in persistent and severe asthma associated with corticosteroid-resistance[54], it will be interesting to further investigate in mice whether allergic asthma developed following d.c. sensitization is

more resistant to corticosteroid, and to explore in patients the possible link of skin IL-1β in AD lesions with the development of corticosteroid-resistant asthma with neutrophilic inflammation.

Our data point to a role for neutrophils for the development of skin allergic sensitization and the atopic march. These cells are early recruited in the skin, and their numbers are positively correlated with the depth of barrier disruption and the expression of chemoattractant factors in the skin. Notably, neutrophils are recognized to for their importance in healing tissue injury[55] and in host defence against microbial pathogens including *S. aureus*[56]. As AD skin is commonly colonized with *S. aureus*, one may expect that neutrophils and their derived IL-1β act as double-sided sword, on the one hand contributing to *S. aureus* clearance, and on the other hand, as shown in this study, contributing to the promotion and exacerbation of skin allergic sensitization, which provides one plausible explanation for the association of *S. aureus* in AD with the development of atopic march[57]. In keeping with our data, an early recruitment of neutrophils was shown to promote contact allergic sensitization of hapten[21]. The role of neutrophils in airway allergic sensitization was also reported, showing that recruitment of neutrophils to the lung promotes ragweed pollen extract allergic airway inflammation [58]. Interestingly, neutrophils were found to mediate the recruitment of DCs to the site of L. major infection[59], for DCs migration to the draining LNs in contact dermatitis [21], and for skewing T cells towards a Th2 phenotype in a model of L. major infection [60]. Whether this is the case in our model remains to be determined. Finally, even though our study has suggested that IL-1β is one key factor mediating the promoting effect of neutrophils in allergic sensitization and the subsequent allergic asthma, it is possible that other factors derived from neutrophils including neutrophil extracellular traps[61] may also have a contribution.

To conclude, our study points to the importance of different microenvironmental factors that are induced by the barrier disruption at different anatomic depth in regulating the allergen sensitization through skin. It reveals that TSLP and IL-1β, produced by different cellular compartments, represent two important players in promoting skin allergen sensitization and atopic march in a context-dependent manner. Our data highlight the importance towards precision medicine in order to achieve better therapeutic/preventative efficiency in individual AD patients. In addition, it points to the necessity to consider and to further test the combined treatment, for example the blockade of TSLP (Tezepelumab) and IL-1β signaling (Canakinumab, Anakinra or new developed reagents) in moderate to severe AD, which could be beneficial not only for AD inflammation, but also for allergic sensitization, thus preventing or reducing the risk of the atopic march. Certainly, given their multiple functions for example IL-1β in host defence

against microbial pathogens, careful design of targeting strategies including administration routes and evaluation of benefits will be crucially required.

Methods

Mice

Breeding and maintenance of mice were performed under institutional guidelines, and all experimental protocols were approved by the animal care and ethics committee of animal experimentation of the IGBMC n°017 and by the Ministère de l'enseignement supérieur, de la recherche et de l'innovation. Balb/c and *Rag1*$^{-/-}$ mice (strain #. 002216) were purchased from the Jackson Laboratory. *Tslp*$^{-/-}$ mice were previously described[23]. 4C13R dual reporter mice[15] were kindly provided by Dr. W.E. Paul (NIH, USA). All the mouse lines used in the study were backcrossed to Balb/c background (> 10 generations). 4C13R^{Tg} reporter mice were bred with *Tslp*$^{-/-}$ mice to generate *Tslp*$^{-/-}$ 4C13R^{Tg} mice (in Balb/c background). Ten to fifteen weeks old female mice were used in all experiments. Mice were housed at a temperature of 22°C, humidity of 40-60% in a 12h light/12h dark cycle, with unlimited access to food and water.

HDM cutaneous sensitization and airway challenge

P.L.E.A.S.E portable (Pantec Biosolutions) laser-assisted skin microporation (LMP) was performed on the dorsal side of mouse ears. Two sets of parameters were optimized. For the depth of 30µm (LMP_30µm): 2 pulses per pore, with fluence of 7.5 J/cm^2, pulse length of 75 µs, RepRate of 500 Hz and power of 1.0 W; for the depth of 91µm (LMP_91µm): 2 pulses per pore with fluence of 22.7 J/cm^2, pulse length of 175 µs, RepRate of 200 Hz and power of 1.2 W. In both cases, pore array size was 14 mm and pore density was 15%.

To induce HDM cutaneous sensitization, 10 µl solution containing sterile PBS containing 2µg of HDM (Greer, Item: XPB82D3A2.5, Lot No.151776) and /or 1µg of recombinant mouse IL-1β (Biolegend, Cat No. 575106), or 1µg of recombinant mouse TSLP (R&D System, 555-TS-010/CF) were applied on ears immediately following LMP of 30µm (for e.c. sensitization) or of 91µm (for d.c. sensitization) at the time points indicated in the experimental schemes in the Figures. Non-sensitized or PBS-treated mice were used as controls. All the mice were then challenged intranasally (i.n.) with HDM (2 µg) for 4 consecutive days.

Antibody administration

To deplete Gr-1^{hi} and / or Gr-1^{int} cells, wildtype Balb/c mice were intraperitoneally (i.p.) injected with 100 µg of NIMP-R14 [clone NIMP-R14, depleting Gr-1^{hi} (Ly-$6G^{+}$) and Gr-1^{int} (Ly-$6C^{+}$/Ly-$6G^{-}$) cells] or 200 µg of anti-Ly6G antibody [clone 1A8, BioXCell, depleting Gr-1^{hi} (Ly-$6G^{+}$) cells] at the time points shown in Figures. As control, mice were injected with PBS.

Bronchoalveolar lavage cell analyses

BAL was taken in anaesthetized mice, by instilling and withdrawing 0.5 ml of saline solution (0.9% NaCl, 2.6mM EDTA) in the trachea. After six times lavages, BAL fluid was pooled and centrifuged, and cell number was counted using a Neubauer hemocytometer. 200µl of BAL fluids with $2.5x10^5$ cells/ml were used to prepare cytospin slides, which were then stained with Hemacolor kit (Merck, Cat No. 1116740001) to identify macrophages, lymphocytes, neutrophils and eosinophils. After counting for each cell type to obtain their frequencies, number of each cell type was calculated according to total BAL cell numbers.

Airway Responsiveness to Methacholine

Airway responsiveness to aerosolized methacholine (MCh; A2251, Sigma-Aldrich) were assessed using the forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada)[62]. Mice were anesthetized with an i.p. injection of xylasine (15 mg/kg), followed 10 min later by an i.p. injection of pentobarbital sodium (54 mg/kg). The trachea was exposed and an 18-gauge metal needle was inserted into the trachea. Airways were connected to a computer-controlled small animal ventilator, and quasi-sinusoidally ventilated with a tidal volume of 10 mL/kg at a frequency of 150 breaths/min and a positive end expiratory pressure of 2 cm H2O to achieve a mean respiratory volume close to that of spontaneous breathing. For baseline measurement, each mouse was challenged for 10 s with an aerosol of PBS generated with an in-line nebulizer and administered directly through the ventilator. Then, aerosolized MCh at 50 mg/mL was administered for 10 s. The effect of MCh was calculated as the peak response, i.e., the mean of the three maximal values integrated for the calculation of lung resistance (R_L, cm H2O.s.mL-1).

Quantitative RT-PCR

Total RNA was extracted from BAL cells using NucleoSpin RNA XS kit (Macherey-Nagel, Cat No. 740902.50) according to manufacturer's instructions. RNA was reverse transcribed by using random oligonucleotide hexamers and amplified by means of quantitative PCR with a LightCycler 480 (Roche Diagnostics, Indianapolis, Ind) and the LightCycler 480 SYBR Green kit (Roche, Cat No. 04707516001), according to the manufacturer's instructions. Relative RNA levels were calculated with hypoxanthine phosphoribosyl- transferase (HPRT) as an internal control. For analyses of each set of gene expression, an arbitrary unit of 1 was given to the samples with the highest level, and the remaining samples were plotted relative to this value. Sequences of PCR primers are: HPRT (TGGATACAGGCCAGACTTTG; GATTCAACTTGCGCTCATCTTA, 161bp) ; IL-4 (GGCATTTTGAACGAGGTCAC; AAATATGCGAAGCACCTTGG, 132 bp) ; IL-5 (AGCACAGTGGTGAAAGAGACCTT; TCCAATGCATAGCTGGTGATTT, 117 bp) ; IL-13 (GGAGCTGAGCAACATCACACA; GGTCCTGTAGATGGCATTGCA, 142 bp), MCPT8 (GTGGGAAATCCCAGTGAGAA; TCCGAATCCAAGGCATAAAG, 160 bp) ; CCR3 (TAAAGGACTTAGCAAAATTCACCA; TGACCCCAGCTCTTTGATTC, 150 bp).

Serum immunoglobulin determination

For HDM-specific immunoglobulins, microtiter plates were coated with HDM and then blocked with BSA. Serum samples were incubated in the coated plates overnight at 4°C followed by incubation with a biotinylated rat anti-mouse IgE (1:250, BD Biosciences; Cat No. 553419; clone R35-118) or IgG1 (1:250, BD Biosciences; Cat No. 553441; clone A85-1). Extravidin horseradish peroxidase (1:1000, Sigma, Cat No. E2886) and TMB (tetramethylbenzidine) Substrate Reagent Set (BD Biosciences, Cat No. 555214) were used for detection. Serum levels of HDM-specific IgG1 and HDM-specific IgE were calculated relevant to a pre-prepared serum pool from HDM-sensitized and challenged mice and expressed as arbitrary units.

TSLP and IL-1β protein level determination

Mouse skin was chopped and homogenized with a Mixer Mill MM301 (Retsch, Dusseldorf, Germany) in lysis buffer (25 mmol/L Tris pH 7.8, 2 mmol/L EDTA, 1 mmol/L dithiothreitol, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche, Cat No. 11873580001). Protein concentrations of skin extract were quantified by using the Bio-Rad Protein

Assay (Bio-Rad Laboratories, Hercules, Calif, Cat No. 500-0006). TSLP and IL-1β levels in skin extracts were determined using the DuoSet ELISA Development Kits (R&D Systems, Minneapolis, Minn, Cat No. DY555 for TSLP, Cat No. DY401 for IL-1β).

Cell preparation for flow cytometry analyses

For preparation of dermal cells, ears were split into ventral and dorsal halves and incubated 1h at 37°C with 4 mg/ml Dispase (Gibco). Dermis was separated from epidermis and incubated 1h at 37°C with 1mg/ml collagenase D (Roche), 0.25 mg/ml DNase I (Sigma) and 2.5% of foetal calf serum in PBS. Cells were passed through a 70 µm strainer (Falcon) and resuspended in FACS buffer (1% of FCS + 2 mM EDTA in PBS) and used for FACS staining.

For cell preparation of whole skin, ears were cut and incubated 1h30 at 37°C with 0.25 mg/ml Liberase TL (Roche), 0.5 mg/ml DNase I in RPMI basic medium. Cells were passed through a 70µm strainer, resuspended in FACS buffer and used for staining.

For cell preparation of EDLNs, EDLNs were dissociated with piston, passed through a 70 µm strainer and resuspended in FACS buffer, counted and used for FACS staining.

Flow cytometry analyses

For surface staining, $2x10^6$ skin cells or LN cells were first incubated with anti-CD16/CD32 antibody (0.5:25, clone 93, eBioscience) to block unspecific binding, followed by surface staining with the following fluorochrome-conjugated antibodies in FACS buffer : CD45 APC-eFluor780 (0.06:25, clone 30-F11), CD45R/B220 APC (1.2:25, clone RA3-6B2), GL7 PE (1.25:25, clone GL-7), Gr-1 PE (Ly-6G/Ly-6C) (0.02:25, clone RB6-8C5), CD8a PerCP-Cy5.5 (0.5:25, clone 53-6.7), TCRβ PerCP-Cy5.5 (1:25, clone H57-597), CD3 FITC (1:25, clone 145-2C11), Ly-6C PE-Cy7 (0.3:25, clone HK1.4), Ly-6G APC (1:25, clone 1A8-Ly6g), CD49b biotin (0.5:25, clone DX5) and streptavidin APC (0.5:25) were from eBioscience; Gr-1 FITC (0.05:25, Ly-6G/Ly-6C) (clone RB6-8C5), Siglec-F PE (0.5:25, clone E50-2440), CD95 PE-Cy7 (1:25, clone Jo2), CD19 FITC (1:25, clone 1D3), CXCR5 biotin (1.5:25, clone 2G8), IgE biotin (0.5:25, clone R35-72) and streptavidin BV605 (0.5:25) were from BD Biosciences; TCRβ PE-Cy7 (0.5:25, clone H57-597), CD4 BV421 (0.5:25, clone GK1.5), PD-1 PE-Cy7 (2:25, clone RMP1-30), CD45R/B220 PE-Cy7 (1.2:25, clone RA3-6B2), IgG1 PerCP-Cy5.5 (1:25, clone RMG1-1) were from Biolegend.

For IL-1β intracellular staining, cells were first stained for surface markers and then stained for IL-1β using Fixation/Permeabilization Kit (BD Biosciences, Cat No. 554715). Briefly, cells were fixed and permeabilized with Fixation/Permeabilization solution for 20 minutes. After wash and centrifugation, cells were resuspended in Perm/Wash buffer containing anti-IL-1β PE antibody (2:100, clone 166931, R&D Systems) for 30 minutes. Cells were washed and resuspended in FACS buffer for analyses.

To eliminate dead cells, propidium iodide was used for surface staining, and Fixable Viability Dye eFluor 506 (0.1:100, eBioscience Cat. 65-0866-18) was used for intracellular staining. Samples were passed on LSRFortessa X-20 (BD) and data were collected with BD FACS DIVA v8 and analysed with FlowJo.

Histopathology

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

Immunohistochemistry

For immunohistochemistry (IHC) staining of major basic protein (MBP) and mast cell protease 8 (MCPT8), 5µm paraffin sections were treated with 0.6% H_2O_2 to block endogenous peroxidase activity before antigen retrieval with either Pepsin (Life technologies; for IHC of MBP) or citric buffer (10 mmol/L citric acid, pH 6; for IHC of MCPT8). Slides were then blocked with normal rabbit serum (Vector Laboratories) and incubated overnight with rat anti-mouse MBP (1:2000, provided by Dr James J Lee, Mayo Clinic, Rochester) and rat anti-mouse MCPT8 (1:500, clone TUG8, Biolegend). Slides were then incubated with biotinylated rabbit anti-rat IgG (1:300) and treated with AB complex (Vector Laboratories, Cat No. PK-6104). Staining was finally visualized with AEC high-sensitivity substrate chromogen solution (Dako) and counter-stained with hematoxylin. Slides were scanned with Nanozoomer 2.0 HT (Hamamatsu) using the program NDP.scan, and images were viewed with NDP.view 2.

