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Ruicheng WEI

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Etudes des mécanismes cellulaires et moléculaires de la réponse immunitaire de type 2 dans la dermatite atopique

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THÈSE dirigée par :
  Dr. LI Mei   Directeur de recherches–CNRS，Université de Strasbourg

RAPPORTEURS :
  Dr. HENRI Sandrine   Chargé de Recherches HdR, Centre d'Immunologie Marseille-Luminy
  Dr. BAYRY Jagadeesh   Chargé de Recherches HdR, INSERM

AUTRES MEMBRES DU JURY :
  Dr. MUELLER Christopher   Directeur de recherches - CNRS
  Dr. BONIFACE Katia   Chargé de Recherches HdR, INSERM
  Prof. Chambon Pierre   Directeur de recherches – CNRS

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Abbreviations

Abbreviation	Definition
TSLP	Thymic stromal lymphopoietin
MHCII	Major histocompatibility complex-II
APCs	Antigen-presenting cells
NK cell	Natural killer cell
PRRs	Pattern recognition receptors
TLR	Toll-like receptors
NLR	Nucleotide oligomerisation receptors
CLR	C-type lectin receptors
RLR	RIG-1 like receptors
PAMPs	Pathogen-associated molecular patterns
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
NODs	Nucleotide-binding oligomerization domain proteins
ER	Endoplasmic reticulum
LPS	Lpopolysaccharide
CTLs	Cytotoxic T lymphocytes
LN	Lymph nodes
TCR	T cell receptor
T-bet	T-box transcription factor
STAT1	Signal transducer and activator of transcription1
SOCS-1	Suppressor of cytokine signaling-1
Th17	T helper type 17
RORγt	Orphan receptor gamma-T
Treg	Regulatory T cell
ICOS	Inducible costimulator
CXCR5	CXC-chemokine receptor 5
Bcl6	B cell lymphoma 6
PD-1	Programmed cell death protein 1
SLAM	Signalling lymphocytic activation molecule
SAP	SLAM-associated protein
BLIMP1	B lymphocyte-induced maturation protein 1
ASCL2	Achaete-scute homologue-2
CCR7	C-C chemokine receptor type 7
ICOSL	ICOS ligand
FDCs	Follicular dendritic cells
CXCR4	C-X-C chemokine receptor type 4
EBI2	G protein coupled receptor 2
GC	Germinal center

IgV	Immunoglobulin variable region
SHM	Somatic hypermutation
BCRs	B cell receptors
BM	Bone marrow
Ig	Immunoglobulin
AD	Atopic dermatitis
FLG	Filaggrin
HDM	House dust mites
VSV	Vesicular stomatitis virus
VDR	Vitamin D receptor
pDC	Plasmacytoid dendritic cells
NETs	Neutrophil extracellular traps
KCs	Keratinocytes
TNF	Tumor necrosis factor
dDCs	Dermal dendritic cells
LCs	Langerhans cells
dsDNA	Double-stranded DNA
ssRNA	Single-stranded RNA
PSORS1	Psoriasis susceptibility region 1
IMQ	Imiquimod
Tfh cells	Follicular helper T cells
CARD	C-terminal repressor domain

Résumé de la these de doctorat

Partie I : Etude de mécanismes de la réponse immunitaire de type 2 dans la dermatite atopique

La dermatite atopique (DA) est l'une des maladies inflammatoires de la peau les plus communes; elle est caractérisée par une inflammation chronique de la peau, un syndrome hyper IgE et une réponse de type T helper 2 (Th2). La prévalence de la DA a augmenté, le nombre d'enfants souffrant d'AD ayant triplé dans les pays industrialisés au cours des trente dernières années, atteignant maintenant 15-30% des enfants et 2-10% des adultes. De plus, la DA forme souvent les prémices d'une progression vers d'autres maladies atopiques, telles que l'asthme. L'accent a donc été porté sur l'acquisition d'une meilleure compréhension de la DA ainsi que sur le développement de stratégies thérapeutiques et prophylactiques efficaces.

Il a été reconnu que la réponse Th2 est impliquée dans la pathogenèse de la DA. Les recherches précédentes au sein de mon laboratoire ont établi un rôle central de la lymphopoiétine stromale thymique (TSLP), une cytokine qui est exprimée par les kératinocytes de l'épiderme, dans la promotion de la réponse Th2 et le déclenchement de la pathogenèse de la DA. Récemment, un nouveau sous-type de lymphocytes T CD4$^+$, les lymphocytes T folliculaires helpers (Tfh), ont été reconnus comme des éléments essentiels de l'immunité humorale grâce au support qu'ils offrent aux lymphocytes B. Les lymphocytes Tfh, avec les lymphocytes B, forment les centres germinatifs (CG) à l'endroit où les lymphocytes B de haute affinité sont sélectionnés et se différencient en lymphocyte B mémoire ou en plasmocyte à longue durée de vie. Toutefois, le rôle pathogénique joué par les lymphocytes Tfh dans la DA et les autres maladies atopiques reste inconnu à ce jour.

Mon travail, lors de ma thèse, avait pour but d'étudier la différentiation des lymphocytes Tfh, ainsi que leur fonction et leur régulation dans la pathogenèse de la DA. Pour cela, j'ai utilisé un modèle murin précédemment établi au sein de notre laboratoire consistant en l'application topique de MC903 (un analogue de la vitamine D3) induisant la production de TSLP par les kératinocytes et, par conséquent, la réponse immunitaire Th2 et la pathogenèse de la DA. Nous avons, dans un premier temps, identifié et caractérisé les

lymphocytes Tfh dans les ganglions lymphatiques drainant la peau dans le modèle murin de DA induit par le MC903 grâce à des techniques de cytométrie en flux. Les résultats ont montré la fréquence des lymphocytes Tfh au sein des lymphocytes T CD4$^+$ ainsi que leur nombre augmentait au fil du temps. Cet accroissement se fait en parallèle de la formation de centres germinatifs comme nous avons pu l'observer par cytométrie en flux ainsi que par marquage immunofluorescent dans les ganglions lymphatiques. J'ai, par la suite, analysé l'expression des cytokines Th1/2/17 dans les lymphocytes Tfh à l'aide de marquage intracellulaire et/ou de souris rapportrices pour ces cytokines, montrant que les lymphocytes Tfh au sein de souris souffrant de DA produisaient de l'IL-4, une cytokine clé de la réponse de type 2 qui régule la commutation isotypique vers les classe d'immunoglobulines IgE et IgG1 dans la réponse humorale. En réalisant des études de cinétique, j'ai identifié deux étapes distinctes dans la génération des lymphocytes Tfh : une étape d'initiation, au jour 7, quand l'engagement vers la lignée Tfh a lieu et une étape de maintenance/expansion, lorsque le nombre de lymphocytes Tfh, de lymphocytes B des CG, de lymphocytes B IgG1$^+$ et IgE$^+$ augmentent.

J'ai alors vérifié l'implication de la TSLP dans la différenciation des lymphocytes Tfh. Les résultats ont montré que, au sein de souris TSLP$^{-/-}$ traitées au MC903, le nombre de lymphocytes Tfh ainsi que de lymphocytes B des CG décroissaient au jour 11, en plus de la diminution de la réponse Th2 déjà observée dans un article précédent. De façon intéressante, la génération des lymphocytes Tfh à l'étape d'initiation n'était pas affectée, mais l'induction d'IL-4 dans ces cellules était diminuée.

J'ai, par la suite, étudié le besoin des cellules dendritiques de la peau pour contrôler la différentiation en lymphocytes Tfh. J'ai trouvé que lorsque les cellules langerine$^+$, incluant les cellules de Langerhans, étaient supprimées dans les souris LangDEP, la différentiation initiale en lymphocytes Tfh, leur maintenance/expansion, ainsi que la formation des CG étaient sensiblement diminués, suggérant un rôle essentiel des cellules dendritiques langerine$^+$ dans la différentiation des lymphocytes Tfh.

Finalement, j'ai examiné l'implication de la signalisation OX40L dans la différentiation des lymphocytes Tfh, celle-ci ayant été rapportée comme étant en aval de TSLP, mais son rôle dans les réponses de Type 2 reste hautement controversé. J'ai montré que le blocage de la

signalisation OX40L à l'aide d'un anticorps neutralisant mène à une diminution du nombre de lymphocytes Tfh et B des CG, ainsi qu'à une réduction de l'expression d'IL-4 dans les lymphocytes Tfh à jour 11. Mais, de façon similaire à ce qui a été observé dans le souris TSLP$^{-/-}$, le blocage de la signalisation OX40L n'affecte pas l'engagement initial des lymphocytes T CD4$^+$ naïfs dans la lignée Tfh. Ceci suggère un rôle important joué par la signalisation TSLP-OX40L dans la maintenance des lymphocytes Tfh plutôt que dans leur différentiation initiale.

Dans son ensemble, la première partie de mon travail doctoral s'est portée sur la différentiation des lymphocytes Tfh, leur production cytokinique ainsi que la formation des centres germinatifs dans le contexte d'un modèle murin de DA induite par le MC903. Par l'exploration du rôle de TSLP, des cellules dendritiques langerine$^+$ et de la signalisation OX40L, mes études ont permis l'acquisition d'une meilleure compréhension des mécanismes sous-tendant les la réponse immunitaire de type 2 dans la pathogenèse de la DA.

En se basant sur ces découvertes, il sera nécessaire de répondre à ces questions : Quels sont les mécanismes moléculaires sous-tendant le rôle joué par les cellules dendritiques langerine$^+$ dans la différentiation des lymphocytes Tfh ? Comment les cellules dendritiques langerine$^+$ ainsi que les autres sous-populations de cellules dendritiques orchestrent-elles la balance entre la différentiation Tfh et Th2 ? La signalisation OX40L importante pour le développement des lymphocytes Tfh est-elle donnée par les cellules dendritiques ou bien par les lymphocytes B ?

Part II : Le MC903 (Calcipotriol) inhibe l'axe IL-23/IL-17/IL-22 dans l'inflammation psoriatique de la souris

Le psoriasis est une maladie de peau commune dont souffre environ 2% de la population mondiale. Elle est caractérisée par une hyperplasie de l'épiderme, une hyperprolifération ainsi qu'une différentiation aberrante des kératinocytes et une infiltration leucocytaire (notamment des lymphocytes T, des macrophages et des neutrophiles). Il a été démontré que l'axe IL23/IL-17/IL-22 joue un rôle crucial dans la pathogenèse du psoriasis. De façon intéressante, le MC903, aussi appelé calcipotriol, a été utilisé en traitement des plaques de psoriasis de gravité faible ou modérée. Bien qu'il ait été suggéré que le MC903 inhibe la

prolifération des kératinocytes et induise leur différentiation terminale, les mécanismes d'actions précis du MC903 dans le psoriasis ne sont pas encore réellement connus à ce jour. Dans la mesure où le MC903 induit la production de TSLP par les kératinocytes et entraine, par là même, une réponse de type Th2, je me suis initialement demandé si le MC903 inhibait l'axe IL23/IL-17/IL-22, et si c'était bien le cas, si cela se faisait par l'intermédiaire de TSLP.

Afin de répondre à cette question, nous avons utilisé un modèle murin de psoriasis induit par l'application topique d'Imiquimod (IMQ), un ligand de TLR7 et TLR8. Il a été démontré précédemment que le psoriasis induit par l'IMQ se développe via l'axe IL23/IL-17/IL-22. Nous avons trouvé que le MC903 inhibe l'axe IL23/IL-17/IL-22 stimulé par l'IMQ de façon dose-dépendante, induisant une diminution de l'infiltration des neutrophiles dans la peau. De façon inattendue, ce rôle joué par le MC903 n'était pas lié à la TSLP puisque les effets du MC903 étaient identiques sur des souris TSLP$^{-/-}$. Toutefois, l'action du MC903 sur l'axe IL23/IL-17/IL-22 était abrogée au sein de souris VDR$^{-/-}$, indiquant que son action est dépendante du VDR. De plus, à l'aide de souris permettant la suppression du VDR de façon sélective dans les kératinocytes (VDR$^{ep-/-}$), nous avos trouvé que l'inhibition de l'axe IL23/IL-17/IL-22 était abrogée au sein de souris VDR$^{ep-/-}$ indiquant que des facteurs produits par les kératinocytes sont impliqués dans l'inhibition de l'axe IL23/IL-17/IL-22.