RNAscope *in situ* hybridization

To localize TSLP and IL-1β RNA in the skin, *in situ* hybridization was performed on freshly prepared 5µm paraffin sections with the RNAscope 2.5 FFPE Red detection Kit (Advanced Cell Diagnostics, Hayward, CA, USA, Cat No. 322360), according to the manufacturer's protocol. Mm-Ppib probe (Mus musculus peptidylprolyl isomerase B; Cat No. 313917) was used as a positive control, and DapB probe (Bacterial Bacillus subtilis dihydrodipicolinate reductase; Cat No. 310043) was used as negative control. Probe-Mm-TSLP (Cat No. 432741) and Probe- Mm-Il1b (Cat No. 316891) were used for detection of TSLP and IL-1β, respectively.

Statistical analyses

Data were analysed using GraphPad Prism 9. Comparison of two groups was performed either by Student’s two-tailed unpaired t-test with Welch's correction or the two-tailed Mann–Whitney rank sum nonparametric test depending on results from the Kolmogorov–Smirnov test for normality. Comparison of more than two samples was performed by ordinary one-way ANOVA followed by Tukey's post-hoc test. Data show values from individual mice and are presented with mean ± SEM (for Student’s t-test or one-way ANOVA), or with median (for Mann-Whitney rank sum nonparametric test). The p values are marked in the Figures. $p>0.05$ is considered as non significant.

Data availability

Source data are provided with this paper.

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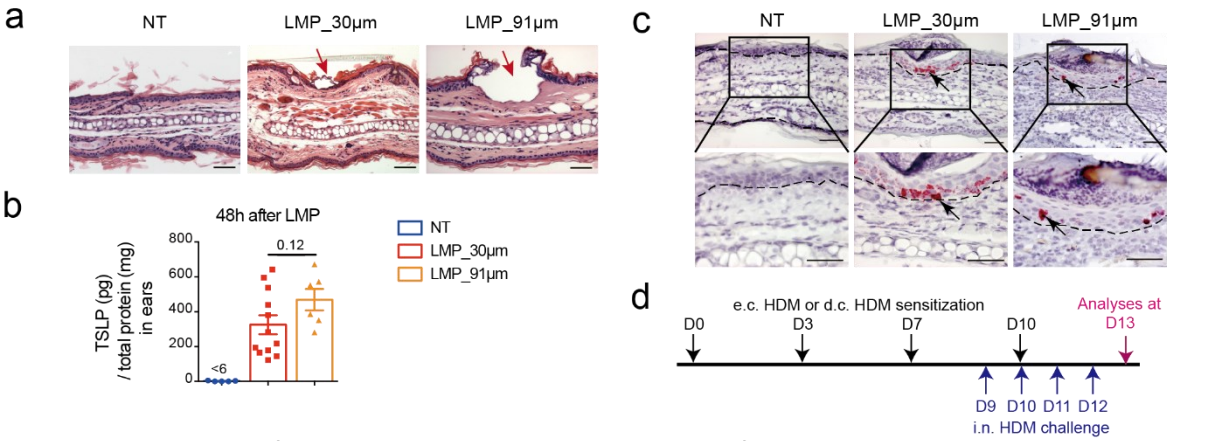
Author contributions

JS, WY and ML conceived and designed the study. JS and WY conducted most experiments and acquired data. P Marschall contributed to the establishment of e.c and d.c sensitization models and the set up for flow cytometry analyses; FD and CL performed airway function study; BG contributed to IL-1 detection by RNAscope and ELISA analyses; BG, P Meyer, PH, CH and EF contributed to the analyses for BAL cells and immune cells by flow cytometry; MG and LC contributed to the experiments during the revision; SM contributed NIMP-R14 clone; MO-A prepared NIMP-R14 Ab for in vivo administration. JS, WY and ML analysed and interpreted data. JS, WY and ML wrote and revised the manuscript. ML directed the study and supervised the work.

Competing interests

The authors declare no competing interests.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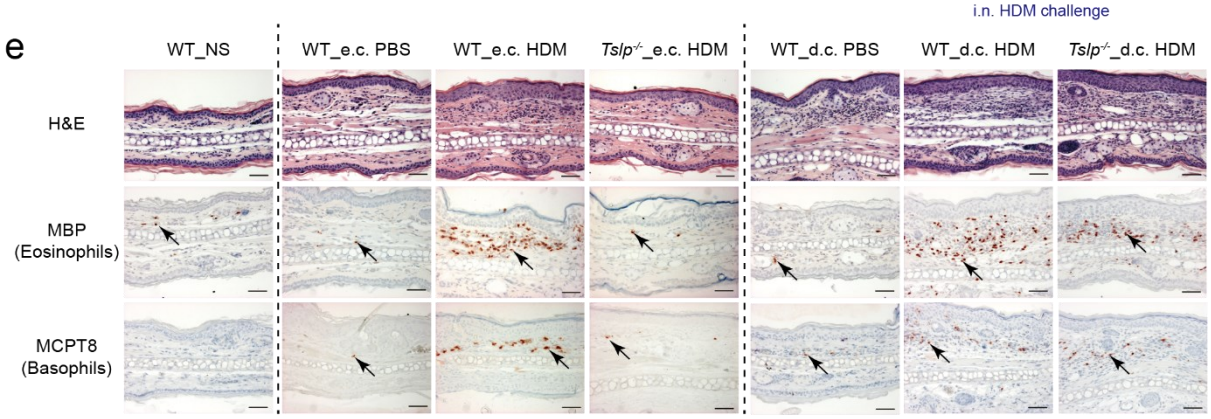
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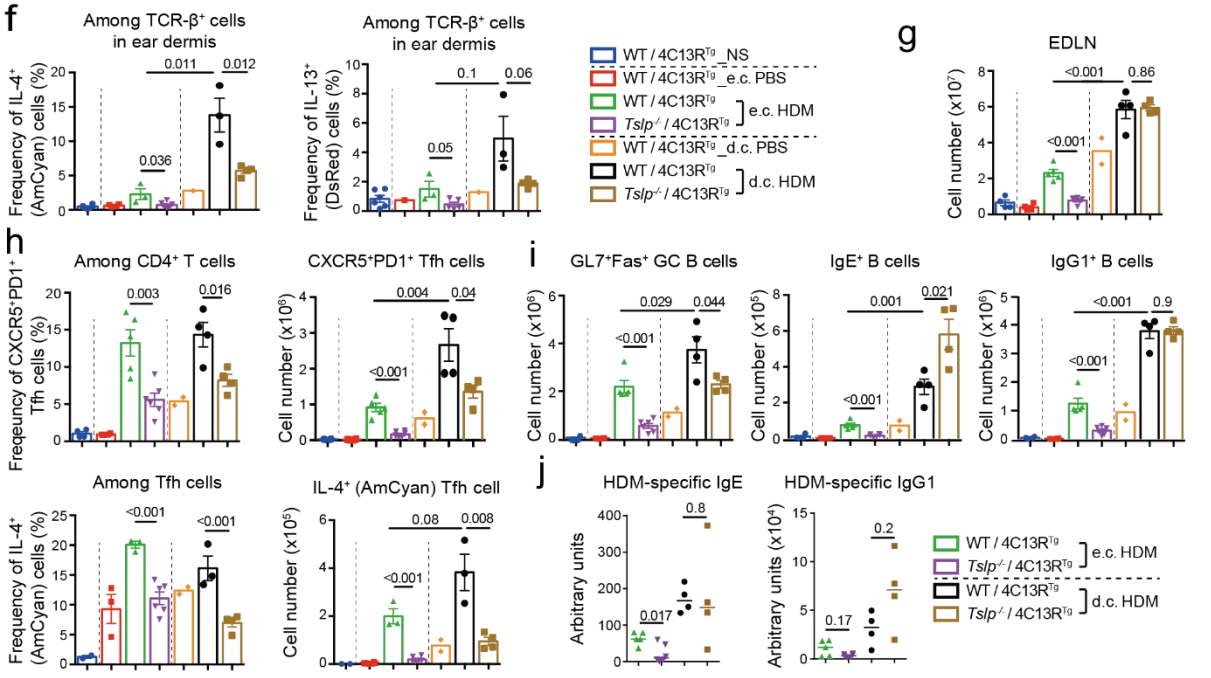

f
Among TCR-β+ cells in ear dermis
Frequency of IL-4+ (AmCyan) cells (%)
Among TCR-β+ cells in ear dermis
Frequency of IL-13+ (DsRed) cells (%)
WT / 4C13RTg_NS
WT / 4C13RTg_e.c. PBS
WT / 4C13RTg
Tslp-/- / 4C13RTg
e.c. HDM
WT / 4C13RTg_d.c. PBS
WT / 4C13RTg
Tslp-/- / 4C13RTg
d.c. HDM
g
EDLN
Cell number (x10⁷)
h
Among CD4+ T cells
Freqeuncy of CXCR5+PD1+ Tfh cells (%)
CXCR5+PD1+ Tfh cells
Cell number (x10⁵)
Among Tfh cells
Frequency of IL-4+ (AmCyan) cells (%)
IL-4+ (AmCyan) Tfh cell
Cell number (x10⁵)
i
GL7+Fas+ GC B cells
Cell number (x10⁶)
IgE+ B cells
Cell number (x10⁵)
IgG1+ B cells
Cell number (x10⁶)
j
HDM-specific IgE
Arbitrary units
HDM-specific IgG1
Arbitrary units (x10⁴)
WT / 4C13RTg
Tslp-/- / 4C13RTg
e.c. HDM
WT / 4C13RTg
Tslp-/- / 4C13RTg
d.c. HDM

Figure 1. TSLP is differentially implicated for Th2 / Tfh responses induced by epicutaneous (e.c.) or dermacutaneous (d.c.) HDM sensitization.

a Hematoxylin & eosin (H&E) stained sections of ears collected immediately after laser microporation (LMP). Red arrow points to a micropore. NT, non-treated. **b** TSLP protein level in ears (*n*=5, 12, 6 mice). **c** RNAscope in situ hybridization for TSLP mRNA. Black arrows point to one of the positive signals. Dashed lines indicate the dermal/epidermal junction. **d** Experimental protocol. House dust mites (HDM) or PBS was applied on LMP_30µm ears to realize e.c. sensitization, or on LMP_91µm ears to realize d.c. sensitization, at day (D) 0, 3, 7 and 10. Mice were challenged intranasally (i.n.) with HDM every day from D9 to D12, and analyzed at D13. **e** H&E staining, immunohistochemistry (IHC) staining with anti-MBP antibody (specific for eosinophils), or anti-MCPT8 antibody (specific for basophils) of ear sections. Black arrows point to one of the positive signals. Scale bar = 50µm (**a**, **c**, **e**). NS, non-sensitized. **f** Frequency of IL-4$^+$ (AmCyan) or IL-13$^+$ (DsRed) cells among CD45$^+$ TCR-β$^+$ cells in ears of mice (*n*=6, 4, 3, 5, 1, 3, 4 mice). **g** Total cell number in ear-draining lymph nodes (EDLNs). **h** Frequency and cell number of Tfh cells and IL-4$^+$ Tfh cells in EDLNs. **i** Numbers of GL7$^+$Fas$^+$ GC B cells, IgE$^+$ B cells and IgG1$^+$ B cells in EDLNs. For **g-i**, *n*=4, 4, 5, 6, 2, 4, 4 mice. **j** Serum levels of HDM-specific IgE and IgG1 (*n*=5, 6, 4, 4 mice). Graphs in **b**, **f-i** show mean±SEM, two-sided Student's t-test. Graphs in **j** show median, two-sided Mann Whitney rank sum test. All data are representative of 3 independent experiments with similar results. Source data are provided as a Source Data file.

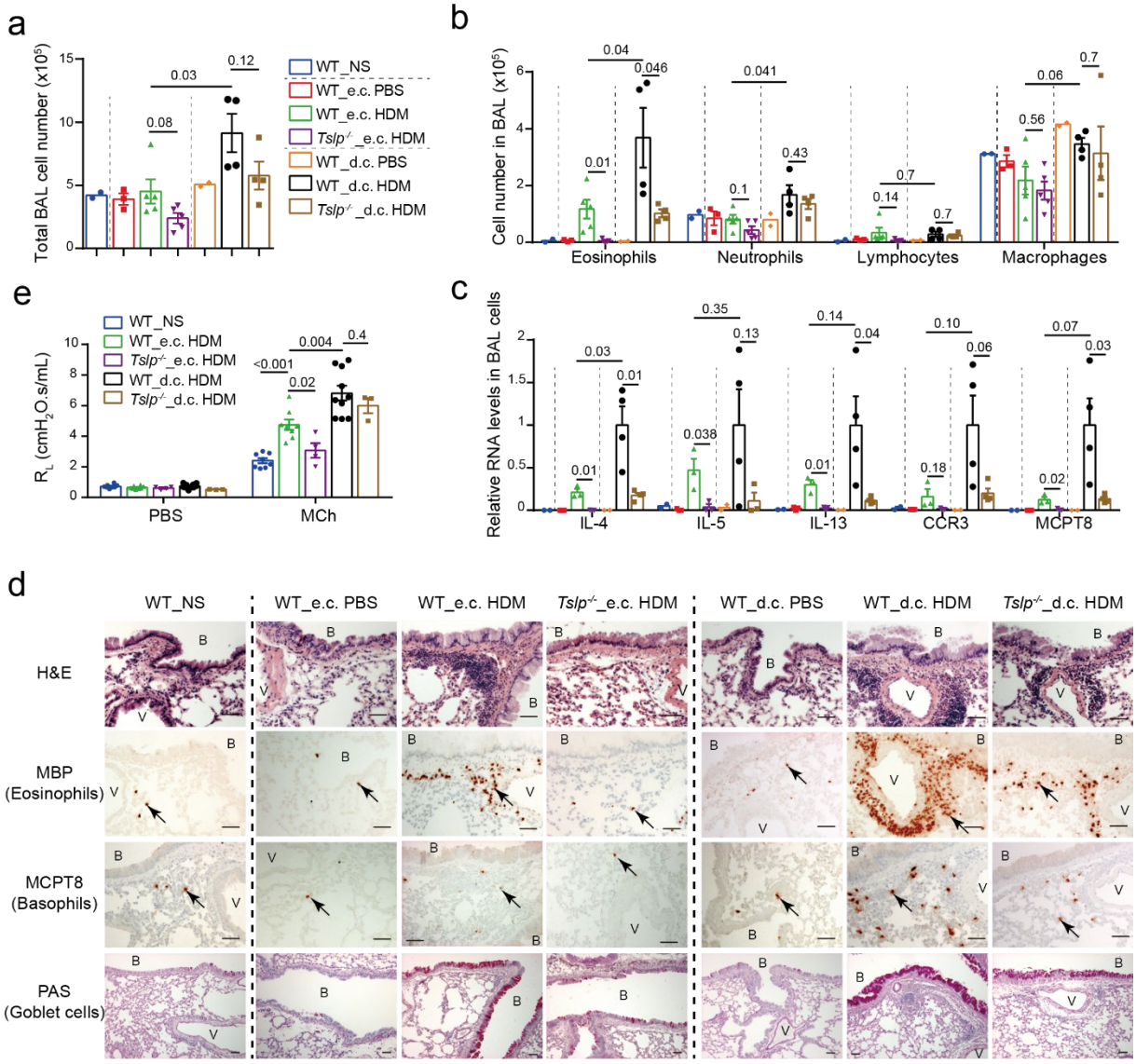
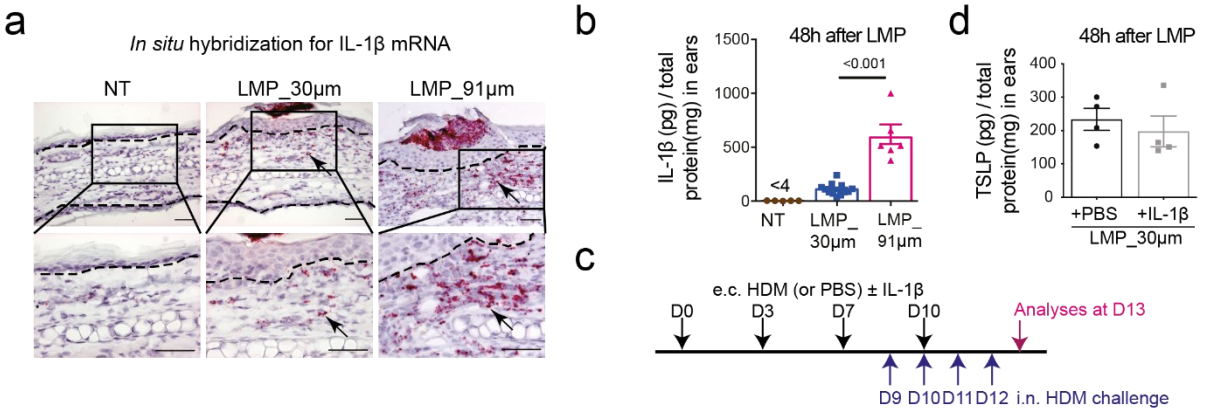
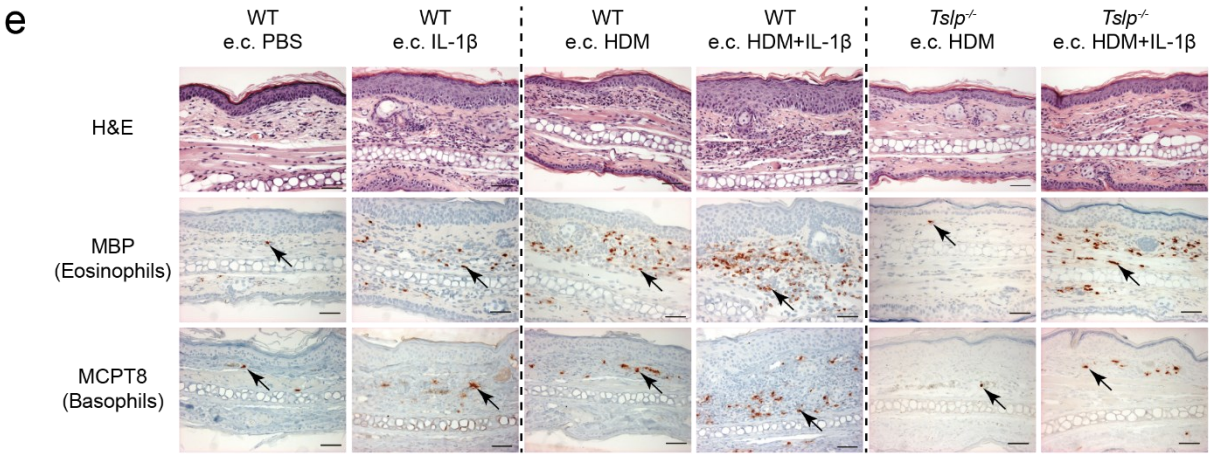
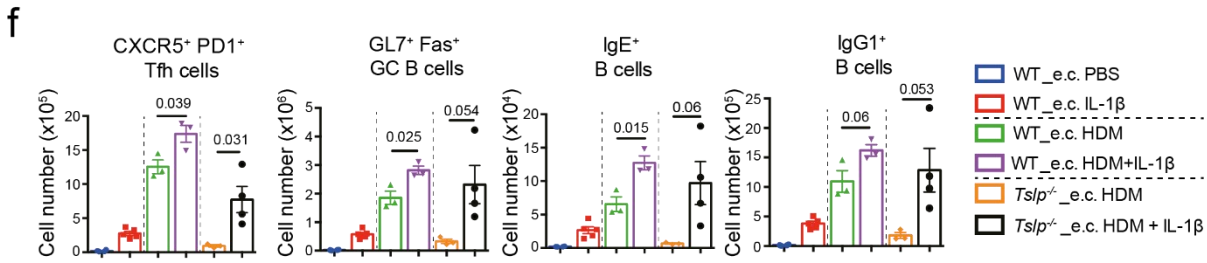

a
Total BAL cell number (x10⁵)
WT_NS
WT_e.c. PBS
WT_e.c. HDM
Tslp⁻/⁻_e.c. HDM
WT_d.c. PBS
WT_d.c. HDM
Tslp⁻/⁻_d.c. HDM
b
Cell number in BAL (x10⁵)
Eosinophils
Neutrophils
Lymphocytes
Macrophages
e
R_L (cmH2O.s/mL)
PBS
MCh
c
Relative RNA levels in BAL cells
IL-4
IL-5
IL-13
CCR3
MCPT8
d
WT_NS
WT_e.c. PBS
WT_e.c. HDM
Tslp⁻/⁻_e.c. HDM
WT_d.c. PBS
WT_d.c. HDM
Tslp⁻/⁻_d.c. HDM
H&E
MBP (Eosinophils)
MCPT8 (Basophils)
PAS (Goblet cells)

Figure 2. TSLP is differentially implicated for e.c. and d.c. HDM sensitization-induced asthmatic inflammation.

a Total cell number in bronchoalveolar (BAL) fluid. NS, non-sensitized. **b** Cell number of eosinophils, neutrophils, lymphocytes and macrophages in BAL fluid. For **a**, **b**, *n*=2, 3, 5, 5, 2, 4, 4 mice. **c** Quantitative RT-PCR analyses of BAL cells (*n*=2, 3, 3, 3, 2, 4, 4 mice). **d** Lung paraffin sections were stained with hematoxylin & eosin (H&E), analyzed by immunohistochemistry (IHC) staining with anti-MBP antibody (for eosinophils) or MCPT8 antibody (for basophils), or by Periodic Acid Schiff (PAS) staining for mucus-producing goblet cells (stained as purple). B: bronchiole. V: blood vessel. Black arrows point to one of the positive cells. Bar = 50μm for all pictures. **e** Lung resistance (R_L) at the baseline (aerosol of PBS) and in response to aerosolized methacholine (Mch; 50mg/ml), measured by FlexiVent system (*n*=8, 8, 4, 10, 3 mice). Graphs in **a-c**, **e** show mean±SEM. Two-sided Student's t-test. Data are representative of 3 (**a-d**) or 2 (**e**) independent experiments with similar results. Source data are provided as a Source Data file.


a
In situ hybridization for IL-1β mRNA
NT
LMP_30µm
LMP_91µm
b
48h after LMP
IL-1β (pg) / total protein(mg) in ears
<0.001
<4
NT
LMP_ 30µm
LMP_ 91µm
d
48h after LMP
TSLP (pg) / total protein(mg) in ears
+PBS
+IL-1β
LMP_30µm
c
e.c. HDM (or PBS) ± IL-1β
D0
D3
D7
D10
Analyses at D13
D9 D10 D11 D12 i.n. HDM challenge


e
WT e.c. PBS
WT e.c. IL-1β
WT e.c. HDM
WT e.c. HDM+IL-1β
Tslp-/- e.c. HDM
Tslp-/- e.c. HDM+IL-1β
H&E
MBP (Eosinophils)
MCPT8 (Basophils)


f
CXCR5+ PD1+ Tfh cells
GL7+ Fas+ GC B cells
IgE+ B cells
IgG1+ B cells
Cell number (x10⁵)
Cell number (x10⁶)
Cell number (x10⁴)
0.039
0.031
0.025
0.054
0.015
0.06
0.06
0.053
WT_e.c. PBS
WT_e.c. IL-1β
WT_e.c. HDM
WT_e.c. HDM+IL-1β
Tslp-/- _e.c. HDM
Tslp-/- _e.c. HDM + IL-1β

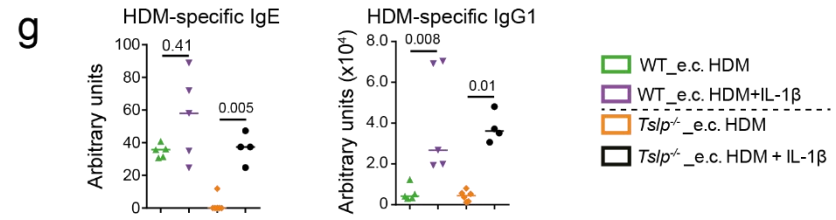

g
HDM-specific IgE
HDM-specific IgG1
Arbitrary units
Arbitrary units (x10⁴)
0.41
0.005
0.008
0.01
WT_e.c. HDM
WT_e.c. HDM+IL-1β
Tslp-/- _e.c. HDM
Tslp-/- _e.c. HDM + IL-1β

Figure 3. Co-administration of IL-1β enhances e.c. HDM-induced Th2/Tfh responses in a TSLP-independent manner.

a RNAscope in situ hybridization for IL-1β mRNA in non-treated (NT), LMP_30µm and LMP_91µm ears at 48h after the microporation. Dashed lines indicate the dermal/epidermal junction. **b** ELISA measurement of IL-1β protein levels in ears at 48 hours after LMP_30µm and 91µm (*n*=5, 12, 6 mice). **c** Experimental protocol. HDM with or without IL-1β was applied on LMP_30µm ears (e.c. HDM ± IL-1β), at day (D) 0, D3, D7 and D10. Mice were intranasally (i.n.) challenged with HDM every day from D9 to D12 to induce allergic asthma, and analyzed at D13. **d** ELISA measurement of TSLP protein levels in 30µm-LMP ears co-administrated with recombinant IL-1β or PBS (*n*=4 mice). **e** Hematoxylin & eosin (H&E) staining and immunohistochemistry (IHC) staining for MBP or MCPT8 on ear sections. Black arrows point to one of the positive signals. Scale bar = 50µm for all pictures. **f** Comparison of $CXCR5^+PD1^+$ Tfh cells, $GL7^+Fas^+$ GC B cells, IgE^+ B and $IgG1^+$ B cells in EDLNs (*n*=4, 5, 3, 3, 3, 4 mice). **g** Serum levels of HDM-specific IgG1 and IgE in HDM-treated mice (*n*=5, 5, 6, 4 mice). Graphs in **b**, **d**, **f** show mean±SEM. Two-sided Student's t-test. Graphs in **g** show median. Two-sided Mann Whitney rank sum test. All data are representative of 2 independent experiments with similar results. Source data are provided as a Source Data file.

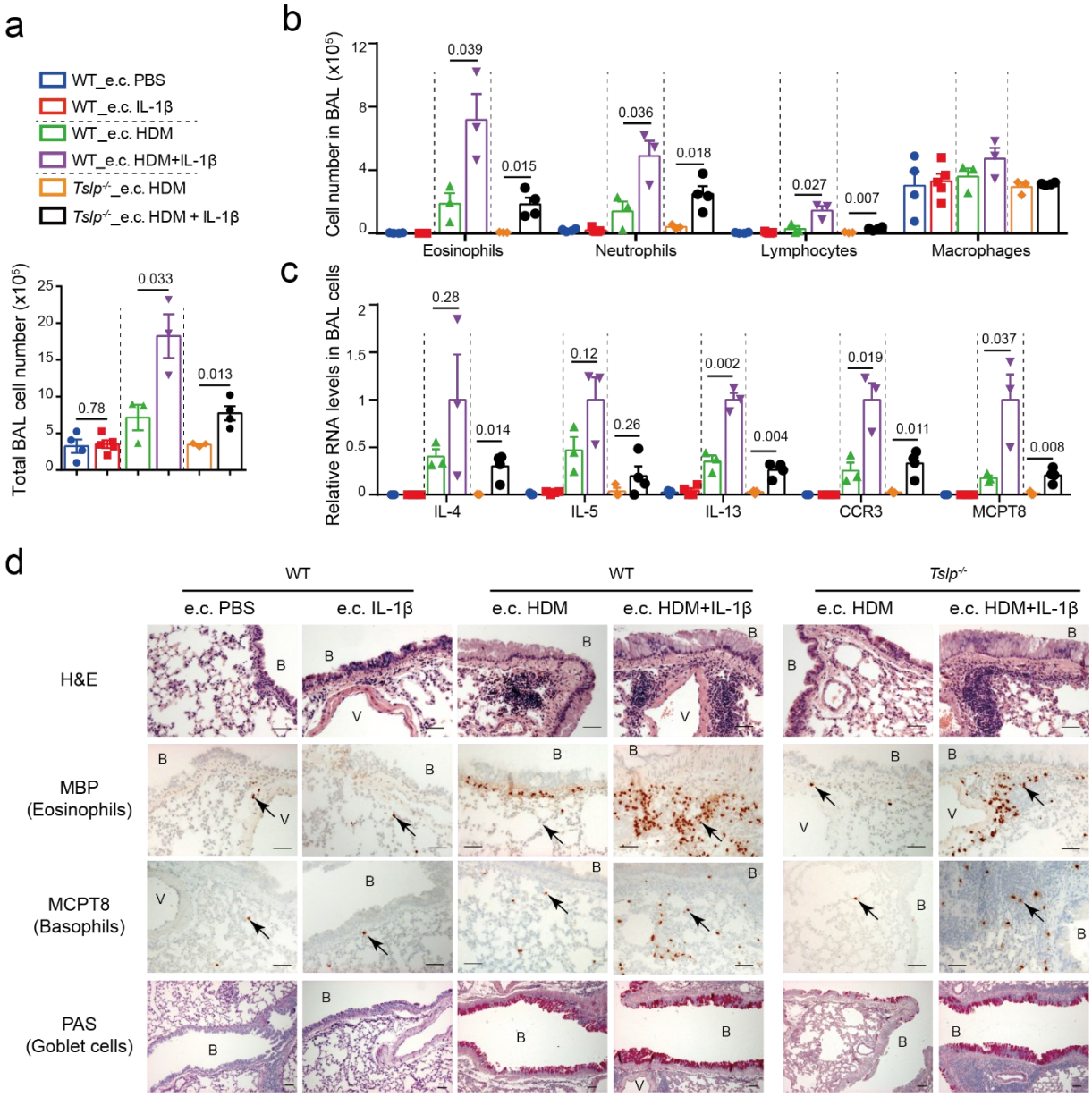

a
WT_e.c. PBS
WT_e.c. IL-1β
WT_e.c. HDM
WT_e.c. HDM+IL-1β
Tslp-/-_e.c. HDM
Tslp-/-_e.c. HDM + IL-1β
Total BAL cell number (x10^5)
0.78
0.033
0.013
b
Cell number in BAL (x10^5)
0.039
0.015
0.036
0.018
0.027
0.007
Eosinophils
Neutrophils
Lymphocytes
Macrophages
c
Relative RNA levels in BAL cells
0.28
0.014
0.12
0.26
0.002
0.004
0.019
0.011
0.037
0.008
IL-4
IL-5
IL-13
CCR3
MCPT8
d
WT
WT
Tslp-/-
e.c. PBS
e.c. IL-1β
e.c. HDM
e.c. HDM+IL-1β
e.c. HDM
e.c. HDM+IL-1β
H&E
MBP
(Eosinophils)
MCPT8
(Basophils)
PAS
(Goblet cells)

Figure 4. Co-administration of IL-1β exacerbates e.c. HDM-induced asthmatic inflammation in a TSLP-independent manner.

a Total cell number in BAL fluid. **b** Cell number of eosinophils, neutrophils, lymphocytes and macrophages in BAL fluid. For **a, b** *n*=4, 5, 3, 3, 3, 4 mice. **c** Quantitative RT-PCR analyses of BAL cells (*n*=3, 4, 3, 3, 3, 4 mice). Graphs in **a**-**c** show mean±SEM. Two-sided Student's t-test. **d** Lung sections were stained with hematoxylin & eosin (H&E), analyzed by immunohistochemistry (IHC) staining for MBP or MCPT8 (stained as dark red, pointed by black arrows), or by Periodic Acid Schiff (PAS) staining (stained as purple). B: bronchiole. V: blood vessel. Scale bar = 50μm for all pictures. All data are representative of 3 independent experiments with similar results. Source data are provided as a Source Data file.