Summary of thesis

Part I Study of the mechanisms underlying the type 2 immune response in atopic dermatitis pathogenesis

Atopic dermatitis (AD) is one of the most common inflammatory skin diseases, characterized by chronic cutaneous inflammation, hyper IgE and T helper type 2 (Th2) response. The prevalence of AD has increased, with the number of children suffering from AD tripled in industrialized nations in the past 30 years, now affecting 15-30% of the children and 2-10% of adult. Moreover, AD often progresses to other atopic diseases such as asthma. It has been thus urged to achieve a better understanding of AD and to develop effective prevention and treatment strategies.

It has been recognized that Th2 cell response is critically implicated in the pathogenesis of AD. The previous studies in my lab have established a central role of cytokine thymic stromal lymphopoietin (TSLP), which is expressed by epidermal keratinocytes, in promoting Th2 cell response and driving the pathogenesis AD. Recently, a new CD4+ helper cell subset, T follicular helper (Tfh) cell, has emerged to be a critical player in humoral immunity by providing help to B cells. Tfh cells together with B cells form germinal centers (GCs) at the site where high-affinity B cells are selected and differentiated into either memory B cells or long-lived plasma cells. However, the pathogenic role of Tfh cells in the context of AD and other atopic diseases remains unexplored.

The part I of my thesis aimed at studying the Tfh cell differentiation, function and regulation in AD pathogenesis. To this aim, I employed our previously established AD mouse model in which MC903 (a vitamin D analog) topical treatment on the skin induces TSLP production by keratinocytes, promotes Th2 cell response and drives the pathogenesis of AD. I first identified and characterized Tfh cells in the skin-draining lymph nodes (LNs) by flow cytometry analysis. Results showed that the frequency of Tfh cells in CD4+ T cells, as well as the Tfh cell number, increased with time. This is accompanied by GC formation, as showed by flow cytometry analysis and fluorescent immunohistochemical staining in the LNs. I then analyzed the expression of Th1/2/17 cytokines in Tfh cells using intracellular staining or/and cytokine reporter mice, showing that Tfh cells in the AD mice produced IL-4,

a key type 2 cytokine that regulates class switch recombination to IgE and IgG1 in humoral immune response. By performing kinetic analyses, I identified two stages of Tfh cell generation: an " initiation" stage (at D7) when Tfh cell lineage commitment has taken place, and an "expansion/maintenance" stage when the number of Tfh cells, GC B cells, IgG1$^+$ B cells and IgE$^+$ B cells all increases.

Next, I addressed whether TSLP is required for Tfh cell differentiation. Results showed that in MC903-treated TSLP$^{-/-}$ mice, Tfh and GC B cell numbers were all diminished at D11, in addition to the decreased Th2 cell response as previously reported. Interestingly, the generation of Tfh cells at the "initiation" stage was not affected, but IL-4 induction in Tfh cells was impaired.

In exploring the requirement of skin DCs in driving Tfh cell differentiation, I found that when Langerin+ DCs, including epidermal Langerhans cells, were depleted in Lang^{DEP} mice, the initial Tfh cell differentiation and the expansion/maintenance of Tfh cells, as well as GC formation, were all markedly impaired, suggesting a critical role of Langerin+ cells in promoting Tfh cell response.

Finally I examined the implication of OX40L signaling in Tfh cell differentiation, as OX40L has been reported to be a TSLP-downstream signaling but its role in type 2 responses remains highly controversial. I showed that the blockade of OX40L signaling using OX40L neutralizing antibody led to a decrease in Tfh cell and GC B cell number, as well as a reduction of IL-4 expression in Tfh cells at D11. But similar to what was observed in TSLP$^{-/-}$ mice, blockade of OX40L signaling did not affect the initial commitment of naïve CD4+ T cell into Tfh lineage, suggesting that TSLP-OX40L signal may play an important role in the maintenance of Tfh cells rather than the initiation of Tfh cell differentiation.

Taken together, my thesis work investigated Tfh cell differentiation, its cytokine expression and germinal center formation using MC903-induced AD mouse model. By exploring the role of TSLP, Langerin+ DCs and OX40L signaling in Tfh cell differentiation and regulation, my study provides novel insights into the mechanisms underlying the type 2 immune responses in AD pathogenesis.

It will be necessary to further address the following questions: What is the molecular mechanism underlying Langerin+ DCs in Tfh cells differentiation? How do Langerin+ DCs and other dermal DCs orchestrate Tfh versus Th2 polarization? Is OX40L derived from DCs or from B cells implicated in Tfh cell response?

Part II: MC903 (Calcipotrol) inhibits the IL-23/IL-17/IL-22 in mouse psoriatic inflammation

Psoriasis is a common skin disorder affecting 2% of the worldwide population. It is characterized by hyperplasia of the epidermis, hyperproliferation and aberrant differentiation of keratinocytes, infiltration of leukocytes (such as T cells, macrophages and neutrophils). A critical role of the IL-23/IL-17/IL-22 axis has been recognized in the pathogenesis of psoriasis. Interestingly, MC903, also called calcipotriol, has been used in the treatment of mild to moderate plaque psoriasis. Although it has been suggested that MC903 inhibits keratinocytes proliferation and induces the terminal differentiation of keratinocytes, the exact mechanism of MC903 in regulating psoriasis inflammation is not clear. As MC903 triggers keratinocytes to produce TSLP that can induce Th2 cell response, our initial question was whether MC903 could inhibit IL-23/IL-17/IL-22 axis, and if yes, whether it is through TSLP.

To address this question, we used a psoriasis mouse model induced by topical application of Imiquimod (IMQ), a TLR7/8 ligand. It has been shown that IMQ-induced psoriasis is mediated via the IL-23/IL-17/IL-22 axis. We found that MC903 inhibited the IMQ-induced IL-23/IL-17/IL-22 axis in a dose-dependent manner. This led to an attenuated neutrophil infiltrate in the skin. Unexpectedly, this inhibiting role of MC903 was not mediated by TSLP, as IMQ-treated TSLP$^{-/-}$ mice exhibited similar inhibitory effect of L-23/IL-17/IL-22 axis by MC903, as shown in IMQ-treated wildtype mice. However, MC903-induced inhibition of L-23/IL-17/IL-22 axis was abolished in VDR$^{-/-}$ mice, indicating that it is VDR-dependent. Further, by using keratinocyte-selective VDR knockout mice (VDR$^{ep-/-}$), we found that the inhibition of IL-23/IL-17/IL-22 axis by MC903 was abolished in IMQ-treated VDR$^{ep-/-}$ mice, suggesting that epidermal keratinocyte-derived factor(s) is(are) implicated in MC903-induced inhibition of L-23/IL-17/IL-22 axis.

Introduction

1. The immune system

Humans and other mammals are living in a world with pathogenic and nonpathogenic microbes and a variety of toxic or allergenic substances, which threat normal host homeostasis. Pathogenic microbes possess a set of mechanisms by which they replicate, spread, and jeopardize normal host functions. During the history of evolution, humans and other mammals developed a sophisticated immune system to control and eliminate these organisms and toxins. At the same time, the immune system developed a complex array of protective mechanisms to avoid responses that induce excessive damage of self-tissues. Over the past decades, there have been numerous advances in our understanding of the immune system and how it functions to protect the body from invading pathogens or self-reactive antigens.

The immune system in mammals can be divided in two branches: innate immunity and adaptive immunity. Innate immunity represents the first line of defense against an invading pathogen. It is an antigen-independent defense mechanism for the host to clear pathogens immediately or within hours after encountering of antigens. The primary function of innate immunity is the recruitment of different kinds of immune cells to infection and inflammation sites through the production of cytokines and chemokines. Cytokine production promotes the production of antibodies. The innate immune system also clears dead cells and antibody complexes and removes foreign substances that are present in tissues, blood and lymphoid tissues. Most importantly, it can present antigens to T cells to mount an adaptive immune response. Numerous cells are involved in the innate immune response, for example, phagocytes (macrophages and neutrophils), basophils, eosinophils, dendritic cells, mast cells and natural killer (NK) cells. Innate immunity exerts its function in four types of defensive barriers: (a) physical barriers, which are epithelial cell layers that express tight cell-cell contacts; (b) secreted mucus layer, which overlays the epithelium in the respiratory, gastrointestinal, and genitourinary tracts; (c) biological fluids, comprising soluble proteins and bioactive small molecules that are constitutively present, such as the complement proteins, or induce defensins, and ficolins; and (d) innate immune cells, with membrane-bound receptors and cytoplasmic proteins, which can bind molecular patterns expressed on the surfaces of invading microbes [1-3].

Adaptive immunity, on the other hand, is antigen-dependent. Adaptive immunity is induced when innate immunity is ineffective to eliminate infectious agents. The primary function of the adaptive immune response is to recognize specific "non-self" antigens and to generate antigen-specific antibody to eliminate specific pathogens or pathogen-infected cells. The hallmark of adaptive immunity compared to innate immunity is the capacity of generating memory T cell and B cells that enables the host to mount a rapid and efficient immune response upon subsequent encounter with the same antigen. The cells of the adaptive immune system include T cells and B cells. Adaptive immune responses require the antigen-specific receptors expressed on the surfaces of T and B lymphocytes. The antigen-specific receptors expressed on T cells and B cells are encoded by germline gene elements that have been assembled by somatic rearrangement. The assembly of antigen receptors from a few hundred germline-encoded gene elements allows the generation of millions of different antigen receptors, and each antigen receptor potentially has unique specificity for a different antigen.

Innate and adaptive immunity are not mutually exclusive mechanisms of host defense, but rather complementary. The innate response represents the first line of host defense and presents antigens to T cells, and the components of the innate system contribute to activation of the antigen-specific T cells. The adaptive immune responses become prominent as soon as antigen-specific T and B cells have undergone clonal expansion. And the antigen-specific cells amplify their responses to pathogens by recruiting innate immune cells to clear the invading pathogens. Thus, although the innate and adaptive immune responses are fundamentally different in nature of actions, synergy between them is critical for a fully effective immune response against invading pathogens.

1.1 Innate immunity

Innate immune response does not require prior exposure to antigens, it can respond immediately to invading pathogens. It recognizes antigen (Ag) molecules that are broadly distributed on different organisms or cells. Components of innate immunity include polymorphonuclear leukocytes , mononuclear leukocytes and natural killer (NK) cells . Phagocytic cells (neutrophils and dendritic cells in blood and tissues, monocytes in blood, macrophages in tissues) engulf and destroy invading pathogens. Polymorphonuclear

leukocytes (including neutrophils, eosinophils, basophils and mast cells) and mononuclear cells (including monocytes and macrophages) release inflammatory mediators. Natural killer cells kill virus-infected cells and tumor cells. The innate immune system senses pathogen infection and recognizes pathogens through a variety of germline-encoded recognition receptors. Pattern recognition receptors (PRRs) are proteins expressed by cells of the innate immune system to identify molecules from pathogens. The PRR are divided into four families: Toll-like receptors (TLR), Nucleotide oligomerisation receptors (NLR), C-type lectin receptors (CLR) and RIG-I like receptors (RLR). They recognize conserved specific pathogen-associated molecular patterns (PAMPs) from a broad spectrum of microbial components. In addition, PRRs can also sense endogenous 'danger' signals by recognizing danger-associated molecular patterns (DAMPs). The recognition of PAMPs or DAMPs by the PRRs leads to an inflammatory response [4].