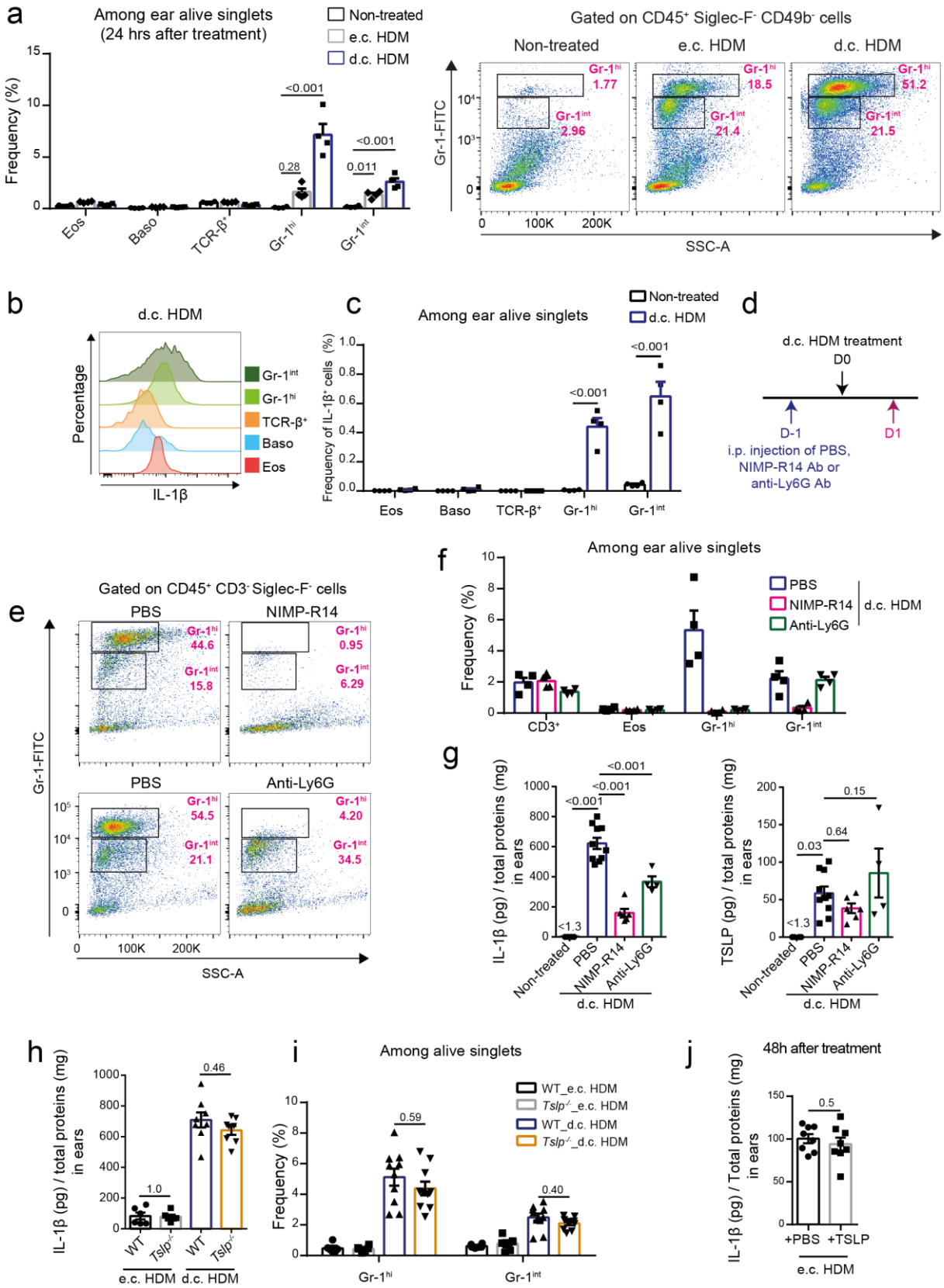

a
Among ear alive singlets
(24 hrs after treatment)
Non-treated
e.c. HDM
d.c. HDM
Frequency (%)
<0.001
0.28
<0.001
0.011
Eos
Baso
TCR-β+
Gr-1hi
Gr-1int
Gated on CD45+ Siglec-F- CD49b- cells
Non-treated
e.c. HDM
d.c. HDM
Gr-1-FITC
SSC-A
Gr-1hi 1.77
Gr-1int 2.96
Gr-1hi 18.5
Gr-1int 21.4
Gr-1hi 51.2
Gr-1int 21.5
b
d.c. HDM
Percentage
IL-1β
Gr-1int
Gr-1hi
TCR-β+
Baso
Eos
c
Among ear alive singlets
Non-treated
d.c. HDM
Frequency of IL-1β+ cells (%)
<0.001
<0.001
d
d.c. HDM treatment
D0
D-1
D1
i.p. injection of PBS,
NIMP-R14 Ab or
anti-Ly6G Ab
e
Gated on CD45+ CD3- Siglec-F- cells
PBS
NIMP-R14
PBS
Anti-Ly6G
Gr-1hi 44.6
Gr-1int 15.8
Gr-1hi 0.95
Gr-1int 6.29
Gr-1hi 54.5
Gr-1int 21.1
Gr-1hi 4.20
Gr-1int 34.5
Gr-1-FITC
SSC-A
f
Among ear alive singlets
Frequency (%)
PBS
NIMP-R14
Anti-Ly6G
d.c. HDM
CD3+
Eos
Gr-1hi
Gr-1int
g
IL-1β (pg) / total proteins (mg) in ears
<0.001
<0.001
<0.001
<1.3
Non-treated
PBS
NIMP-R14
Anti-Ly6G
d.c. HDM
TSLP (pg) / total proteins (mg) in ears
0.15
0.64
0.03
<1.3
Non-treated
PBS
NIMP-R14
Anti-Ly6G
d.c. HDM
h
IL-1β (pg) / total proteins (mg) in ears
0.46
1.0
WT
Tslp-/-
WT
Tslp-/-
e.c. HDM
d.c. HDM
i
Among alive singlets
WT_e.c. HDM
Tslp-/-_e.c. HDM
WT_d.c. HDM
Tslp-/-_d.c. HDM
Frequency (%)
0.59
0.40
Gr-1hi
Gr-1int
j
48h after treatment
IL-1β (pg) / Total proteins (mg) in ears
0.5
+PBS
+TSLP
e.c. HDM

Figure 5. The d.c. HDM treatment induces the infiltration of IL-1β-expressing Gr-1^{hi} and Gr-1^{int} cells in the skin.

(**a-c**) Analyses of IL-1β-expressing cells in the skin. Wildtype Balb/c mouse ears were subjected to d.c. or e.c. HDM treatment and were analysed 24 hrs later. **a.** left, frequencies of eosinophils (Eos), basophils (Baso), TCR-β^{+} T, Gr-1^{hi} and Gr-1^{int} cells among alive singlets. Right, representative FACS plots. *n*=4 mice. **b** Histogram comparison of IL-1β in cells from the d.c. HDM-treated ears. **c** Frequencies of IL-1β^{+} cells among alive singlets (*n*=4 mice). (**d-g**) Depletion of neutrophils and monocytes / macrophages reduces IL-1β in d.c. HDM-treated ears. **d** Experimental protocol. Wildtype Balb/c mice were intraperitoneally (i.p.) injected with PBS, NIMP-R14 (anti-Gr-1) or anti-Ly6G antibody (Ab) at day (D) -1. Ears were d.c. HDM-treated at D0 and analysed at D1. **e** Representative FACS plots showing the depletion of both Gr-1^{hi} and Gr-1^{int} cells by NIMP-R14 Ab, or of Gr-1^{hi} cells by anti-Ly6G Ab. **f** Frequencies of cells among ear alive singlets (*n*=4 mice). **g** IL-1β and TSLP protein levels in ears (*n*=4, 10, 6, 4 mice). (**h-j**) Infiltration of IL-1β-expressing cells is TSLP-independent. Ears of WT or *Tslp*$^{-/-}$ mice were treated with e.c. HDM or d.c. HDM at D0 and analysed at D1 for IL-1β (**h**) and for Gr-1^{hi} and Gr-1^{int} cells (**i**). For **h**, *n*=6, 6, 8, 8 mice. For **i**, *n*=6, 6, 10, 10 mice. **j** Recombinant TSLP or PBS were administrated on e.c. HDM-sensitized ears and IL-1β levels were measured (*n*=8 mice). Graphs in **a**, **c**, **f**-**j** show mean±SEM. **a**, **f**, **g**, **h**, **i**, one-way ANOVA test; **c**, **j**, Two-sided Student's t test. Data are representative of 3 (**a-c, e-i**) or 1 (**j**) independent experiments with similar results. Source data are provided as a Source Data file.

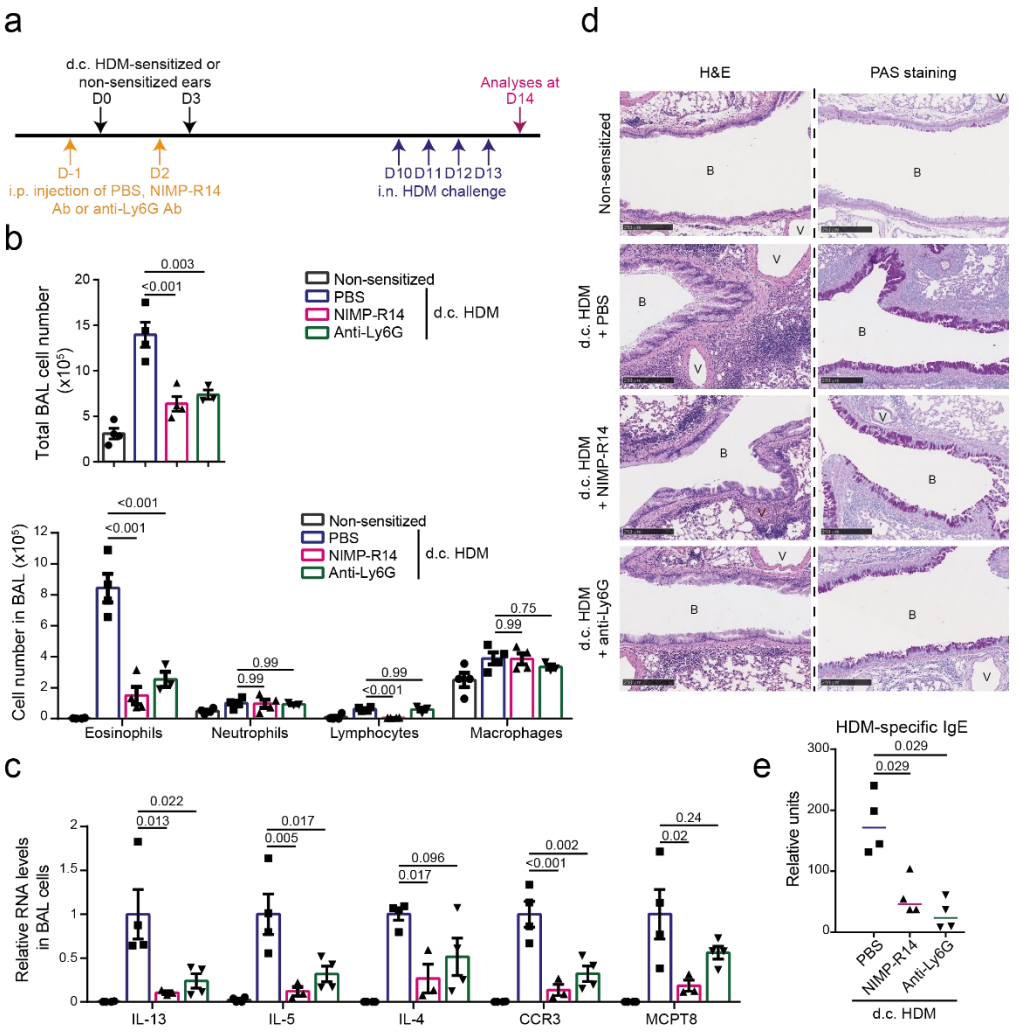

a
d.c. HDM-sensitized or non-sensitized ears
D0
D3
Analyses at D14
D-1
D2
i.p. injection of PBS, NIMP-R14 Ab or anti-Ly6G Ab
D10 D11 D12 D13
i.n. HDM challenge
b
Total BAL cell number (x10⁵)
Non-sensitized
PBS
NIMP-R14
Anti-Ly6G
d.c. HDM
0.003
<0.001
Cell number in BAL (x10⁵)
<0.001
<0.001
0.99
0.99
0.99
<0.001
0.75
0.99
Eosinophils
Neutrophils
Lymphocytes
Macrophages
c
Relative RNA levels in BAL cells
0.022
0.013
0.017
0.005
0.096
0.017
0.002
<0.001
0.24
0.02
IL-13
IL-5
IL-4
CCR3
MCPT8
d
H&E
PAS staining
Non-sensitized
d.c. HDM + PBS
d.c. HDM + NIMP-R14
d.c. HDM + anti-Ly6G
B
V
e
HDM-specific IgE
Relative units
0.029
0.029
PBS
NIMP-R14
Anti-Ly6G
d.c. HDM

Figure 6. Depletion of IL-1β-expressing cells during d.c. HDM sensitization reduces the subsequent asthmatic phenotype.

a Experimental protocol. Wildtype Balb/c mice were intraperitoneally (i.p.) injected with PBS, NIMP-R14 or anti-Ly6G antibody (Ab) at day (D) -1 and D2. Mice were d.c. sensitized with HDM on LMP_91µm ears at D0 and D3 or non-sensitized. All mice were intranasally (i.n.) challenged with HDM from D10 to D13 and analysed at D14. **b** Total cell number and differential cell counting in BAL fluid (*n*=4, 4, 4, 3 mice). **c** Relative mRNA levels of genes in BAL cells (*n*=4, 4, 3, 4 mice). **d** Lung sections were stained with hematoxylin-eosin (H&E) for histological analyses or Periodic Acid Schiff (PAS) for goblet cell hyperplasia analyses. B: bronchiole. V: blood vessel. Bar=250 µm for all pictures. **e** Serum level of HDM-specific IgE measured by ELISA (*n*=3 mice). Graphs in **b**, **c** show mean±SEM, One-way ANOVA test. Graph in **e** marks median, two-sided Mann Whitney rank sum test. All data are representative of 2 independent experiments with similar result. Source data are provided as a Source Data file.

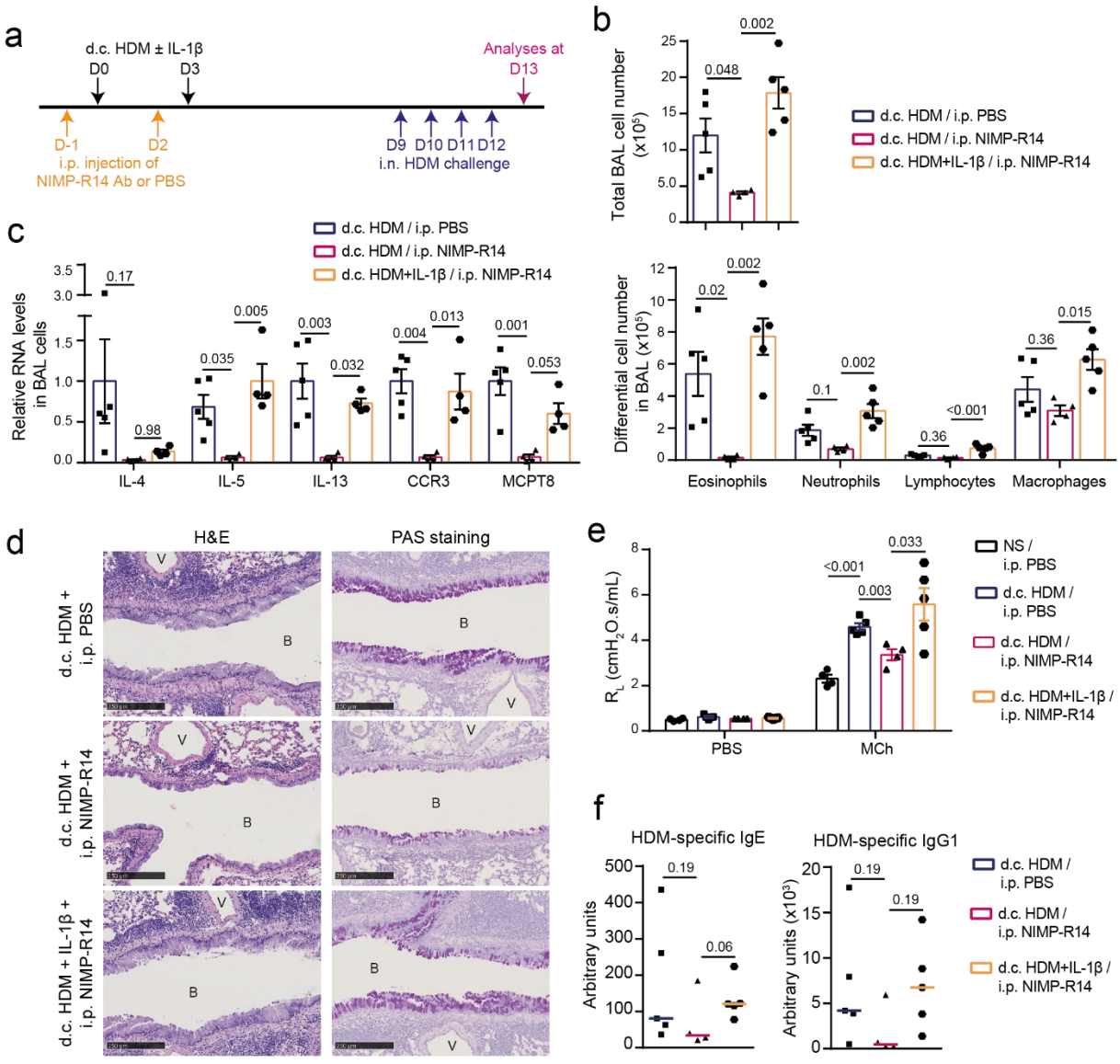

a
d.c. HDM ± IL-1β
D0
D3
Analyses at
D13
D-1
i.p. injection of
NIMP-R14 Ab or PBS
D2
D9 D10 D11 D12
i.n. HDM challenge
b
Total BAL cell number
(x10⁵)
0.048
0.002
d.c. HDM / i.p. PBS
d.c. HDM / i.p. NIMP-R14
d.c. HDM+IL-1β / i.p. NIMP-R14
c
Relative RNA levels
in BAL cells
0.17
0.98
0.035
0.005
0.003
0.032
0.004
0.013
0.001
0.053
IL-4
IL-5
IL-13
CCR3
MCPT8
Differential cell number
in BAL (x10⁵)
0.02
0.002
0.1
0.002
0.36
<0.001
0.36
0.015
Eosinophils
Neutrophils
Lymphocytes
Macrophages
d
H&E
PAS staining
d.c. HDM +
i.p. PBS
d.c. HDM +
i.p. NIMP-R14
d.c. HDM + IL-1β +
i.p. NIMP-R14
V
B
e
R_L (cmH2O.s/mL)
<0.001
0.003
0.033
PBS
MCh
NS /
i.p. PBS
d.c. HDM /
i.p. PBS
d.c. HDM /
i.p. NIMP-R14
d.c. HDM+IL-1β /
i.p. NIMP-R14
f
HDM-specific IgE
HDM-specific IgG1
Arbitrary units
0.19
0.06
Arbitrary units (x10³)
0.19
0.19
d.c. HDM /
i.p. PBS
d.c. HDM /
i.p. NIMP-R14
d.c. HDM+IL-1β /
i.p. NIMP-R14

Figure 7. Administration of IL-1β restores the d.c. HDM-induced asthmatic phenotype in NIMP-R14-treated mice.

a Experimental protocol. Wildtype Balb/c mice were intraperitoneally (i.p.) injected with PBS or NIMP-R14 antibody at day (D) -1 and D2. Mice were d.c. sensitized with HDM ± IL-1β on LMP_91µm ears at D0 and D3. All mice were intranasally (i.n.) challenged with HDM from D9 to D12 and analysed at D13. **b** Total cell number and differential cell counting in BAL fluid (*n*=5, 4, 5 mice). One-way ANOVA test. **c** Relative RNA levels of genes in BAL cells (*n*=5, 4, 4 mice). One-way ANOVA test. **d** Lung sections were stained with hematoxylin-eosin (H&E) or Periodic Acid Schiff (PAS). B: bronchiole. V: blood vessel. Bar=250 µm for all pictures. **e** Lung resistance (R_L) at the baseline (aerosol of PBS) and in response to aerosolized methacholine (Mch; 50mg/ml), measured by FlexiVent system (*n*=4, 5, 4, 5 mice). Two-sided Student's t test. **f** Serum level of HDM-specific IgE and IgG1 measured by ELISA (*n*=5, 4, 5 mice). Two-sided Mann Whitney rank sum test. NS, non-sensitized. Data are representative of 2 (**b-d, f**) or 1 (**e**) independent experiments with similar results. Source data are provided as a Source Data file.

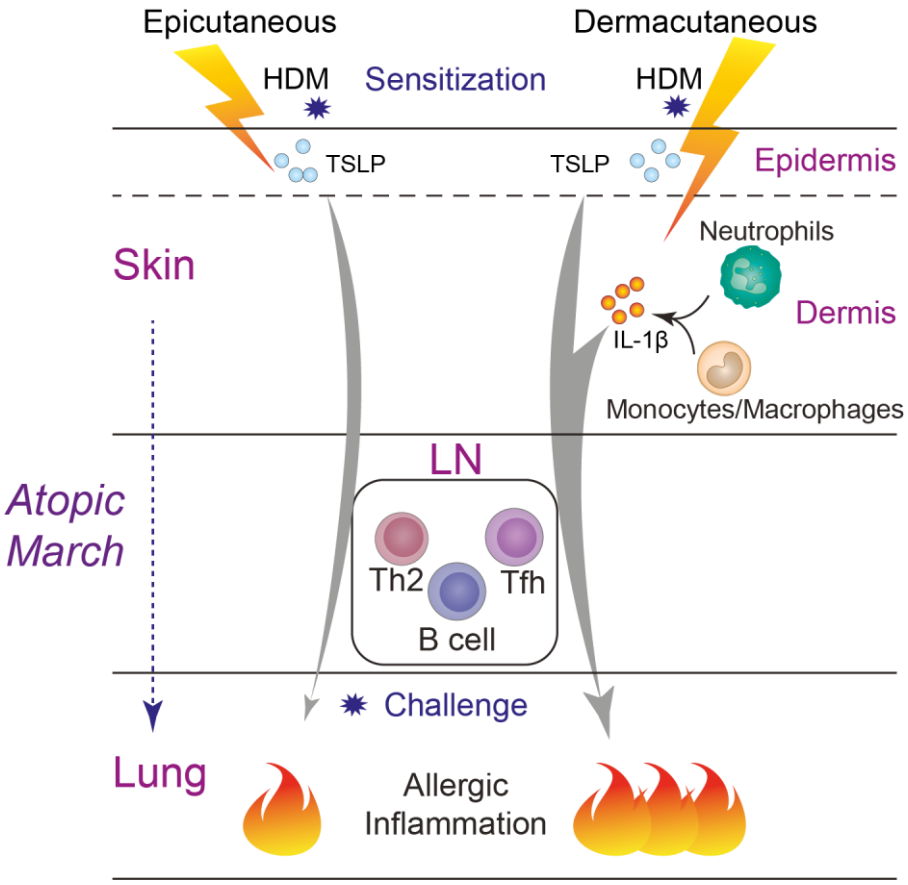
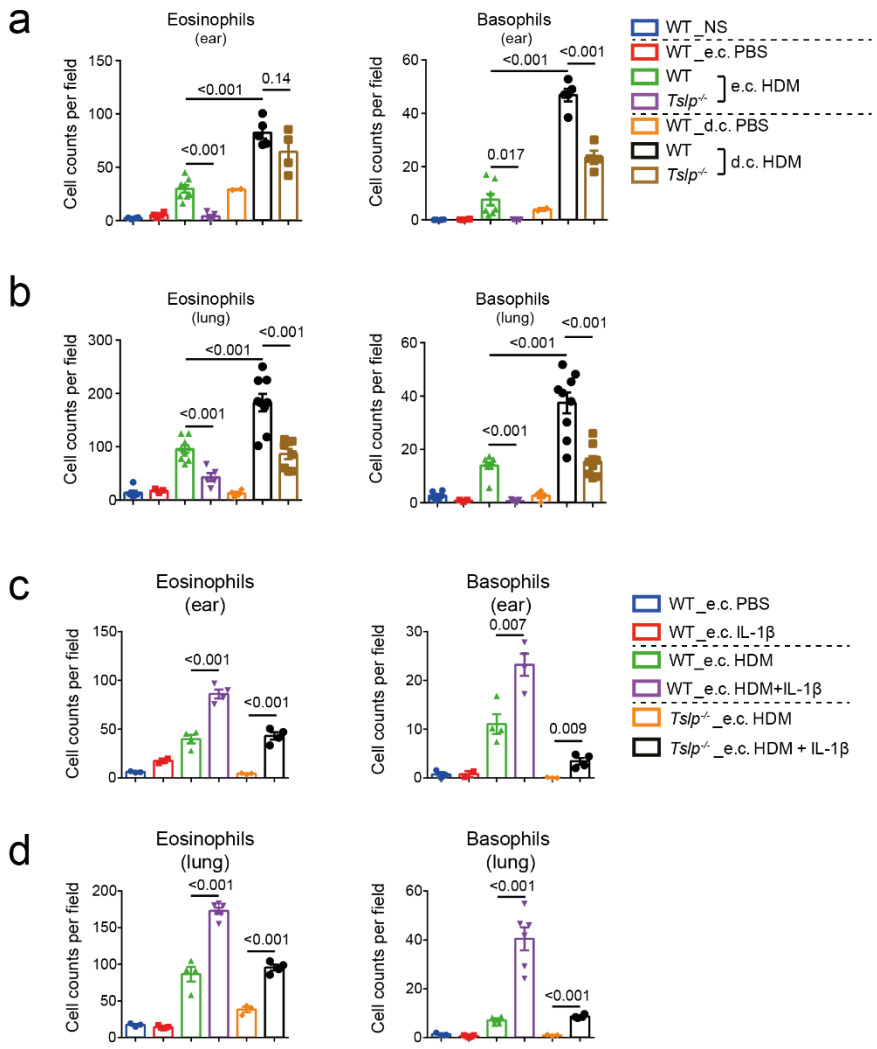

Epicutaneous
Dermacutaneous
HDM
Sensitization
HDM
TSLP
TSLP
Epidermis
Skin
Neutrophils
Dermis
IL-1β
Monocytes/Macrophages
LN
Atopic
March
Th2
Tfh
B cell
Challenge
Lung
Allergic
Inflammation

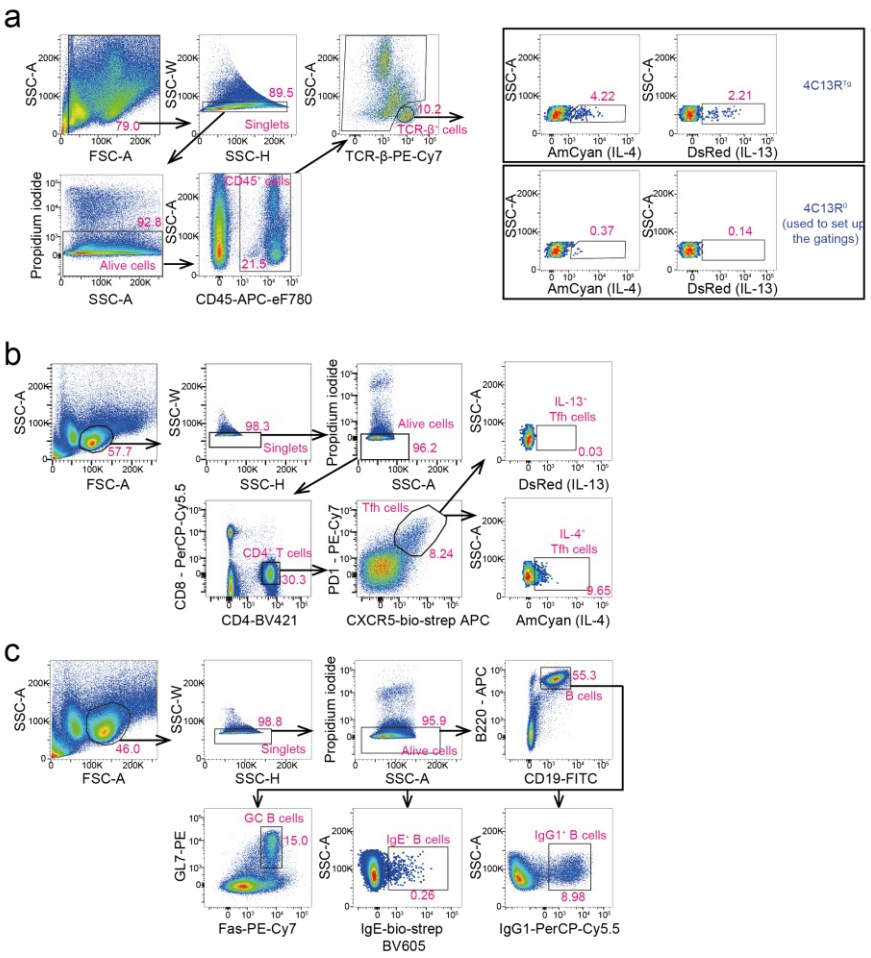
Figure 8. A schematic representation of the context-dependent role of TSLP and IL-1β in promoting skin allergic sensitization and the atopic march.