1.1.1. Cells of the innate immune system

Innate immune cells comprise of natural killers (NKs), dendritic cells (DCs), macrophages, mast cells, neutrophils, basophils and eosinophils. These cells express a set of PRRs that are able to recognize PAMPs and DAMPs and induce a rapid inflammatory response, which stimulate the killing of infected or transformed cells, phagocytosis, apoptosis and antigen presentation [5, 6].

a. Mononuclear phagocytes

The mononuclear phagocytes are macrophages and dendritic cells. Macrophages are distributed throughout the body of the host. Therefore macrophage will encounter the invading pathogens immediately wherever infection is introduced via any route. Macrophages are capable of engulfing and destroying microbes through the generation of a respiratory burst [7]. Macrophages also activate signaling through the adaptor MyD88 and promote the formation of inflammasome, which stimulates the production of cytokines, such as TNF and IL-1β, to enhance the innate antimicrobial activity [8].

DCs are present in the peripheral organs, lymphatic system and blood system. DCs can be subdivided into subsets that differ in their tissue distribution, phenotype, function and ontogeny. DCs express a set of PRRs, including the mannose receptor (CD206), TTLRs,

dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), nucleotide-binding oligomerization domain proteins (NODs) and dectin-1, to sense the danger signals and pathogen-associated molecular patterns [9]. Activation of TLRs on DCs results in increased expression of MHC–peptide complexes and co-stimulatory molecules and the production of different cytokines, which have a profound effect on T-cell priming and differentiation [10]. Phagocytic function of DCs plays key roles in the adaptive immune response. DCs take up microbial antigens, and process them by means of proteolysis into peptide fragments, and then present them to naïve T cells to trigger subsequent differentiation.

b. Polymorphonuclear phagocytes

The polymorphonuclear phagocytes comprise neutrophils, basophils and eosinophils. They are very important in the suppression of pathogen infection and repair of tissue injury. Neutrophils are released into the blood after maturation in bone marrow. Neutrophils accumulate at sites of pathogen infection and tissue injury, and they eliminate pathogenic microbes by engulfing microbe and release of granules [11]. Neutrophils have been reported to produce substantial amounts of the cytokines TNF and IL-12, as well as chemokines.

Eosinophils are multifunctional leukocytes involve in the generation of inflammatory response. The maturation of eosinophils takes place in the bone marrow, and their survival in peripheral tissues is enhanced by IL-5. Eosinophils can be induced in a great number in the course of parasitic infections, or allergic immune responses. The physiological function of eosinophils is to protect the host from parasitic infections. Eosinophils are not phagocytic, but they have large granules that contain eosinophilic cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin. These toxic molecules and enzymes are highly cytotoxic when released onto the surface of organisms [12].

Basophils are basophilic granulocytes circulating in the peripheral blood, which are relatively few in number compared with the other white cells. The cell surface of basophils express high-affinity receptor for IgE (FcεRI), and they are key initiators of hypersensitivity responses to helminthic parasites and allergies, which release histamine and other

preformed mediators from their granules and produce large amount of lipid mediators to induce tissue inflammation, and smooth muscle contraction [13].

Mast cells are derived from hematopoietic stem cells, and they are widely distributed throughout tissues. Mast cells and basophils are morphologically similar cells that represent distinct lineages. Mast cells also bear high-affinity receptors for FcεRI. Crosslinking of FcεRI by the binding of antigen to IgE leads to degranulation and release of preformed mediators (vasoactive amines, histamine and serotonin), membrane derived mediators (leucotrienes B4, C4, D4 and E4), prostaglandins and platelet activating factor, many of which can potentially mediate proinflammatory, anti-inflammatory and/or immunosuppressive [14].

c. NK cells

NK cells are large granular lymphocytes, and they play a major role in the rejection of tumors and the destruction of cells infected by viruses. Destruction of infected cells is achieved through the release of perforins and granzymes to induce apoptosis. NK cells bear immunoglobulin receptors FcR and bind antibody- coated cells and lead to antibody-dependent cellular cytotoxicity. In addition, NK cells express receptors on their surface for MHC I. Normal host cells are MHC I positive, the binding of this MHC I to its receptor on the natural killer cell will lead to programmed cell death. Tumor cells often downregulate MHC I expression, and viruses often cause downregulation of MHC I expression on host cells. In these cases, the receptors for MHC I cannot be bound, therefore the NK cell can secret perforins and granzymes to lyse the target [15, 16].

1.1.2 PRRs and PAMPs

The recognition of PAMPs or DAMPs by the PRRs triggers different inflammatory response, such as the secretion of cytokines/chemokines, the induction of antimicrobial peptides, pyroptotic cell death and the recruitment of phagocytic cells. Currently, four different classes of PRR families have been identified, and they are the TLRs, NLRs, RLRs, CLRs [6].

The TLR family is responsible for sensing invading pathogens that exist outside of the cell or in intracellular lysosomes and endosomes. TLRs comprise N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain [17]. So far, 13 murine TLRs and 10 human TLRs have been identified. TLR1, TLR2, TLR4, TLR5, and TLR6 are present on the plasma membrane, TLR3, TLR7 (TLR8 in human), and TLR9 are mainly present on the endoplasmic reticulum (ER) membrane [18]. Different TLRs recognize different molecular patterns from different microorganisms and self-components. TLR2 senses various PAMPs from bacteria, mycoplasma, fungi, and viruses. TLR2 forms a heterodimer with either TLR1 or TLR6 to recognize its ligands. Ligation of TLR2 and its ligands induces the production of a variety of pro-inflammatory cytokines in macrophages and DCs. TLR4 together with myeloid differentiation factor 2 (MD2) that were expressed on the cell surface recognizes lipopolysaccharide (LPS) of bacteria. In addition, TLR4 also recognizes viruses by binding to viral envelope proteins. TLR5 is highly expressed in DCs of the lamina propria (LPDCs) in the small intestine, and it is known to recognize bacterial flagellin from invading bacteria. TLR11 recognizes uropathogenic bacteria and a profilin-like molecule derived from the intracellular protozoan Toxoplasma gondii. TLR11 is also required for parasite-induced IL-12 production. Unlike the other TLRs, which are expressed mainly on cell surface, TLR3, 7, 8 and 9, are localized in ER membrane and recognize nucleic acids. TLR3 detects viral double-stranded (ds) RNA in the endolysosome. TLR7 detects RNAs from bacteria. TLR9 senses unmethyated DNA with CpG motifs derived from bacteria and viruses. Activation of these TLRs leads to the production of pro-inflammatory cytokines and type I IFNs. The ligand for TLR10 has not been identified [19].

The RIG-I-like receptor (RLR) family comprise of three members: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology-2 (LGP2) [20], which play a major role in sensing RNA virus infection to initiate antiviral immune response. RLRs recognize viral dsRNAs in the cytoplasm and trigger signaling pathways that induce the production of pro-inflammatory cytokines and type I IFNs. The three RLRs are broadly expressed in most tissues. RIG-I and MDA5 share a number of structural similarities, they comprise three distinct domains: an N-terminal region, a central DExD/H box RNA helicase domain, and a C-terminal repressor domain (CARD). LGP2 lacks the N-terminal CARDs and is thought to function as

a regulator of RIG-I and MDA5 signaling. RIG-I and MDA5 activate Interferon-beta promoter stimulator 1 (IPS-1) on the mitochondrial membrane to promote the expression of type I and type III IFNs.

NLRs family detects the presence of PAMPs and endogenous molecules in the cell cytoplasm. These NLRs have three domains: a c-terminal leucine-rich repeat, an N-terminal signaling domain and a central nucleotide-binding NACHT domain. The c-terminal leucine-rich repeat recognize microbial PAMPs or endogenous host molecules. The N-terminal signaling domain contains a dead effector domain (DED), a Pyrin domain (PyD), a CARD, baculovirus inhibitor repeats (BIRs) and an acidic domain, it is required for homotypic interactions with downstream signaling proteins. The central nucleotide-binding NACHT domain is required for formation of active receptor complexes for activation of downstream signaling [21]. There are four subfamilies in mammalian NLRs, the subfamilies are determined based on different N-terminal effector domains. They are NLRC (known as NODs), NLRP (known as NALPs), NLRB (known as NAIP) and NLRA. The activation of NLRs orchestrates inflammatory response, autophagy or cell death.

1.2 Adaptive immunity

When innate immunity is ineffective to eliminate pathogens, which usually leads to the establishment of infection, adaptive immunity is developed to eliminate invading pathogens. The adaptive immune responses are able to recognize specific "non-self" antigens and generate pathogen-specific immunologic effector pathways to eliminate specific pathogens or pathogen-infected cells. As long as the adaptive immune response has been established, it creates immunological memory after an initial response to a specific pathogen, which can quickly recognize and eliminate a specific pathogen when the body is re-exposed to the same antigen. Adaptive immune responses are based primarily on the antigen-specific receptors expressed on T cell and B cells surfaces. After the infection has been established, antigen-presenting cells (APCs), such as DCs and macrophages, engulf pathogens and present antigens to naïve T cells, leading to T cell priming, activation, and differentiation. The cells of the adaptive immune system are T cells and B cells. T cells are involved in cell-mediated immune responses, whereas B cells play a major role in the humoral immune response. In cell-mediated immune responses T cells recognize infected cells and directly

destroy infected cells together with pathogens, or release cytokines to recruit natural killer cells and phagocytes to destroy infected cells indirectly. In antibody responses, B cells are activated to secrete antibodies, which neutralize the foreign antigens or capture the foreign antigens and deliver them to phagocytes.

1.2.1 Effector T cell subsets

a. CD8+ cytotoxic T lymphocytes (CTLs)

CD8+ T cells are a subset of T lymphocytes that are responsible for the direct killing of intracellular pathogens, including viruses and bacteria, or tumor cells. Shortly after a peripheral infection, naive CD8+ T cells are primed by APCs in secondary lymphoid organs such as lymph nodes (LN) and spleen, leading to CD8+ T cell activation and differentiation into cytotoxic effector cells. Proinflammatory cytokine, IL-12, plays a critical role in terminal differentiation of CD8+ effector T cells. IL-12 activates mechanistic target of rapamycin (mTOR) signaling pathway to induced T-bet expression in CD8+ T cell, which promotes effector CD8+ T cell differentiation subsequently [22]. Multiple extracellular signals cooperate to regulate CD8+ T cell proliferation, survival, acquisition of effector functions and migration to infection sites.

Antigens inside a cell can be bound to MHC Class I molecules, and brought to the surface of the cell by the class I MHC molecule, CD8+ T cells recognize foreign peptides presented by MHC Class I molecules on the surface of infected cell and lyse these infected cells by the secretion of perforin and granzymes. Perforin forms a pore in the membrane of the target cell, which allows the granzymes to enter the infected cell to cleave the proteins inside the cell, destroying viral proteins and ultimately resulting in apoptosis of the target cell. In addition, activated CD8+ T cells express FasL on their cell surface, and FasL binds to Fas that are expressed on the surface of the infected cell, resulting in apoptosis of the target cell [23]. Besides the direct destruction of infected cells, CD8+ T cells can secret TNF-α and IFN-γ, which have anti-tumour and anti-viral effects.

b. CD4+ Helper T cells (Th1/Th2/Th17/Treg)

Lineage-specific differentiation depends on the cytokine milieu of the microenvironment and antigen interaction with T cell receptor (TCR) expressed on naïve CD4 T cell.

Activation of TCR induces an activation of a series of downstream signaling pathways, which eventually lead to naïve cell proliferation and differentiation into specific effector cells. Th1 cells play a key role in the cell-mediated cytotoxic response against intracellular pathogens by the production of IFNγ to enhance macrophage activation and to promote the activation of antigen-specific CTLs. Several transcription factors cooperate together to induce full differentiation of the Th1 cells, such as T-box transcription factor (T-bet), STAT1, STAT4, Runx 3, Eomes and Hlx. Among these transcription factors, T-bet is the master regulator for Th1 differentiation. T-bet activates a set of target genes to promote Th1 differentiation and enhance the production of IFNγ, and it plays an important role in suppressing the development of other CD4+ T cell subsets, such as Th2 and Th17. IL-12 and IFNγ are two critical cytokines that initiate the downstream signaling cascade for the differentiation of Th1 cells. IL12 is secreted mainly by APCs after their activation through the pattern recognition receptors. Subsequently, IL-12 promotes NK cells to produce IFNγ. IFN γ in turn activates signal transducer and activator of transcription 1 (STAT1), which triggers T-bet expression. T-bet further induces IFNγ production by the differentiating cells, thereby amplifying T-bet expression and upregulating the expression of IL12Rβ2. This process enables activated CD4+ T cell to response to DC-derived IL-12. IL-12 induces STAT4 expression, and STAT4 in turn induces IFNγ production, creating a positive feedback loop for complete Th1 cell differentiation.

c. Th2 cells

Th2 cell is required for B cell help in humoral immunity and elimination of extracellular microbes and intestinal helminthes. Once a naïve CD4+ T cell is primed by signals received from an antigen-presenting cell, naïve CD4+ T cell start to initiate differentiation. The expression of the lineage-specifying transcription factor, GATA-3 activates a set of Th2 cytokine cluster to produce IL-4, IL-5, and IL-13, which are critical for IgE production that can provoke or sustain allergic immune response, recruitment of eosinophil, recruitment of basophils, and clearance of extracellular parasites. IL-6, also produced by DCs, enhances IL-4 production by activated CD4+ cells. In addition, IL-6 upregulates the expression of suppressor of cytokine signaling-1 (SOCS-1) for the inhibition of Th1 cell differentiation. STAT5 also play an important role in the Th2 lineage commitment, the cooperation of STAT5 and GATA3 is required for the complete Th2 cells differentiation. STAT5 activation

by IL-2 is independent of IL4 signaling, and it does not induce GATA3 expression. However, the IL-4/STAT6 pathway is not completely essential for Th2 cell differentiation as Th2 cell differentiation can also occur independently of the STAT6/IL-4 axis, and it probably require additional cytokines including IL-2, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP).

d. Th17 cells

T helper type 17 (Th17) cells play a critical role in eliminating extracellular pathogens, such as bacteria and fugi, by the recruitment of neutrophils and macrophages to infected sites. However, aberrant upregulation of Th17 cells and production of IL-17 are responsible for the pathogenesis of multiple inflammatory and autimmune disorders, including rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disorders. Upon TCR activation, naïve CD4+ T cells differentiate into Th17 cells in the presence of TGF-beta and IL-6 [24, 25]. These cytokines activate STAT3 to induce IL-21 expression, thereby establish early commitment to the Th17 lineage [26]. IL-21 signaling promotes the expression of IL-23R on cell surface, IL-17A, and the transcription factor, orphan receptor gamma-T (RORγt). RORγt is the master transcription factor for Th17 differentiation, and it directly promotes the TH17-lineage-specific cytokines, *Il17a* and *Il17f* [26, 27]. In addition, IL-22 is also one of hallmark cytokines secreted by Th17 cells. These cytokines stimulate the expression of pro-inflammatory mediators and anti-microbial peptides. IL-23, mainly produced by APCs, maintains STAT3-dependent expression of IL-23 R, ROR gamma t, and IL-17A, which stabilize the Th17 differentiation.

e. Tregs

Regulatory T (Treg) cells are a subpopulation of CD4+ T cells，and they are also known as suppressor of T cells. The primary function of regulatory T cells is to maintain self-tolerance and immune homeostasis by suppressing the activation, proliferation, and effector functions of immune cells that are involved in immune responses [28]. There are two origins of Tregs, one is derived from thymus with the expression of CD25 and the transcription factor FOXP3, which are usually called natural Tregs (nTregs). Another one, known as induced Treg cells (iTreg), are derived from the peripheral, which is arise from naïve CD4+CD25-FOXP3- conventional T cells after antigen priming in a specific cytokine milieu, such as TGF-β [29]. After clearance of pathogens, they negatively regulate the immune

response. Treg cells mediate their suppressive function through a variety of different mechanisms. One mechanism is mediated via IL-10, secreted by Treg cells, which directly or indirectly inhibit effector T cell responses and thus limits tissue damage by the excessive inflammatory responses [30]. Another mechanism is mediated via IL-35 and TGFβ that are secreted by Treg cells to induce naive CD4+ T cells to differentiate into Treg cells, thereby altering the ratio of Treg to other T helper cell subsets. In addition, CTLA-4, a cell surface molecule, participates in Treg cell-mediated suppression. CTLA-4 binds to CD80 and CD86 that are expressed on DCs, leading to downregulation of these molecules on the DC and blockage of the co-stimulatory signal transduction between CDs and T cells that are important for CD4+ T cell activation and differentiation [31].

1.2.2 T follicular helper cells

Production of high-affinity class-switched antibodies and memory B cells are essential for clearance of pathogens and vaccine efficacy. The antibodies produced can clear invading pathogens through neutralization, opsonization, and antibody dependent cell cytotoxicity. The T helper cell subset that is best capable of providing help to B cells remained controversial until the past decade. Several groups that studied human B cell activation found the preferential ability of CXC-chemokine receptor 5 (CXCR5) + T cells to help B cells to produce antibodies [32, 33]. CXCR5 is a chemokine receptor whose ligand is CXCL13 expressed in B cell follicles. B and T cells that express CXCR5 can migrate toward B cell follicles in response to CXCL13 [34, 35]. These CXCR5+ T cells were considered to be Follicular helper T (Tfh) cells. Gene-expression profiling of human and mouse Tfh cells showed that Tfh cells are very different from Th2 cells and other effector CD4+ T cell subsets, which neither express T-bet nor Gata-3 [36, 37]. The combination of these human and mouse gene-expression profile revealed a set of genes upregulated in Tfh cells, including B cell lymphoma 6 (Bcl6), Achaete-scute homologue-2 (ASCL2), IL-21, programmed cell death protein 1 (PD-1), and inducible costimulator (ICOS), which have subsequently been shown to be key molecules for regulating Tfh cell development, migration, homeostasis, and function. Thereafter, high expression of PD-1 and CXCR5 on T cells is considered to be a reliable way to identify Tfh cells [38, 39]. These results have increased our understanding of Tfh cell biology and pathology.

Upon exposure to a foreign antigen, DCs present antigen to naïve CD4+ T cells and initiate Tfh cell differentiation. Among DCs, both monocyte-derived DCs and conventional DCs were reported to initiate Tfh cell differentiation. In addition, Langerhans cells can effectively promote Tfh cell differentiation and germinal center (GC) formation in LNs upon exposure to cutaneous antigen or parasites such as *Leishmania* [40-42]. After antigen stimulation, naïve CD4 T cells differentiate into early Tfh cells, and then these cells accumulate at the T cell–B cell border, where they engage activated B cells. Subsequently, they migrate into B cell follicles and initiate GC reactions [43].A series of molecules contribute to T cell help to B cells. Adhesion molecules expressed by Tfh cells (or B cells) are necessary for cell contact, because co-stimulatory ligand interactions and directional cytokine production can take place between T cells and B cells during cognate interactions. For example, SAP expressed on Tfh cells binds to the intracellular domains of signaling lymphocyte activation molecule (SLAM) family surface receptors expressed on B cells, which facilitates cell–cell adhesion [44, 45]. CD40L is expressed on Tfh cell surface, and provides pro-mitotic signaling to B cells, which enables proliferation, differentiation, and isotype switching [46]. GC B cells are exquisitely pro-apoptotic [47]. Tfh cells produce IL-4 to trigger pro-survival signals in GC B cells, which function as anti-apoptosis [46]. Besides, IL-4 supports immunoglobulin class switching to IgG1 in mice. Tfh cells can also produce IFN-γ, which supports immunoglobulin class switching to IgG2a in mice [48, 49]. Somatic hypermutation is crucial for GC B cells for the affinity maturation of B cells. Somatic hypermutation undergo sequential rounds of immunoglobulin gene mutation and selection. Immunoglobulin gene mutation is achieved by DNA damage induced by the enzyme activation-induced cytidine deaminase (AID) and DNA repair performed by DNA repair enzymes [47]. The GC B cells with AID must express Bcl-6 to repress the DNA damage response program, otherwise it would trigger self-destruction of the cell [50]. CD40L, IL-4 and IL-21 produced by Tfh cells contribute to the signals that induce AID and Bcl-6 expression by B cells [51]. IL-21 efficiently induces IgM to IgG class-switch recombination, whereas IgE recombination is induced by a high IL-4 to IL-21 ratio [52, 53]. ICOS signaling is essential for T cell help to B cells. ICOS co-stimulatory receptor is essential for T-cell activation and function, and it is critical for CD40-mediated antibody class switching. ICOS is also required to maintain Tfh cells, within the GC, Tfh cells and B cells require Icos:Icos ligand for productive engagement [54-56].

1.2.2.1 Tfh cell differentiation

Tfh cell differentiation is a multistage, multifactorial process. The Tfh cell differentiation process begins with an initial DCs priming of a naive CD4+ T cell, which undergoes a cell-fate decision with acquisition of master transcription factor Bcl6 expression and chemokine receptor CXCR5 expressed on cell surface to become early Tfh cells. And then the early Tfh cells are able to migrate to the border of the B cell follicle and undergo further Tfh cell differentiation. Bcl6 is the master transcription factor for Tfh cell differentiation. Upon CD28 activation and STAT1 or STAT3, CD4+ T cells upregulate Bcl6 expression [57]. Overexpression of Bcl6 leads to upregulation of various Tfh cell effector molecules, such as PD-1, CXCR5, CXCR4, and SAP [58-60]. In addition, Bcl6 also represses promoters and enhancers of Th1 (IFNGR1, TBX21, and STAT4), Th2 (i.e., GATA3), or Th17 (i.e., RORA, IL-17A–F) differentiation [45, 58, 61, 62]. Moreover, Bcl6 represses B lymphocyte-induced maturation protein 1 (BLIMP1), which directs differentiation away from the Tfh pathway [45]. DCs present antigen to the TCR of a naïve CD4+ T cell to activate TCR signaling. TCR signal strength can bias T cell differentiation *in vivo*. A single naïve CD4+ T cell can give rise to multiple different effector T helper subsets upon stimulation with the same pathogen, suggesting that non-TCR signals also participate in Tfh cell differentiation. Non-TCR signals, such as IL-6, inducible ICOS and IL-2, have been reported to involve in Tfh cell differentiation. There is no single event that defines Tfh cell differentiation. Non-TCR and TCR signals have to cooperate together to determine T cell differentiation fates.
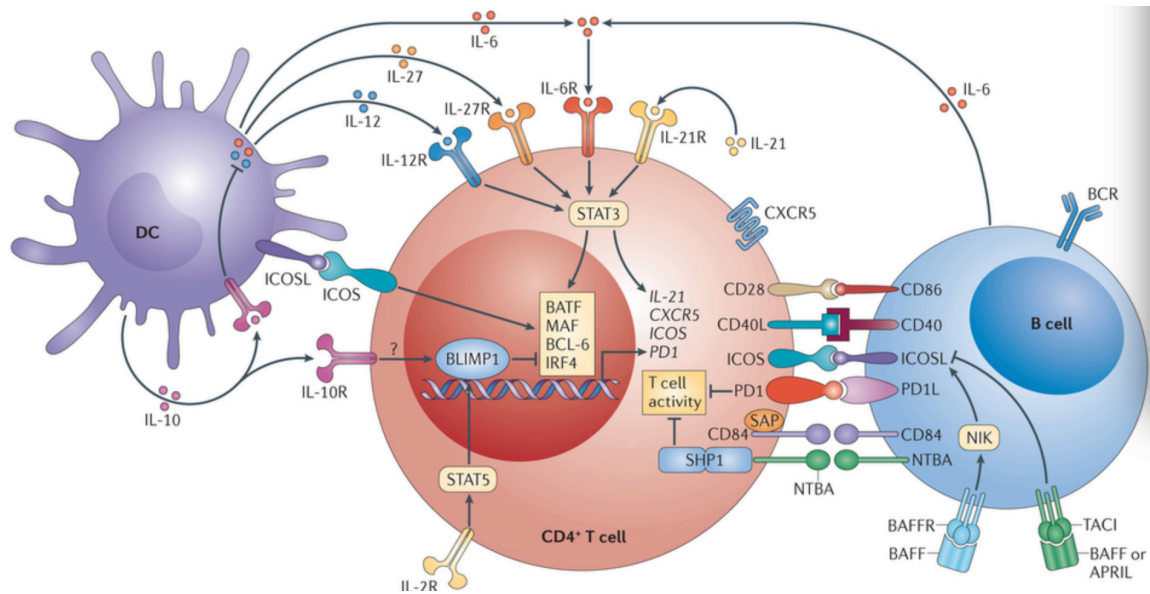
Figure 1. Overview of Tfh Cell Differentiation. Naive CD4+ T cells interact with antigen-presenting DCs in the interfollicular or T cell zones; DC-primed CD4+ T cells acquire expression of CXCR5 and Bcl6 to become early Tfh cells. These cells then migrate to the T cell–B cell border and — following interactions with cognate B cells — differentiate into GC Tfh cells. This differentiation process is governed by signals provided by STAT3-activating cytokines, including IL-6, IL-12, IL-21 and IL-27. These cytokines are secreted by DCs (which produce IL-6, IL-12 and IL-27), B cells (which produce IL-6 and possibly IL-27) and CD4+ T cells (which produce IL-21). Cytokine-mediated activation of STAT1 might also contribute to this process (not shown). These cytokines operate individually or collectively to induce or enhance expression of the transcription factors BCL-6, MAF, BATF and IRF4, which then imprint the Tfh cell fate on a T cell by inducing the transcription of signature genes, including CXCR5, ICOS, IL21 and PD-1. Cell–cell interactions among activated CD4$^+$ T cells, antigen-presenting DCs and B cells also promote Tfh cell formation. CD28–CD86, CD40 ligand (CD40L)–CD40 and ICOS–ICOS ligand (ICOSL) interactions are central to this process. Notably, ICOSL expression on B cells is controlled through the opposing effects of BAFF signaling via the distinct receptors BAFFR and TACI. In addition, SLAM family receptors have a dual role in Tfh cell generation: the recruitment of SAP to these receptors facilitates and maintains conjugate formation between T and B cells, whereas recruitment of inhibitory phosphatases (such as SHP1) suppresses these interactions, thereby influencing the ability of CD4+ T cells to form Tfh cells and to support B cell responses. Tfh cell generation is restricted by BLIMP1, which is induced by IL-2 in a STAT5-dependent manner; BLIMP1 functions by repressing the expression of Bcl6. IL-10 also suppresses Tfh cell formation, but it is unknown whether this is also mediated via BLIMP1. APRIL, a proliferation-inducing ligand; BCR, B cell receptor; IL-10R, IL-10 receptor; NIK, NF-κB-inducing kinase; NTBA, natural killer, T and B cell antigen (also known as LY108 in mice); PDL1, PD1 ligand 1. **[taken from (Elissa K. Deenick, et al. 20013)].**