When allergen HDM sensitization occurs superficially in the skin (epicutaneous sensitization), TSLP derived from keratinocytes located in the epidermis plays a dominantly crucial role for allergic sensitization through the lymph node (LN; generating Th2, Tfh and B cell responses) and the subsequent allergic inflammation in the lung. When allergen sensitization occurs deeply in the skin (dermacutaneous sensitization), IL-1β derived from the infiltrated neutrophils and monocytes/macrophages contributes together with TSLP, to generate a stronger allergen sensitization and subsequently a more severe lung allergic inflammation.

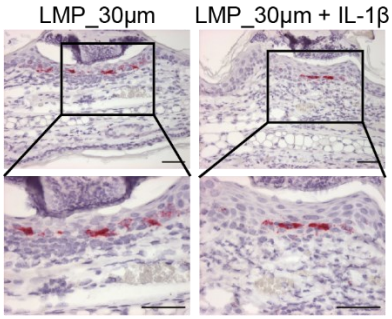
Supplementary Figures



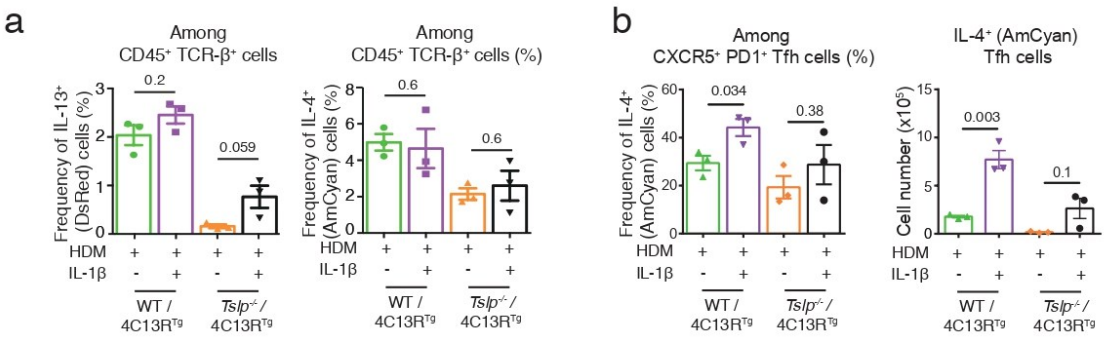
Supplementary Fig.1 Summary of cell counts for skin- or lung-infiltrating eosinophils and basophils from immunostained paraffin sections. One point corresponds to the average number of positively stained cells per microscopic field (at x 20 magnification) from 5 microscopic fields of the section of one mouse biopsy. (**a-b**) Mice were treated as described in **Fig. 1d**. Cell counts of eosinophils and basophils in ears (**a**; related to **Fig. 1e**; n=4, 4, 8, 5, 2, 5, 4 mice) or lungs (**b**; related to **Fig. 2d**; n=6, 3, 9, 5, 4, 9, 8 mice). (**c-d**) Mice were treated as described in **Fig. 3c**. Cell counts of eosinophils and basophils in ears (**c**; related to **Fig. 3e**; n=3, 2, 4, 4, 3, 4 mice) or lungs (**d**; related to **Fig. 4d**; n=3, 5, 4, 6, 3, 4 mice). Graphs show mean±SEM. Two-sided Student's t-test. p values are indicated. Source data are provided as a Source data file.



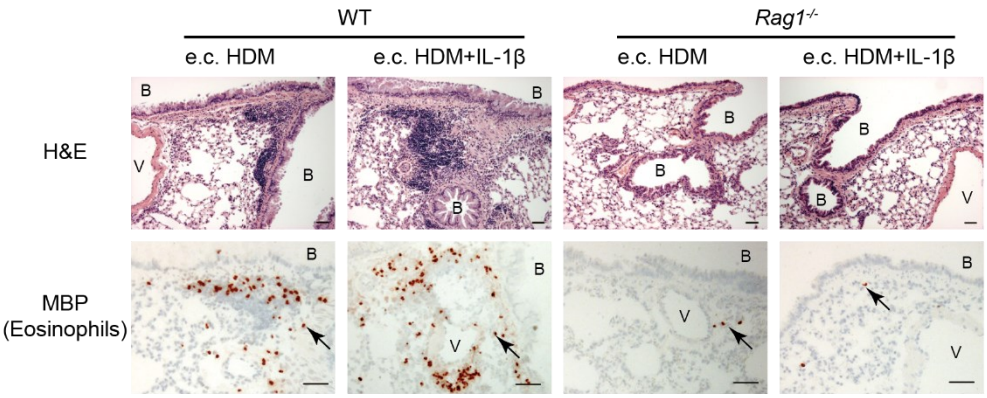
Supplementary Fig. 2. Gating strategy for cells from e.c HDM- or d.c. HDM-treated 4C13R^{Tg} mice. **a** Gating strategy for IL-4 and IL-13 expression by TCRβ⁺ cells in skin dermis, using Il4/Il13 dual reporter 4C13R^{Tg} mice. After excluding debris, doublets and dead cells, hematopoietic cells were gated as CD45⁺. CD45⁺TCR-β⁺ cells were further gated for AmCyan (IL-4)⁺ and DsRed(IL-13)⁺ to examine cytokine production. Gates for Amcyan(IL-4) and DsRed(IL-13) are set with 4C13R^{0} mice. **b** Gating strategy for Tfh cells in ear-draining lymph nodes (EDLNs). Alive single cells are gated for CD4 to identify CD4⁺ T cells, Tfh cells are futher gated as CXCR5⁺PD1⁺ population. AmCyan (IL-4) or DsRed (IL-13) expressed in Tfh cells is gated in 4C13R^{Tg} mice, showing that Tfh cells express AmCyan (IL-4) but not DsRed (IL-13), as expected. **c** Gating strategy for B cells in EDLNs. B cells are gated as CD19⁺B220⁺ cells. Inside B cells, germinal center (GC) B cells are gated as GL7⁺Fas⁺ population. B cells are also gated for IgE⁺ and IgG1⁺ cells.



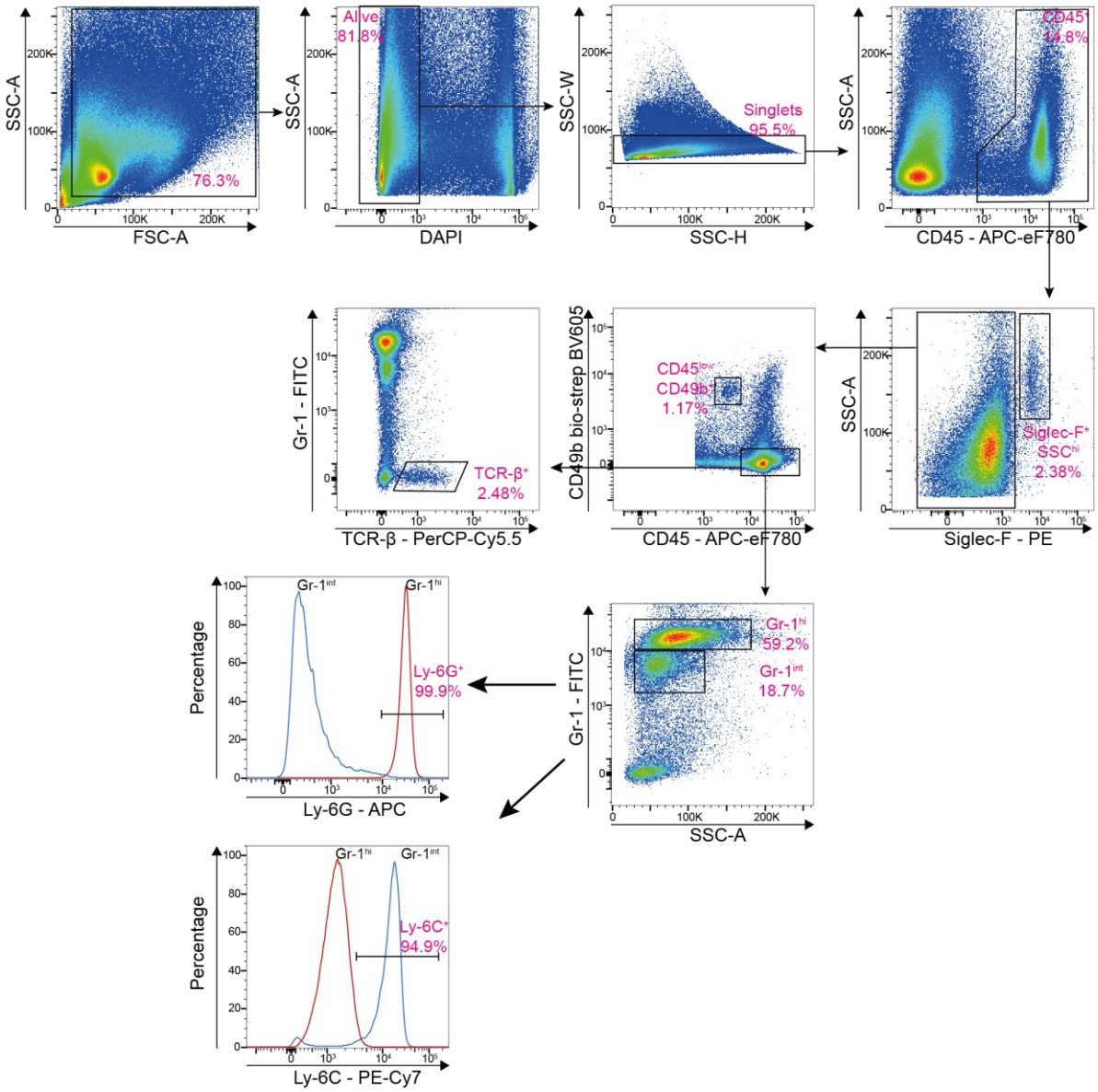
Supplementary Fig. 3. Administration of IL-1β on LMP_30µm ears does not induce TSLP expression in ears. Ears from wildtype Balb/c mice were treated with LMP_30µm or LMP_30µm + 1µg of IL-1β (Cat No. 575106, Biolegend). Ears were collected 48h after treatment. RNAscope in situ hybridization for TSLP mRNA. Bar = 50µm for all pictures. Data are representative of 2 indep-dent experiments with similar results.



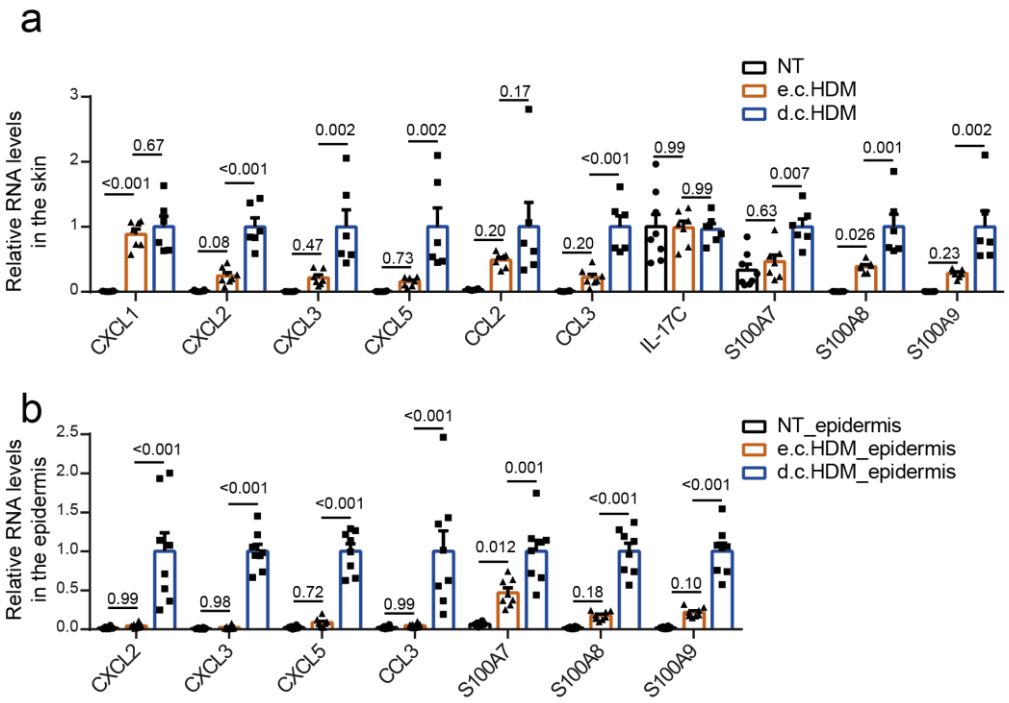
Supplementary Figure 4. Co-administration of IL-1β with e.c. HDM enhances IL-13 expression by Th2 cells in the skin and IL-4 expression by Tfh cells in ear-draining lymph nodes (EDLNs), in a TSLP-independent manner. Mice were treated with an experimental protocol as shown in **Fig. 3c**. **a** Frequency of IL-13$^+$ (DsRed) or IL-4$^+$ (AmCyan) cells among CD45$^+$ TCR-β$^+$ cells in ears, analyzed in WT and *Tslp*$^{-/-}$ mice in 4C13R^{Tg} background, showing that IL-1β administrated with e.c. HDM appears to enhance IL-13 expression by skin TCR-β$^+$ cells in both WT and *Tslp*$^{-/-}$ mice. **b** Administration of IL-1β promotes IL-4-expressing Tfh cells in EDLNs. Graphs show mean±SEM; n=3 mice per group; Two-sided Student's t-test. p values are indicated. Data are representative of 2 independent experiments with similar results. Source data are provided as a Source data file.