a. T cell receptor (TCR) signal strength

Following stimulation via the TCR, naïve CD4 T cells selectively differentiate into different T helper subsets. The mechanisms controlling the induction of this differentiation program are less clear, however, the TCR signal strength have been implicated in Tfh cell differentiation. TCR signal strength has been reported to regulate Tfh cell differentiation. Cumulative TCR receptor signal strength is affected by both antigen affinity and antigen dose. It was reported that a small dose of antigen induces Th2 or Tfh cells differentiation, and an intermediate dose of antigen induces Th1 cells [63-65]. It has been reported that high affinity of Ag favors Tfh cell differentiation [66]. Similarly, Deenick and colleagues found that boosting ovalbumin-immunized mice with the immunodominant peptide resulted in an increased generation of Tfh cells [67]. High affinity TCR signaling has been reported to promote more stable T-cell contacts with APCs compared with low affinity TCR signals [68]. However, contradictory results were found in a Friend virus infection model [69]. Tubo et al found that TCR: major histocompatibility complex-II (MHCII) dwell time is a more accurate parameter for prediction of cell-fate preference. However, the relationship between TCR: MHCII dwell time and cell-fate preference is not correlated in a linear manner [65]. Collectively, it appears that there is no simple relationship between TCR signal strength and Tfh cell differentiation.

b. Dendritic Cells

Antigen presenting cells are essential for priming naïve CD4+ T cells to initiate CD4+ T helper subsets differentiation. DCs and B cells are two most important antigen-presenting cells that cooperate to induce Th cell differentiation and GC formation. It was reported that antigen presentation by B cells is not necessarily required for Tfh cell differentiation, as prolonged peptide presentation by DCs is able to induced Tfh cell differentiation in the absence of B cells [67]. It was also shown that B cells alone neither prime naive T cells nor induce Tfh cell differentiation efficiently [70]. Therefore, effective Tfh cell development and GC responses may require sequential antigen presentation by DCs and then B cells [70]. In mouse immunized with protein antigen coupled with adjuvant CpG, both monocyte-derived DCs and conventional DCs have been reported to initiate Tfh cell differentiation. However, the efficiency of induction of Tfh cell differentiation are different between these two populations [71]. Monocyte-derived DCs promotes Tfh cell differentiation more effectively

than conventional DCs [71]. In skin, Langerhans cells were shown to present *Leishmani*-derived cutaneous antigens or *Leishmania major* parasites and effectively initiate Tfh cell differentiation and GC formation [72]. There in on other skin-resident DCs has been reported to present cutaneous antigens to initiate Tfh cell differentiation.

The receptor/ligand interactions between CD4+ T cells and DCs have been implicated in Tfh cell differentiation. OX40 signaling in CD4+ T cells can upregulate CXCR5 expression, which leads to the migration of activated CD4+ T cells to the border of the T-zone and the B-cell follicle in the secondary lymphoid organs [73-76]. However, little is known about how OX40L upregulates CXCR5 expression. OX40L is the ligand of OX40, and DCs is one of cellular sources of OX40L. Over-expression of OX40L on CD11c+ DCs in a transgenic mouse line leads to the migration of activated CD4+ T cells from T cell zone to B cell follicle, highlighting the importance of OX40L/OX40 interactions between CD4+ T cells and DCs in the Tfh cell differentiation. B7.1 (CD80), B7.2 (CD86) and CD40 that are expressed on the cell surface of DCs participate in the initial activation of CD4+ T cells. B7.1 (CD80) and B7.2 (CD86) expressed on the cell surface of DCs binds to CD28 expressed on the cell surface of CD4 T cells provide an important co-stimulatory signal for the activation of CD4+ T cells [77]. CD40 is expressed on the cell surface of DCs, and its ligand, CD40L, is expressed on T cells. T cell activates DCs via CD40, which facilitate the ability for DCs to support ongoing T cell activation [78].

In addition to receptor/ligand interactions, DCs secrete cytokines that promote Tfh cell differentiation. IL-6 is the earliest non-TCR signal that initiates Tfh cell differentiation. Bcl6 is the master transcription factor essential for Tfh cell differentiation. It has been reported that IL-6 signaling transiently induces Bcl6 expression by newly activated CD4+ T cells [61]. In addition, IL-6 is a potent inducer of IL-21 expression by murine Tfh cells [79]. Many DC subsets are robust cellular sources of IL-6.

c. Transcription factors involve in early Tfh cell differentiation

The commitment of naïve CD4+ T cells into the Tfh cell differentiation depends on the expression of transcription factor. Bcl6 has been identified as a master transcription factor essential and specific for Tfh cell differentiation [45, 61, 80]. After Bcl6 has been identified as the transcription factor required for Tfh cell differentiation, an increasing list of

transcriptional factors have been identified to participate in different aspects of Tfh cell development, migration, and function. Among these transcription factors, ASCL2, Bcl6, and c-MAF regulate most of the key targets required for Tfh cell differentiation. Bcl6 is upregulated upon CD28 activation and IL-6 via activation of STAT1 [57, 81]. The transcription factor, Basic leucine zipper transcription factor ATF-like (BATF), has also been reported to promote Bcl6 expression in Tfh cells [82]. Early growth response gene 2 (EGR2) and EGR3 have also been reported to directly induce the expression of Bcl6 in Tfh cells [83]. The transcription factor Batf has been found to bind to the Bcl6 locus to activate Bcl6 expression. BLIMP1 is a transcriptional repressor for Tfh cell differentiation, and BCL6 represses BLIMP1 to facilitate Tfh cell differentiation. In addition, Bcl6 expression results in upregulation of various molecules that foster Tfh phenotype, including PD-1, CXCR5, CXCR4, and SAP [59, 60, 80]. To maintain the Tfh cell differentiation, Bcl6 represses the promoters and enhancers of IFNGR1, TBX21, STAT4, GATA3, RORA, which contain Th1, Th2 and Th17 differentiation [59, 62, 84, 85]. ASCL2 is a basic helix-loop-helix transcription factor, and it has been reported to direct promote early Tfh cell differentiation. ASCL2 upregulates CXCR5 expression and downregulates PSGL1 and CCR7 expression, which promote the migration of Tfh toward B cell follicles [86]. In addition, ASCL2 also inhibits expression of genes that are involve in Th1 and Th2 differentiation, such as *Ifng*, *Tbx21*, *Il2*, and *Rorc* [86]. TCF-1 and LEF-1 play important roles in early Tfh cell differentiation. TCF-1 and LEF-1 can induce Bcl6 expression, and they can also enhance IL-6R and ICOS expression on Tfh cells and suppresses IL-2R expression on Tfh cells, thus coordinate Tfh cell differentiation and the formation of GCs [87, 88].

d. T:B interaction at the border of T cell zone and B cell follicle

After T cell priming by DCs, Tfh cell precursors acquire early Tfh cell phenotype, but they have to receive persistent antigen stimulation to become fully differentiated Tfh cells and maintain Tfh cell phenotype. Early Tfh cells acquired expression of CXCR5 and sphingosine-1-phosphate receptor-2 (S1PR2) on the cell surface downregulation of C-C chemokine receptor type 7 (CCR7), which facilitate migration toward B cell follicles [89, 90]. At this stage, antigen-specific B cells experience clonal expansion and accumulate in the T-B junction, and these B cells present cognate antigens to early Tfh cells for further differentiation [91]. Antigen presentation is extremely critical for the maintenance CD4+ T

cells differentiation, as every cell division of antigen-specific CD4 T cells require antigen recognition. Because DCs last for only a few days before dying during an acute infection or immunization in secondary lymphoid organs, therefore antigen-specific B cells are the primary APCs available. During this process, Tfh cells continue to upregulate the expression of CXCR5, PD-1, and ICOS on the cell surface, which enable them to differentiate into GC Tfh cells. Antigen-specific B cells not only serve as antigen-presenting cells, but also serve as a source of ICOS ligand (ICOSL). ICOSL-ICOS interaction has roles in both Tfh cell differentiation and migration, it has been reported that ICOS receptor instructs Tfh cell differentiation via induction of Bcl6 expression [60]. Further more, activated B cells express CD80, CD86, and other costimulatory ligands to support the development of GC Tfh cells [92-94]. Tfh cells provide help to GC B cells are also in the form of cytokine signals. Tfh cells are able to produce IL-21, IL-4 and IFN-γ in response to different stimuli. *In vivo*, Tfh cell-derived IL-21 can maintain Bcl6 expression in GC B cells, which controls the maintenance and optimal affinity maturation of the GC response [91, 95]. Th2-type stimuli induce a population of Tfh cells in which IL-4 is a dominant cytokine. While Th1-type stimuli induce Tfh cells that express IFN-γ as a dominant cytokine [46]. IL-4 and IFN-γ promote class switching to IgG1 or IgG2a in mice, respectively [48, 49].

e. GC formation

After the cognate T:B interaction, the T:B conjugates subsequently migrate into B cell follicles and initiate GC reactions. The GC is a distinct structure that is comprised of GC Tfh cells, GC B cells, follicular dendritic cells (FDCs), macrophages and stroma cells. The majority of GC Tfh cells are $CXCR5^{hi}PD1^{hi}Bcl6^{hi}Maf^{hi}SAP^{hi}$ [46]. GC Tfh cells also have very high expression of C-X-C chemokine receptor type 4 (CXCR4), and very low expression of CCR7, sphin-gosine 1-phosphate 1 receptor (S1P1R) and PSGL1. GC Tfh cells lose the expression of Epstein-Barr virus-induced G protein coupled receptor 2 (EBI2). Loss of EBI2 expression by both GC B cells and GC Tfh cells facilitate the proper localization to GCs [96]. Besides, adhesion molecules expressed on both GC Tfh cells and GC B cells regulate their interaction localization. SLAM-associated protein (SAP), an SH2-domain adaptor protein, is specifically upregulated in GC Tfh cells. SAP expression is essential for GC Tfh cell development and GC development [97]. The SLAM family receptors SLAMF6 is expressed on GC Tfh cells and GC B cells. SLAM-SLAMR6 signaling

limits T:B adhesion. SAP binds to the cytoplasmic tails of SLAM family receptors, which prevent signaling through SLAMF6, thus enabling the formation of stable T:B conjugates [98, 99].

The T:B conjugates migrate from the interfollicular region into the center of the follicle. Migrating antigen-specific B cells begin to proliferate and push the resident follicular B cells aside to form the early GC, which consists of B cell blasts surrounded by the mantle zone. Thereafter GC expands rapidly as a result of the fast proliferation of the B cell blasts and form dark zones and light zones. The dark zone mainly consists of densely packed B cell blasts, whereas the light zone contains TFH cells and FDCs. Inside the GC, GC B cells first proliferate in the dark zone, and then migrate to the light zone. At the light zone, FDCs deliver antigen to GC B cells, and GC B cells present this antigen to Tfh cells subsequently. GC Tfh cells provide CD40 ligation and cytokine signals to GC B cells to promote GC B cell development. CD40L is expressed on GC Tfh cells, and CD40L-CD40 interaction provides survival signal to GC B cells and promote GC B cell differentiation into memory B cells [100, 101]. In addition, CD40L-CD40 interaction also maintains GC responses and promotes GC B cell differentiation into high-affinity plasma cells, and memory B cells [102, 103].

In addition, Tfh cells also produce cytokines to promote activated B cells differentiate into GC B cells. IL-4 is a dominant cytokine produced by Tfh cells, and IL-4 is dispensable for GC B cell development, immunoglobulin class switching, and the initiation of follicular and extrafollicular antibody responses [104]. IL-21 is also a B cell helper cytokine produced by GC Tfh cells [105-107]. IL-21 can also enhance CD40L-induced GC B cell proliferation [107, 108] and maintain Bcl6 expression in GC B cells to induce optimal affinity maturation of the GC response [108, 109]. Tfh cells are also able to produce IFN-γ in Th1-type stimuli for the support of class switching in mice to IgG2a [49, 110-113].

In the light zone, GC B cells that express high-affinity antigen receptors are positively selected. A small proportion of GC B cells upregulate c-myc expression after receiving help from Tfh cells, and then they return to the dark zone subsequently to undergo further cell division and diversification of immunoglobulin variable region (IgV) genes by somatic hypermutation (SHM), resulting in the generation of mutant clones that have a broad range of affinities for the antigen [111-113]. At the same time, these B cells provide ICOSL to enhance the productive GC Tfh:B cell conjugation, inducing calcium signaling in Tfh cells.

These calcium signals promote the production of IL-4, IL-21 and CD40L, which further promote B undergo immunoglobulin isotype class-switching and antibody affinity maturation [114, 115]. These events facilitate GCs to generate long-lived antibody-secreting plasma cells and long-lived resting memory B cells. So far, it is unclear which signals drive GC B cell differentiation into memory B cells or plasma cells.

1.2. 3. B cells

B cells are a critical component of the adaptive immune response. B cells express B cell receptors (BCRs) on their cell membrane. BCRs allow the B cell to bind a specific antigen, which induces antibody response thereafter to suppress the invading pathogens. B cell development is initiated in the fetal liver before birth and then is initiated from hematopoietic stem cells in the bone marrow (BM) in adults [116-118]. But the functional maturation takes place in secondary lymphoid tissue with the acquisition of multiple cell surface markers expression, such as CD19, CD45R, and surface IgM. B cells have to exit the bone marrow and migrate to the spleen for further maturation to acquire surface expression of markers including CD21, CD23, CD24 and AA4, which eventually reach final maturation stage as "follicular" B cells.

B cells are produced continuously throughout life, initially arising from the fetal liver, as all eukaryotic cells, B cell lineage commitment is based on transcription factor competition and cross-regulation[119, 120]. Accordingly, acquiring B cell identity involves both the onset of B cell transcriptional program and the loss of other immune cell potentials [120].

B cell lineage commitment begins with gene rearrangements at the immunoglobulin (Ig) heavy and light chain loci and the initiation of the expression of several "master" transcription factors, such as Pax5 [121, 122]. Thereafter, these cells become common lymphoid progenitors. As long as common lymphoid progenitors commit to the B lineage, the transcription factor E2A activates EBF and Pax5, which in turn activates a cascade of B cell-specific genes to promote further B cell development. B cells can be divided into B-1 cells and B-2 cells. It is still unclear whether B-1 cells and B-2 cells origin from the same common progenitor [123-125]. Murine B-1 cells origin primarily from the fetal liver and undergo self-renewal to sustain the population in the periphery [124-126]. B-2 cells are

derived from the bone marrow. Antigen recognition by BCR leads to cross-linking of BCR, which initiate a signaling cascade that enables B cell effector activity. With the help provided by Tfh cells, activated B cell develop into GC B cells, which eventually leads to the development of antibody-secreting plasma cells and the generation of memory B cells and in secondary lymphoid organs.

2. Atopic dermatitis

Atopic dermatitis (AD) is the most common, chronic and relapsing inflammatory skin disease that affects mainly children with a characteristic phenotype and typically distributed skin lesion. The cumulative prevalence of AD has been increasing, affecting 30% of children all over the world. A few dry eczematous patches and large areas of erythematous rash present in the AD skin. The AD skin has a disruption of epidermal-barrier function, which result in dry skin and exposure to common environmental allergens such as pollen, house dust mites, and food allergens. Upon exposure to environmental allergens, the immune infiltrates in the AD skin produce IgE antibodies in response to environmental allergens, which result in a chronic skin inflammation with the recruitment of lymphocytes, monocyte macrophages, dendritic cells, and eosinophils to the dermis.

The mechanisms underlying the pathogenesis of AD are not clear. Two hypotheses regarding the mechanism of atopic dermatitis have been proposed. One hypothesis proposes that aberrant IgE-mediated skin immune response causes epithelial-barrier dysfunction, which result in exposure to environmental allergens and induction of sustained immune responses to environmental allergens. The other holds that there is an intrinsic skin barrier defect that causes aberrant immune responses to invading environmental allergens subsequently.

Figure 2. Clinical, Histologic, and Immunohistochemical Aspects of Atopic Dermatitis. Panel A shows initial lesions of early-onset atopic dermatitis involving the cheek and scalp in an infant at 4 months of age. Panel B shows classic head and neck manifestations of atopic dermatitis in an adult. Panel C shows typical chronic, lichenified flexural lesions in adult. The arrow in Panel D (hematoxylin and eosin), which shows the typical histologic aspects of acute lesions, indicates a spongiotic area within the epidermis. The asterisk indicates the prominent perivascular infiltrate. Panel E (hematoxylin and eosin) shows a chronic lesion with thickening of the epidermis. The asterisk indicates the prominent perivascular in infiltrate. [**taken from (Bieber et al. 2008)**].

The previous studies in my lab have established a central role of cytokineTSLP, which is expressed by epidermal keratinocytes (KCs), in promoting Th2 cell response and driving the pathogenesis AD [127, 128]. We found that induction of TSLP expression in KCs result in generating an atopic dermatitis upon application of the active Vitamin D3 analogue MC903 on Mouse Skin, and TSLP act as a master switch for Th2 inflammation.

2.1 Epidemiology

The prevalence of atopic dermatitis has been increasing during the past decades, affecting about one-fifth of all individuals. Atopic dermatitis frequently starts in early infancy. Around 50% of all cases of atopic dermatitis develop symptoms within their first year of life, and probably 85% experience an onset before 5 years of age [129]. About 70% with childhood onset of AD recover spontaneously before becoming adolescence, whereas the remaining 30% cases continue to have AD onset into adulthood or experience a relapse of AD onset after several years of recovery [70].

About 50–70% of children with early-onset of atopic dermatitis have the skin allergic to one or more allergens, such as pollen, house dust mites, and food allergens, however, the children with late-onset of atopic dermatitis are sensitized to less allergens [130]. AD patients with moderate to severe onset of atopic dermatitis tend to develop asthma and even hay fever [131].

2.2 Clinical manifestation

The manifestation of skin lesion in atopic dermatitis looks similar to other eczemas, such as contact eczema. The clinical appearance of atopic dermatitis displays a large variation in the morphology and distribution of the eczema. Patients with AD often display dry and scaly skin. The skin lesion of many patients with atopic dermatitis present with dry skin resulted from the low water content or an excessive water loss through the epidermis. In some patients, the skin lesion is very pruritic, red, and eczematous plaques can be observed on the cheeks and extensor extremities. Scratching could cause skin thickening and darkening, which usually leads to further complications, including bacterial infection. AD patients at different ages tend to have the rash on certain parts of the body. The skin lesion of infants with AD is usually at scalp, face, the front of the knees, and the back of the elbows. The lesions are often present with erythema, papules, vesicles, excoriations, oozing, and formation of crusts. The skin lesion of children with AD is usually at the neck, wrists, legs, ankles, and the creases of elbows or knees. The eczema of these patients becomes drier and lichenified with excoriations, papules, and nodules. In adults, the skin lesion often appears in the creases of the elbows or knees and on the nape of the neck [132].

2.3 Physiopathology

Atopic dermatitis is an IgE-mediated hypersensitivity response to allergens. The pathogenesis of AD is multifactorial process and involves a complex immunologic cascade, such as disruption of the epidermal barrier, IgE dysregulation and defects in the cutaneous cell-mediated immune response. Currently, two hypotheses have been proposed to address the induction of skin lesions in patients with atopic dermatitis. One hypothesis suggests that the disruption of homeostasis of the immune system is responsible for the induction of atopic dermatitis. The disruption of homeostasis of the immune system is due to inappropriate immune responses to antigens encountered in the skin. The other hypothesis suggested skin barrier defect triggers the Atopic dermatitis.