Supplementary Fig. 5. Co-administration of IL-1β does not have any exacerbation of asthmatic phenotype in *Rag1*$^{-/-}$ mice. Mice were treated as decribed in Fig. 3c. H&E and IHC for MBP (for eosinophils) in the lung sections from WT and *Rag1*$^{-/-}$ mice. B: bronchiole; V: blood vessel. Bar = 50μm for all pictures. Data are representative of 2 independent experiments with similar results.

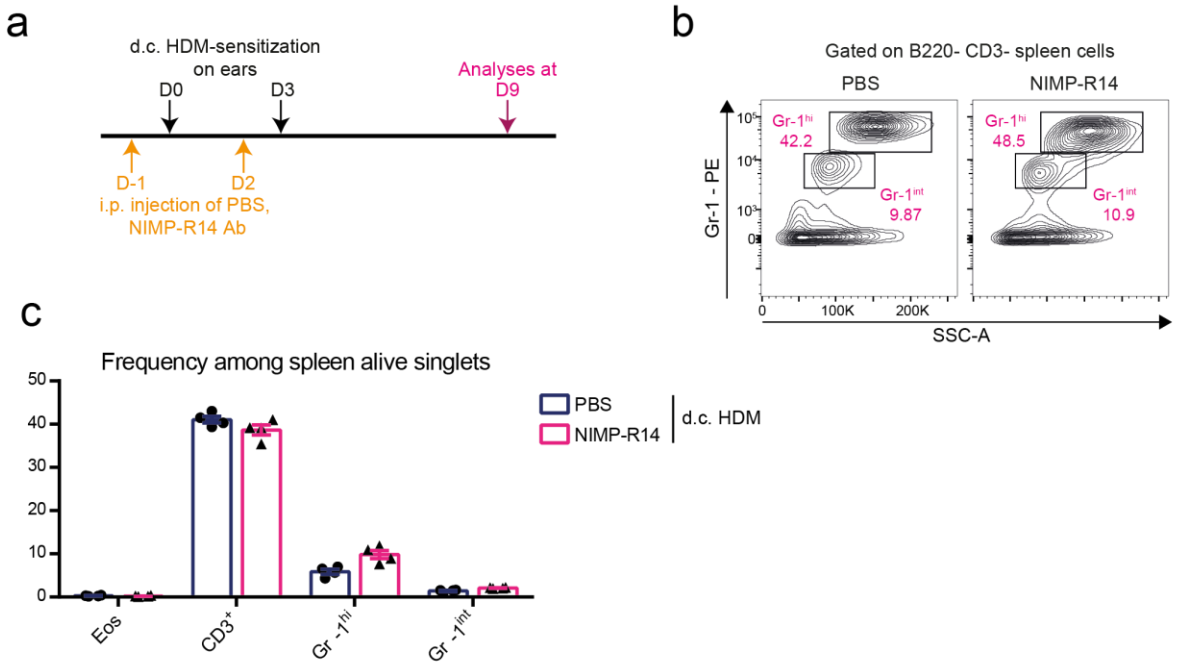


Supplementary Fig. 6. Gating strategy for cells prepared from d.c. HDM-sensitized skin at 24h after treatment. After removing debris, dead cells and doublets, hematopoietic cells were gated as CD45^{+}. Eosinophils were gated as CD45^{+} Siglec-F^{+} SSC-A^{hi}. Basophils were gated as CD45^{low} Siglec-F^{-} CD49b^{+}. TCR-β^{+} T cells were gated as CD45^{+} Siglec-F^{-} CD49b^{-} TCR-β^{+}. Neutrophils were gated as CD45^{+} Siglec-F^{-} CD49b^{-} Gr-1^{hi}. Monocytes / macrophages were gated as CD45^{+} Siglec-F^{-} CD49b^{-} Gr-1^{int}. Gr-1^{hi} and Gr-1^{int} cells were further analysed for their expression of Ly-6G and Ly-6C, showing that as previously reported (Rose, S. et. al. 2012 PMID: 22213571), Gr-1^{hi} represent Ly-6G^{+} Ly-6C^{-} neutrophils, whereas Gr-1^{int} represent Ly6C^{+} Ly-6G^{-} monocytes/macrophages.

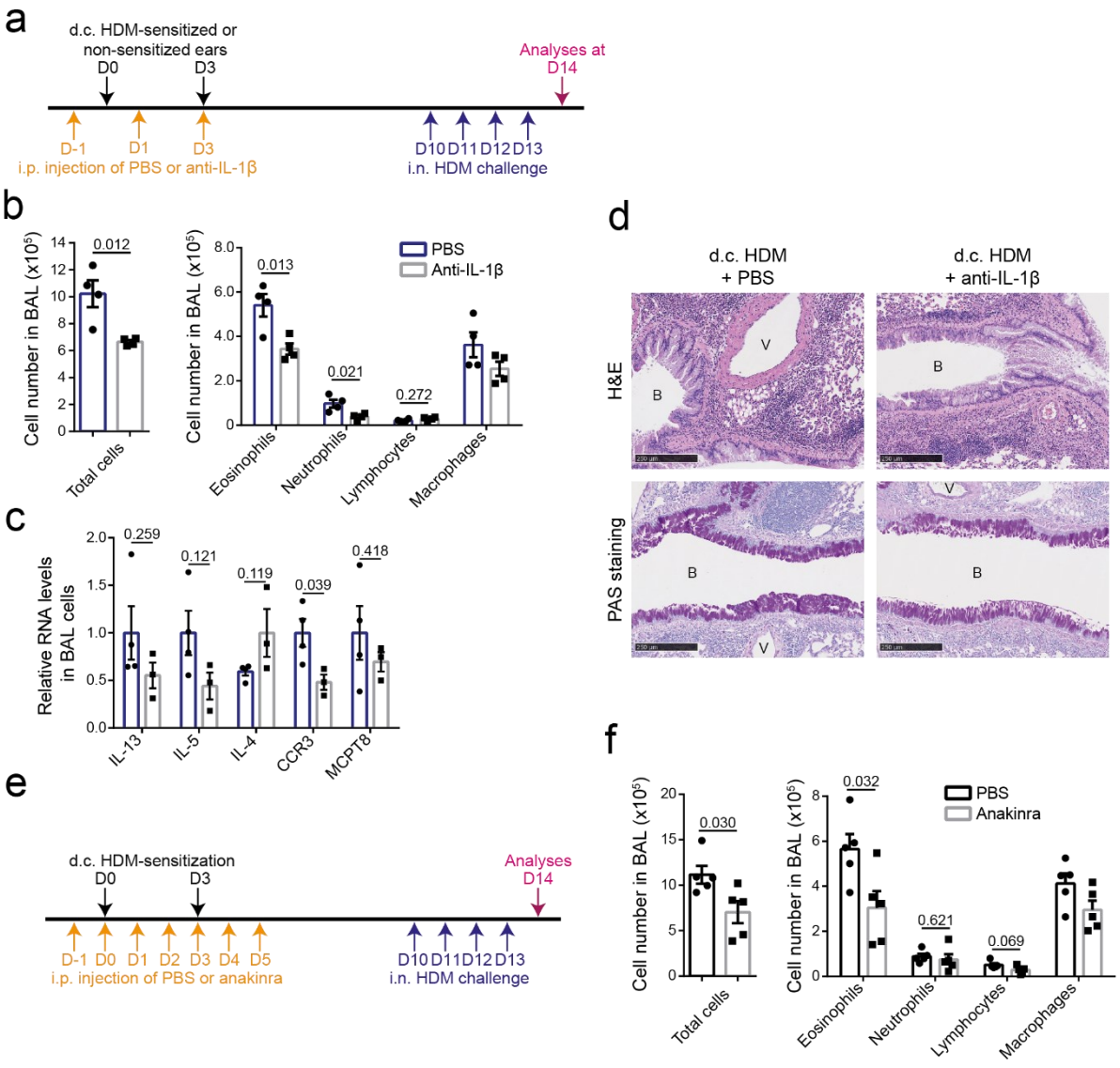


Supplementary Fig. 7. Increased expression of neutrophil-attractant factors in d.c. HDM-treated skin. HDM (2µg) was applied on LMP_30µm (e.c. HDM) and LMP_91µm (d.c. HDM) ears of wildtype mice, and analysed 24 hrs later by quantitative RT-PCR (RT-qPCR). **a** Relative RNA levels of neutrophil chemoattractant factors in the skin from non-treated (NT), e.c. HDM and d.c. HDM-treated mice. n=6, 7 or 8 mice per group. **b** Relative RNA levels of neutrophil chemoattractant factors in the isolated epidermis (following the separation of epidermis and dermis from the ears incubated with 2.5 mg/ml dispase at 4°C overnight) from NT, e.c. HDM and d.c. HDM-treated mice. n=8 mice per group. Graphs show mean±SEM. One-way ANOVA test. p values are indicated. All data are representative of 2 independent experiments with similar results. Source data are provided as a Source data file.

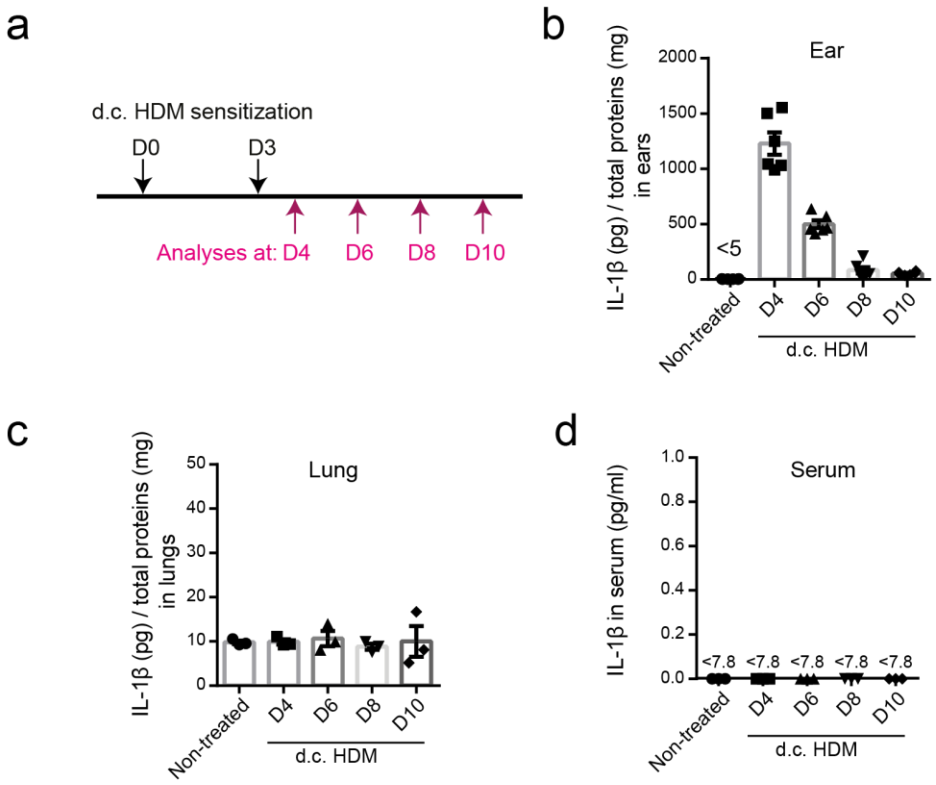
Sequences of PCR primers are: CXCL1 (GCTGGGATTCACCTCAAGAA; AGGTGCCATCAGAGCAGTCT, 208 bp) ; CXCL2 (AGTGAACTGCGCTGTCAATG; TTCAGGGTCAAGGCAAACTT, 153 bp) ; CXCL3 (ATCCAGAGCTTGACGGTGAC; TCATCATGGTGAGGGGCTTC, 189 bp) ; CXCL5 (GTCCACAGTGCCCTACGG; ACTGGCCGTTCTTTCCACTG, 164 bp) ; CCL2 (GGTCCCTGTCATGCTTCTGG; CTTCTTGGGGTCAGCACAGA, 235 bp) ; CCL3 (GCAACCAAGTCTTCTCAGCG; TCTTTGGAGTCAGCGCAGAT, 181 bp) ; IL-17C (TGCTGGAAGCTGACACTCAC; CGTTGATGCATCCACGACAC, 123 bp) ; S100A7 (CTTGTCCCTGGAGGAGTTGA; GCTTGCCCAAGATGTACAGG, 167 bp) ; S100A8 (GGAAATCACCATGCCCTCTA; GAGATGCCACACCCACTTTT, 178 bp) ; S100A9 (AGATGGCCAACAAAGCACCT; TGTGTCCAGGTCCTCCATGA, 208 bp).



Supplementary Fig. 8. Gr-1^{hi} and Gr-1^{int} cells are recovered at D9 following the administration of NIMP-R14 antibody. **a** Experimental protocol. Wild type Balb/c mice were intraperitoneally (i.p.) injected with PBS or NIMP-R14 antibody (100µg) at D-1 and D2. Mice were d.c. sensitized with HDM (2 µg) on LMP_91µm ears at D0 and D3. Spleen was analysed at D9 to examine the recovery of Gr-1^{hi} and Gr-1^{int} cells (the time point before intranasal (i.n.) HDM challenge; see the experimental protocol presented in Figure 6a). **b** Representative FACS plots of Gr-1^{hi} and Gr-1^{int} cells. **c** Comparison of frequency of eosinophils (eos), CD3^{+} cells, Gr-1^{hi} and Gr-1^{int} cells, showing similar Gr-1^{hi} and Gr-1^{int} cell percentages in NIMP-R14 and PBS-administrated mice. Data shown are mean±SEM; n=4 mice per group. Source data are provided as a Source data file.



Supplementary Fig. 9. The administration of anti-IL-1β antibody or Anakinra during d.c. HDM sensitization tends to reduce the subsequent asthmatic phenotypes. a Experimental protocol. Wild type Balb/c mice were intraperitoneally (i.p.) injected either with PBS or 200μg of anti-IL-1β antibody (clone B122, Cat No. BE0246, BioXCell) at D-1, D1 and D3. 2μg of HDM was applied on LMP_91 μm ears at D0 and D3 to induce d.c. HDM sensitization. Mice were then intranasally (i.n.) challenged with 2μg of HDM from D10 to D13. Mice were analysed at D14. **b** Total cell number and differential counting for eosinophils, neutrophils, lymphocytes and macrophages in BAL fluid. n=4 mice per group. **c** Relative RNA levels of genes in BAL cells. n=4, 3 mice per group. **d** Lung sections were stained with haematoxylin & eosin (H&E) for histological analyses or Periodic Acid Schiff (PAS) for goblet cell hyperplasia analyses. B: bronchiole. V: blood vessel. Bar=250 μm for all pictures. **e** Experimental protocol. Wild type Balb/c mice were i.p. injected either with PBS or 10 mg of anakinra (Kineret®, Amgen) from D-1 to D5. 2μg of HDM was applied on LMP_91μm ears at D0 and D3 to induce d.c. HDM sensitization. Mice were then i.n. challenged with 2μg of HDM from D10 to D13, and analysed at D14. **f** Total cell number and differential counting for eosinophils, neutrophils, lymphocytes and macrophages in BAL fluid. n=5 mice per group. Graphs in **b**, **c**, **f** show mean±SEM; Two-sided Student's t-test. All data are representative of 2 independent experiments with similar results. Source data are provided as a Source data file.