The idea of disruption of homeostasis of the immune system claims that atopic dermatitis results from an immunological disorder within the skin. The disruption of homeostasis of the adaptive immune system induces cutaneous inflammation characterized by sequential and progressive patterns of inflammatory cell infiltration, particularly by CD4+ cells. Infiltrating CD4+ T cells then secrete the skin-homing adhesion molecules for the recruitment of dendritic cells, monocytes, macrophages, mast cells, Th1, Th17 and regulatory T cells into cutaneous sites. The interaction of these cells ultimately leads to Th2 dominated immune response with a massive production of interleukin IL-4, IL-5, IL-10, and IL-13 in the skin. These interleukins lead to an increased production of IgE and peripheral eosinophilia [133]. In atopic dermatitis, Langerhans cells and inflammatory dendritic cells within the epidermis present antigens to T cells, and these cells express a trimeric high-affinity receptor for IgE on the cell suface [134]. IgE captures the invading allergens and then bind to Langerhans cells and inflammatory dendritic cells, thus these cells can take up allergens more efficiently. Usually, these IgE bound allergens cause immediate-type allergic reactions. Delivery of these antigens to Langerhans cells and inflammatory dendritic cells can induce T-cell-mediated reactions of the delayed type hypersensitivity response [133]. The theory of skin barrier defects is proposed based on the fact that mutation of genes that encode structural proteins in the stratum corneum and stratum granulosum often leads to skin barrier dysfunction and transepidermal water loss, which causes eczema subsequently. Impaired skin barrier will result in dry skin and leads to increased penetration of allergens into the skin, resulting in allergic inflammation [135].
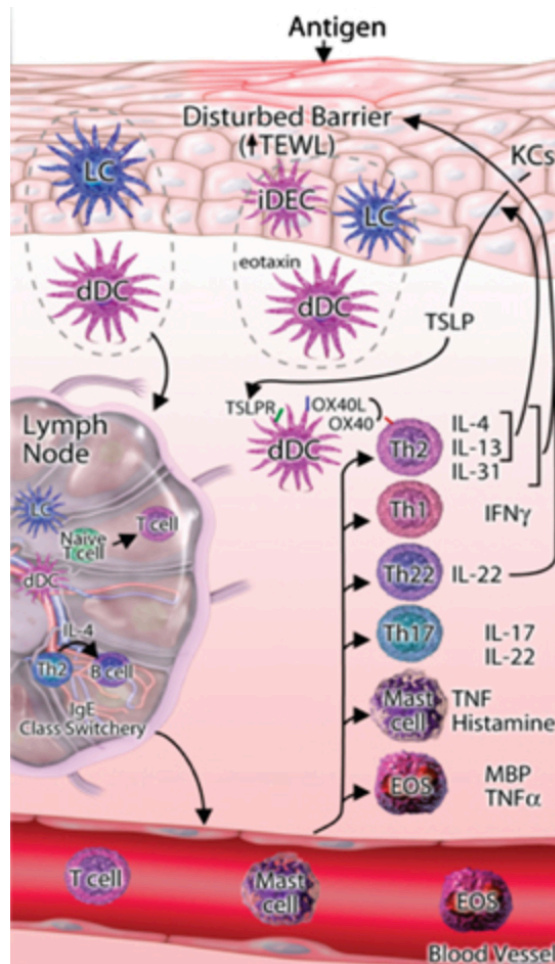
Figure 3. Immune mechanism in the pathogenesis of atopic dermatitis (AD). In patients with AD, a disturbed epidermal barrier leads to increased permeation of antigens, which encounter LCs, iDECs), and dDCs, activating T helper type 2 (Th2) T cells to produce IL-4 and IL-13. DCs then travel to lymph nodes, where they activate effector T cells and induce IgE class switching. IL-4 and IL-13 stimulate KCs to produce TSLP. TSLP activates OX40 ligand–expressing dDCs to induce inflammatory Th2 T cells. Cytokines and chemokines, such as IL-4, IL-5, IL-13, eotaxins, CCL17, CCL18, and CCL22, produced by Th2 T cells and DCs stimulate skin infiltration by DCs, mast cells, and eosinophils. Th2 and Th22 T cells predominate in patients with AD, but Th1 and Th17 T cells also contribute to its pathogenesis. The Th2 and Th22 cytokines (IL-4/IL-13 and IL-22, respectively) were shown to inhibit terminal differentiation and contribute to the barrier defect in patients with AD. Thus, both the barrier defects and immune activation alter the threshold for ICD, ACD, and self-reactivity in patients with AD. TEWL, transepidermal water loss. [**taken from (Gittler et al. 2013)**].

2.4 Pathogenesis

The pathogenesis of AD is not completely understood, it is believed that the disorder seems to result from the complex interaction among skin barrier defects, immune abnormalities and environmental risky factors.

2.4.1 Genetics

Genetic defect has been proved to be an intrinsic risky factor underlying the pathogenesis of AD. Using genetic linkage analysis combined with association studies, several genes linked to either epidermal function or the immune system have been identified. Palmer et al identified two filaggrin (FLG) mutations, R501X and 2282del4, which are associated with the pathogenesis of AD [136]. Filaggrin plays a crucial role in skin barrier integrity. It is an important epidermal structural protein that is essential for skin barrier formation. 20 mutations in the FLG gene have been found so far, and around 50% of AD patients carry mutations in the this gene. SPINK5 and LEKT1 are also skin-related genes, and they have also been identified as genetic factors that associated with the pathogenesis of AD [137, 138]. These skin barrier abnormalities results in transepidermal water loss (passage of water from inside the body through the epidermal layer of the skin to the surrounding atmosphere) and increased penetration of allergens and microbes into the skin.

2.4.2 Environmental factors

In addition to genetic factors, environmental factors are also considered to be the main drivers of AD. Environmental risk factors that contribute to AD are usually climate, diet, obesity, infectious agents, tobacco smoke and pollution. Microbial exposure has increased the susceptibility to AD. Antibiotics are also found to link to an increasing risk of AD [139-141]. Of note, the skin microbiota has been found to play an important role in the homeostasis of skin immune system and the development of AD [142].

2.5. Thymic stromal lymphopoietin (TSLP)

AD is the major risk factor for developing other atopic diseases. Children with AD tend to develop allergic asthma later in life, as well as allergic rhinitis, which is usually referred to

atopic march. TSLP is thought to be a potential factor linking to the atopic march. In epidermis TSLP overexpression mouse models, aggravated allergic responses in the lung induced by OVA was observed, in which elevated circulating TSLP plays a significant role in the process of this phenotypic outcomes [143-145].

TSLP is a type I cytokine belonging to IL-2 family, which is involved in the initiation, development and progression of atopic dermatitis both in mice and in human. The epidermis of lesional skin in AD patients has higher TSLP expression than that of epidermis in non-lesional skin or skin from patients with non-allergic dermatitis [146]. TSLP has also been found to induce Th2-type skin inflammation in mouse models. The previous work in our laboratory, by employing mouse genetic approaches, particularly the targeted conditional somatic mutagenesis in a given cell type and at a given time, has markedly contributed to the research field of atopic diseases, particularly in revealing the crucial role of an skin-derived cytokine TSLP in atopic pathogenesis. We have found that keratinocytic TSLP, which was found over-expressed in skin lesions of AD patients, not only plays a key role in triggering the Th2-type AD inflammation, but also represents important factor in linking AD to asthma. Inducible expression of TSLP in the skin epidermal keratinocyte drives development of a skin inflammatory disease resembling human AD [127, 128, 147, 148]. Increased TSLP production in the epidermis induces the onset of Th2 cytokine–associated inflammation including dermal infiltrates containing lymphocytes, mast cells, eosinophils and elevated circulating levels of IgE. These studies have established the role of TSLP secreted by epidermal keratinocytes as a key cytokine that trigger the Th2 cytokine associated inflammation in AD. Besides AD and asthma, our previous study reveal the role of TSLP in atopic march. In this study, keratinocytic TSLP, the expression of which is induced by skin barrier impairment, is essential for generating AD-like skin inflammation and triggering a subsequent allergic asthma [149, 150]. Here I give an overview of TSLP on the regulation of immune response.

2.5.1 Expression

TSLP is produced predominantly by epithelial cells that reside in the barrier surface of skin, lung and gut [146]. Epithelial cells at barrier surfaces of the skin, lung and gut express TSLP in response to allergen challenge, virus infection, microbe infection, parasite infection,

ligands of pattern recognition receptors and ligands of nuclear receptors. Bronchial EC line of human and mice can produce TSLP in response to protease allergens. Aeroallergen house dust mites (HDM) can induce TSLP expression in lung ECs and in DCs [151-153]. Human airway epithelial cell (ECs) are able to produce TSLP upon stimulation with virus such as rhinovirus RV16 and vesicular stomatitis virus (VSV) [154-156]. Microbes have also been reported to promote TSLP expression in epithelium cells. Gut microbiota containing a variety of commensal bacteria promote TSLP gene expression in gut epithelium cells [157, 158]. Parasite infection can result in TSLP expression. The intestine of mice infected with intestinal parasite *Trichuris muris* or *Trichinella spiralis* displayed an upregulation of TSLP expression [159-161]. TLR ligands have been reported to promote TSLP expression in primary human airway ECs [162, 163]. The vitamin D receptor (VDR) is a member of the nuclear receptor family of transcription factors. The ligand for VDR, (1, 25-dihydroxyvitamin D3), or its low-calcemic analog MC903 can promote mouse skin KCs to produce TSLP [164]. Other cells, such as smooth muscle cells, lung fibroblasts and mast cells, also produce TSLP. The expression of the cytokine is similar between humans and mice.

2.5.2 TSLP Receptor

The functional, high affinity TSLP receptor complex is composed of TSLPR receptor and interleukin 7 receptor-α (IL-7Rα). The human TSLPR and mouse TSLPR are quite different at the protein sequence level (~40% homology), but they share certain functional similarities. In mouse, TSLPR signaling cascade activates the STAT5 transcription factor without the involvement of JAK kinases [165]. In contrast, humans TSLPR signal cascade activates STAT 1,3,4,5, and 6, as well as a robust and sustained activation of JAK-1 and JAK-2 [166-168]. In addition, TSLP is also reported to activate Src and Tec families of non-receptor tyrosine kinases of the TSLP receptor complex signaling cascade [169]. TSLP receptor complex is expressed broadly on a wide variety of hematopoietic lineage cell populations, such as DCs, monocyte, macrophages, B cell progenitors, activated T ells, basophils, and eosinophils [170, 171].

2.5.3 TSLP and immune response

TSLP is originally characterized by its ability to activate B cells and DCs. Subsequently TSLP was found to promote Th2 cell responses associated with the pathogenesis of many inflammatory diseases, including atopic dermatitis and asthma. TSLP can induce Th2 cytokine–associated inflammation by direct or indirect activation of the effector function of Th2 cells, basophils, eosinophils and other granulocyte populations, as well as promoting regulatory T cell responses in peripheral tissues. TSLP can induce phenotypic changes in human DCs, including the upregulation of major histocompatibility complex class II and costimulatory molecules, such as CD86, CD40 and OX40L on the cell surface, and the production of a variety of chemokines [170, 172]. TSLP-stimulated human CD11c+ DCs activated naïve CD4+ T cells to differentiate into Th2 cells via OX40-OX40L interactions, and these Th2 cells acquired the ability to produce pro-inflammatory cytokines IL-4, IL-5, IL-13, and TNF-α to promote Th2 cytokine–associated inflammation [172]. Besides influencing the differentiation of Th2 differentiation, TSLP can also activate basophils, mast cells and NKT cells, which result in increased cytokine production [173, 174].

TSLP plays an important role in the maintenance of peripheral CD4+ T cell homeostasis. At the human peripheral mucosa lymphoid tissues, epithelial cells express TSLP, which play a critical role in DC-mediated proliferation of naïve and memory T cells. TSLP-activated mDCs induced a robust and sustained expansion of autologous naive CD4+ T cells in the absence of any exogenous antigens, cytokines. The expansion of the autologous naive CD4+ T cells is dependent on MHC class II and costimulatory CD80 and CD86 signaling and is a polyclonal expansion with central memory T cell phenotype (CD45R0$^+$CCR7$^+$CD27$^+$ CD62L$^+$), which have the potential to further expand and differentiate into either Th1 or Th2 effector cells [175]. A similar role for TSLP in the maintenance of peripheral CD4+ T cell homeostasis is also found in murine [176].