Supplementary Fig. 10. IL-1β levels in skin, lung and serum following d.c. HDM sensitization. a Experimental protocol. Wildtype Balb/c mice were dermacutaneously (d.c.) sensitized with HDM (2µg) at D0 and D3, and IL-1β levels in ears (**b**), lungs (**c**) and sera (**d**) were measured by ELISA at D4, D6, D8 or D10, showing that an increase in IL-1β was detected in the treated ears, which reduced with the time, but was not detected in the lung or in the serum. Data shown are mean ± SEM. n=6 mouse ears (**b**), 3 mice (**c, d**) per group. Source data are provided as a Source data file.

GENERAL CONCLUSION AND DISCUSSION

My thesis has been focused on elucidating the molecular and cellular mechanisms driving skin allergic sensitization and the atopic march using newly established allergic skin sensitization and atopic march mouse models based on skin barrier disruption by LMP and delivery of an allergen, followed by allergen challenge in the airway. In the first part, I studied the role of TSLP and DCs in Tfh cell response during allergic skin sensitization. In the second part, I investigated how the cutaneous cytokine microenvironment shapes allergic skin sensitization and the atopic march.

The key findings of the first part of my work, and the remaining questions to be investigated are summarized in Figure 7:

- My study demonstrates that TSLP produced by keratinocytes is crucially required for Tfh / GC B cell and Th2 cell responses in allergic skin sensitization in mice. TSLP signals through TSLPR expressed on DCs to induce Tfh cell differentiation, and more precisely on $Lang^-$ DCs, as TSLPR expressed by LCs is not required for the induction of Tfh / GC B cell response. However, which $Lang^-$ DC subpopulation(s) is/are implicated in Tfh cell differentiation remains to be determined. This can be explored by several ways. First, the delivery of fluorochrome-coupled OVA may allow us to determine which DC subset uptakes the allergen. Next, to examine the potential role of the possible DC subpopulation, it would be interesting to employ mouse lines with the impaired DC subpopulations, for example *Irf4*$^{-/-}$, *Dock8*$^{-/-}$ (for cDC2), *Ccr2*$^{-/-}$ (for mo-DCs), or *Batf3*$^{-/-}$ (for cDC1) to test their roles in LMP/OVA-induced Tfh cell differentiation.
- My study suggests that LCs plays an inhibitory role in skin allergen sensitization-induced Tfh / GC B cell response, in skin allergic inflammation and the atopic march. LCs could therefore represent an interesting target for allergen immunotherapy. To identify how LCs inhibit Tfh and Th2 cell response, it will be interesting to deliver fluorochrome-coupled OVA to LC^{DEP} and CT mice to determine whether LCs limit the uptake of the allergen by other DC populations. Moreover, it will be necessary to analyze whether Tregs in the skin are reduced in LMP/OVA-sensitized LC^{DEP}, as it has been previously shown that LCs may promote Treg response in the skin[126].
- My work characterized a new mouse tool to inducibly deplete Tfh cells. This will be a useful tool to study Tfh cells in different contexts, such as cancer, autoimmunity, vaccination, or allergy. To investigate the function of Tfh cells in the atopic march, we can plan to deplete Tfh cells specifically during the sensitization phase of our atopic march protocol, and

compare the skin and airway phenotype of Tfh- and non-depleted mice.

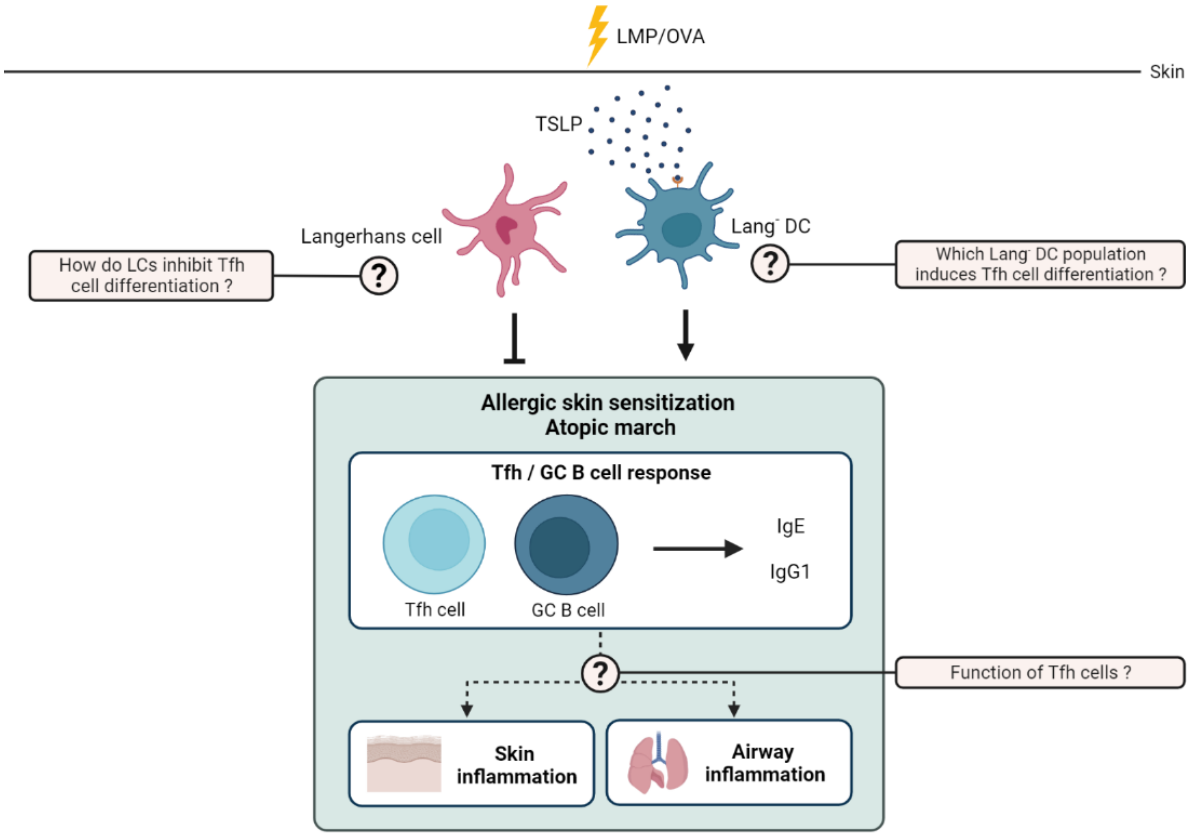


Figure 7. Schematic representation of the role of TSLP and DCs in Tfh / GC B cell response, and skin and airway inflammation. Created with Biorender

In the second part of my thesis, I provided experimental evidence on how TSLP could be differentially implicated in allergic skin sensitization occurring at different anatomic depths which are highly related to the heterogeneity of AD. The key findings and the remaining questions to be investigated are summarized in Figure 8:

- My work demonstrates that TSLP is differentially implicated in epicutaneous and dermacutaneous allergic sensitization. In epicutaneous sensitization, TSLP is crucially required for Tfh / GC B cell response and the atopic march, while TSLP plays only a partial role in deeper sensitization. This could provide one explanation for the non-significant results obtained from clinical trial for Tezepelumab (anti-TSLP) in moderate to severe AD patients[315]. My study reinforces the need for personalized medicine for AD patients. TSLP could be a therapeutic target for some patients whereas more severe AD patients might

benefit from a combined therapy, for example anti-TSLP and anti-IL-1β.

- My study suggests that IL-1β produced by neutrophils and monocytes / macrophages is a key player in dermacutaneous allergic sensitization and the atopic march, independently of TSLP. One limitation of our study is the absence of human exploration. It would be interesting to determine whether TSLP and IL-1β expression in human AD biopsies exhibits a similar pattern as observed in mouse, particularly, whether IL-1β is overproduced in severe AD compared to mild AD. It remains to be tested for the effect of blocking IL-1β, in addition to TSLP, in severe AD patients, using Tezepelumab and IL-1β signalling inhibitors. Several molecules blocking IL-1β signalling exist such as Anakinra (IL-1 receptor antagonist), Canakinumab (anti- IL-1β) which is indicated in the treatment of periodic fevers or gout arthritis, or new therapeutic agents which are under development[316].
- My results also suggest a role for neutrophils and monocytes / macrophages in promoting allergic skin sensitization and the atopic march. I showed that IL-1β-producing neutrophils and monocytes / macrophages infiltrate the skin, however whether IL-1β acts on DCs in the skin or directly on Tfh cells remains to be investigated. Indeed it has also been proposed that monocytes could produce IL-1β in the secondary lymphoid organs to promote Tfh cell differentiation[317]. Therefore, I propose to assess IL-1β-expressing cell recruitment to the LNs by flow cytometry. Furthermore, to determine whether IL-1β acts on DCs to promote the atopic march, it would be interesting to cross CD11c-Cre mice with *Il1r1*$^{fl/fl}$ mice to ablate IL-1R1 specifically in DCs, and to subject these mice to LMP/HDM.
- In addition to TSLP and IL-1β, there could be other factors involved in allergic skin sensitization such as neutrophil extracellular traps (NET) which have been reported to promote allergic airway inflammation[118], or IL-36 cytokines which have been shown to be overexpressed in skin of Netherton syndrome patients characterized by severe AD lesions[318]. My lab has recently generated IL36αγ$^{-/-}$ in which both IL-36α and IL-36γ genes were deleted. It will be interesting to subject these mice to our atopic march protocol and analyse their role in allergen sensitization and the subsequent asthmatic inflammation. To investigate the role of NETs, we may think to perform immunostaining against MPO and citrullinated-H3 (characteristic of NET formation) in skin sections. Then, if we observe NET formation in the skin, it will be interesting to inhibit NETs during skin sensitization using DNAse treatment to determine whether they promote skin allergic sensitization and the atopic march.

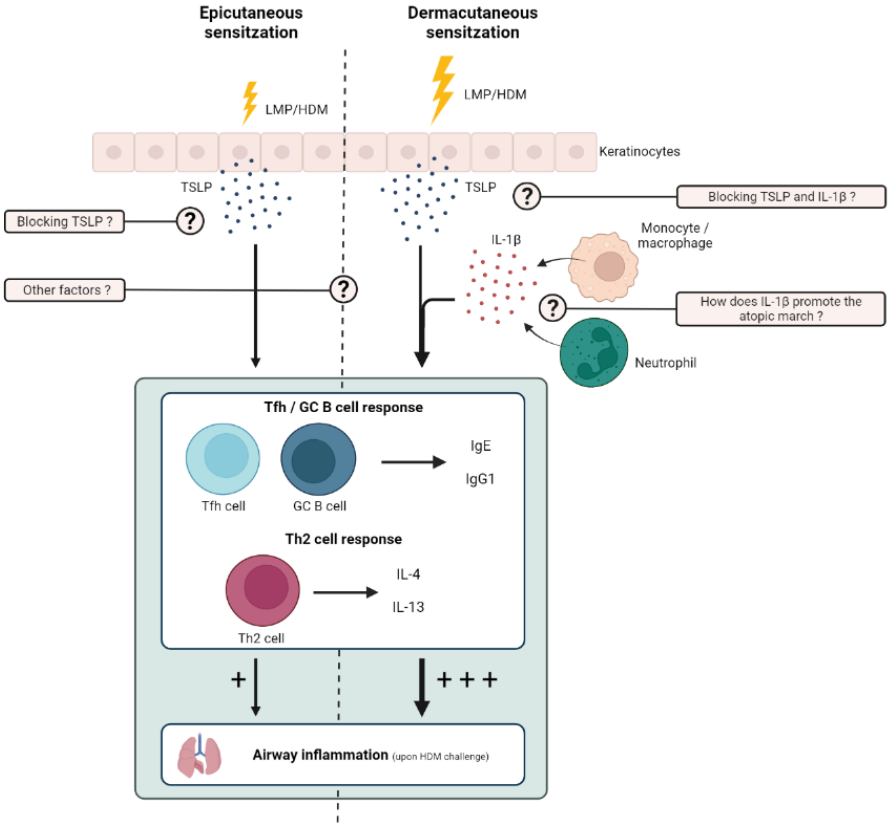


Figure 8. Schematic representation of the context-dependent role of TSLP and IL-1β in allergic skin sensitization and the atopic march. Created with Biorender

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Mécanismes moléculaires et cellulaires impliqués dans la sensibilisation allergique cutanée et la marche atopique

Résumé

Les maladies atopiques, comprenant la dermatite atopique et l'asthme, sont caractérisées par une réponse immunitaire de type Th2 et par une augmentation du taux d'IgE sériques. Il a été montré que les enfants atteints de dermatite atopique sont plus sujets à développer de l'asthme en grandissant. Cette évolution chronologique des maladies atopiques est appelée la marche atopique. Il a été proposé que la sensibilisation allergique pourrait s'effectuer via la peau des patients atteints de dermatite atopique. Mon travail de thèse était d'étudier les mécanismes moléculaires et cellulaires impliqués au cours de la sensibilisation allergique et dans la marche atopique.

Dans la partie 1, j'ai montré que la cytokine TSLP induisait les lymphocytes Tfh et la production d'IgE via TSLPR sur les cellules dendritiques dans un modèle de sensibilisation allergique cutanée. De plus, j'ai montré que les cellules de Langerhans jouaient un rôle inhibiteur dans la réponse Tfh et limitaient l'inflammation cutanée et le phénotype asthmatique.

Dans la seconde partie, j'ai montré que l'implication de TSLP dépendait du contexte de sensibilisation allergique. J'ai également identifié l'IL-1β comme une cytokine clé au cours de la marche atopique, pouvant promouvoir la sensibilisation allergique et l'asthme indépendamment de TSLP.

Mots clés : dermatite atopique, marche atopique, sensibilisation allergique, cellule de Langerhans, lymphocyte T folliculaire helper, TSLP, IL-1β

Résumé en anglais

Atopic diseases, including atopic dermatitis (AD) and asthma, are characterized by Th2 immune response and elevated IgE levels. It has been shown that AD children are more prone to develop asthma later on in life. This progression from atopic dermatitis to other atopic diseases is called the atopic march. It has been proposed that allergic sensitization could occur through barrier-defective skin of AD patients. My thesis work focused on elucidating the molecular and cellular mechanisms driving allergic sensitization and the atopic march.

In the 1st part, I showed that TSLP induces Tfh cells and IgE production through TSLPR on dendritic cells during allergic sensitization through barrier-defective skin. Moreover, I showed that Langerhans cells inhibit Tfh cell differentiation, IgE production and Th2 cutaneous and airway inflammation.

In the 2nd part, I elucidated the context-dependent role of TSLP during skin allergic sensitization, with a crucial role during epicutaneous sensitization. In addition, I identified IL-1β as a key cytokine in atopic march, promoting both skin allergic sensitization and airway inflammation in a TSLP-independent manner.

Keywords: atopic dermatitis, atopic march, allergic sensitization, Langerhans cell, Tfh cell, TSLP, IL-1β