TSLP upregulates the surface expression of MHC class II, CD54, CD80, CD83, CD86, and DC-lamp on human mDCs and promote human mDCs to produce large amounts of t IL-8, eotaxin-2, TARC and MDC, but not Th1-polarizing cytokine, such as IL-12, IL-23, IL-27 and type I IFNs. These cytokines can attract neutrophil, eosinophils and Th2 cells, creating a Th2-permissive microenvironment [177]. Naïve allogeneic CD4+ T cells stimulated with

TSLP-DCs produce the classical Th2 cytokines (IL-4, IL-5 and IL-13) and large amounts of TNF, which mediates the type 2 inflammatory responses. Besides inducing the production of Th2 cytokines and TNF, CD4+ T cells produce very low amount of IL-10 and IFN-γ that counteract Th2 inflammation upon stimulation with TSLP-DCs, which creates a microenvironment that favors Th2 responses [178, 179]. The production of Th2 cytokines and inhibition of IL-10 and IFN-γ in CD4 T cells was induced by signals triggered by OX40L. TSLP triggers human mDCs to express the OX40L at both mRNA and protein levels. OX40L expressed on human mDCs is important for the Th2 cell differentiation, as the production of Th2 cytokines and TNF was inhibited and the production of IL-10 was enhanced in CD4+ T cells when OX40L was blocked using a neutralizing antibody. In addition, naive CD4+ T cells treated with recombinant OX40L produced TNF, but not IL-10 [177]. Signals triggered by OX40L induced the generation of Th2 cells. OX40 signaling directly induces NFATc1 in naïve CD4+ T cells. NFATc1 in turn triggers IL-4 production and then triggers IL-4-dependent GATA-3 transcription, which induce Th2 lineage commitment. In both human and mouse, TSLP can stimulate DCs to generate Th2 cells. More direct evidence that TSLP plays a role in Th2 differentiation comes from both *in vitro* culture systems and transgenic mice. Bone marrow-derived murine DCs treated with TSLP produce CCL17 and upregulate co-stimulatory molecules on the cell surface, with the ability to prime naive T cells to produce IL-4.

There are two pathways for TSLP-driven Th2 differentiation in the mouse, one is via DCs as in human, and the other one is via direct activation of naïve CD4+ T cells to induce Th2 differentiation of naive CD4 T cells. Naive CD4 T cells express both chains of the TSLPR and IL-7R. Therefore TSLP can also directly act on CD4+ T cells. In an *in vitro* T cell activation system, TSLP can directly drive Th2 differentiation without exogenous IL-4 *in vitro* [180]. However, the ability of Th2 differentiation induced by TSLP direct stimulation is dependent on IL-4, as the Th2 differentiation is inhibited by IL-4 blockade, suggesting a role for IL-4 in this process. In this process, TSLP signaling leads to direct IL-4 transcription.

TSLP plays an important role in normal B cell development. TSLP functions as a B cell growth and differentiation factor, pro-B cells derived from fetal liver and bone marrow proliferates in response to TSLP. Besides, aberrant TSLP signaling can have a significant impact on B cells during inflammatory responses [181] In an inducible TSLP transgenic

mouse model in which TSLP is overexpressed in the epidermis displayed an increase of immature and mature B cells, and lose of marginal B cell zone in spleen, as well as expansion of follicular mature B cells and peritoneal B-1b cells. Mature B cells exhibited an activated cell phenotype. These mice developed autoimmune hemolytic anemia *in vivo* subsequently [181, 182].

TSLP can promote basophil hematopoiesis from bone marrow resident precursors. SPC-TSLP Transgenic mice in which TSLP is specifically expressed in the lung epithelial cells displayed elevated basophil numbers, which contributed to Th2 responses by producing Th2 cytokines such as IL-4 and IL-13, and presenting antigens to CD4+ T cells [183-185]. The elevated basophil level is resulted from TSLP-induced basophil differentiation from precursors in the bone marrow. Moreover, TSLP-activated basophils expressed IL-33R in response to IL-33 (another allergy-associated cytokine). Thus, basophils may play a key role in the development of allergic symptoms by producing Th2 cytokines IL-4 and IL-13, as well as increasing a susceptibility to IL-33. However, the induction of Th2 response may be due to the fact that basophils also function as antigen presenting cells for the allergen induced Th2 response, but not due to providing Th2 cytokines [183].

In addition, TSLP plays an important role in allergic airway disease. TSLP expression can be detected in patients with asthma, and it has been reported to correlate with Th2 cytokines and chemokines and inversely with lung function [186, 187]. The involvement of TSLP in human asthma has been validated by a variety of mouse models. In a tissue specific TSLP transgenic mouse model in which TSLP is constitutively expressed in the lung epithelium under control of the SPC promoter developed a progressive asthma-like disease characterized by lung infiltration of eosinophils and Th2 cells, airway remodeling, and airway hyperreactivity as age increased [188].

3. Psoriasis

Psoriasis is a chronic and recurrent inflammatory skin disease，characterized by raised, red scaly itchy plaques that may vary in severity from small and localized to complete body coverage [189]. This disease affects about 2-3% of the world-wide population [190]. Psoriasis originates from chronic interactions between hyperproliferative keratinocytes and

infiltrating immune cells, it is a skin-specific T- cell mediated autoimmune disease. Psoriasis was initially considered only due to dysfunction of keratinocyte proliferation, and infiltration of immune cells was considered to be just a consequence of the hyper-proliferating keratinocytes [191]. Psoriasis is a multifactorial disease resulting from environmental triggers and genetic susceptibility.
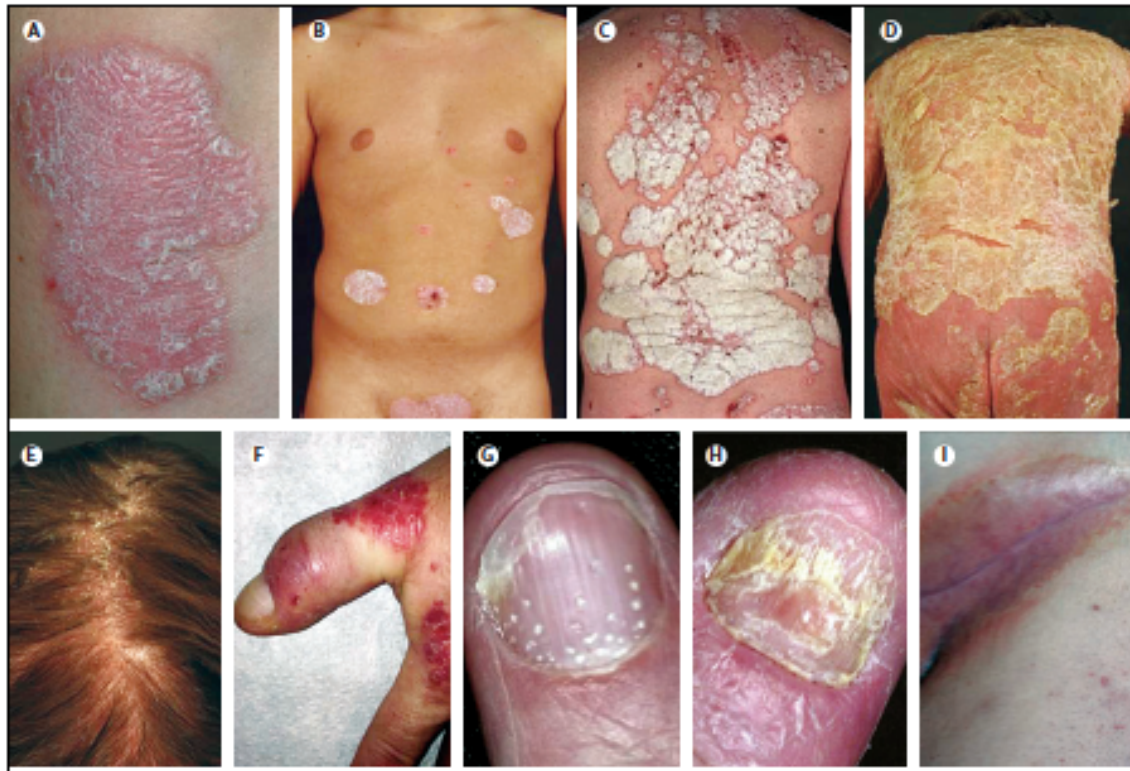

Figure 4: Clinical manifestations of psoriasis. Typical erythematous plaques with silvery scales (A) can be scattered (B, psoriasis nummularis), cover larger areas of the skin (C, psoriasis geographica) or affect the entire body surface (D, erythrodermic psoriasis). Scalp involvement might be accompanied by non-scarring alopecia (E). Psoriatic arthritis affects up to 30% of all patients (F, thumb interphalangeal joint). Nail changes are frequent and range from pitting and yellow or brown discolouration (G) to complete dystrophy (H). Psoriasis inversa occurs in inter- triginous areas and is usually devoid of scales (I). **[taken from (Wolf-Henning Boehncke et al. 2015)]**

3.1 Epidemiology

Psoriasis is universal in occurrence, with a worldwide prevalence varies between 0.6% and 4.8%. The prevalence of psoriasis is about 2% in Europe and North America, however, the incidence in some developed countries accounting for 4.6% of the population [192]. In

certain ethnic groups, such as Africans, African-Americans, Japanese, Alaskans, Australians, and Norwegian Lapps, Psoriasis is a rare case [192, 193]. The prevalence is equal in men and women and can first appear at any age, varied from infancy to elderly. In general, the prevalence of psoriasis increases from 0·12% at the age of 1 year to 1·2% at the age of 18 years, which present in a linear increase [194]. Henseler and Christophers divided the clinical presentations of psoriasis into two types based on the age of onset, namely type I psoriasis and type II psoriasis. Type I psoriasis begins before 40 years old and type II psoriasis begins after the age of 40 years. More than 75% of cases is Type I psoriasis. Patients with type I psoriasis tend to have more severe disease manifestation than patients with type II psoriasis. About 10-30% of patients with psoriasis would develop psoriatic arthritis as a successive condition of psoriasis. Environmental factors and genetic factors, such as climate, ultraviolet and ethnicity, are considered to affect the prevalence of psoriasis.

3.2 Clinical manifestation

Psoriasis is a papulosquamous disease with variable morphology, including the shape, redness and localization of plaques. The lesions of psoriasis are typically distributed on the scalp, elbows, knees, lumbosacral area, and in the body folds. Five types of psoriasis have been reported, including plaque psoriasis, eruptive psoriasis, inverse psoriasis, pustular psoriasis and erythrodermic psoriasis. Plaque psoriasis is the most common form of psoriasis, this form of psoriasis sharply circumscribed, round or oval nummular plaques. The plaques appear to be dry, red and raised lesions covered by silvery white scales. The lesions may initially begin as small plaques, and they can extend over larger areas or even cover the entire body surface. Eruptive psoriasis is common in children and it is developed shortly after an acute group B haemolytic streptococcal infection of the upper respiratory tract. The plaques are smaller in size and less scaly than the plaque type psoriasis, and it is usually distributed in a centripetal fashion and they can also spread to the head and limbs. Inverse psoriasis affects the flexures, including inframammary, perineal and axillary. Inverse psoriasis lesions are devoid of scale and appear as red, shiny, well-demarcated plaques. The pustular psoriasis is typically characterized by disseminated dark erythematous patches with conspicuous sterile pustule, and is often present in patients with

existing or previous psoriasis vulgaris. Erythrodermic psoriasis is manifested by inflammation, itching and painful red rashes, the lesions can cover the entire body of patient with a red, peeling rash.

3.3 Physiopathology

Psoriasis is a complex multifactorial skin inflammatory disorder related to a combination of genetic, environmental and immunological factors. Injury, trauma, infection and medication can trigger the initiation of psoriasis. In addition, TLR ligands can also trigger the initiation of psoriasis. Imiquimod (a TLR7 agonist) has been reported to induce psoriasis-like skin inflammation, which is mediated via the IL-23/IL-17 axis and by activated DCs [191]. So far, it is still not clear whether the pathogenesis of psoriasis is induced by hyperplastic keratinocytes with secondary immune activation or by aberrant immune activation with secondary hyperplastic keratinocytes. Gilliet and coworkers proposed that hyperplastic keratinocytes initiate psoriasis. They found that AMP LL37 is produced by keratinocytes due to the skin injury. AMP LL37 can bind to DNA or RNA released from injured skin cells to became DNA/ LL37 complexes and RNA/ LL37 complex respectively. DNA/ LL37 complexes bind to TLR9 in plasmacytoid dendritic cells (pDCs), which results in the production of type I interferons IFN-α and –β. LL37/RNA complexes can also activate plasmacytoid DCs through TLR7 to produce type I interferons. Type I interferons can activate myeloid DCs. Besides, LL37/RNA complexes also activate myeloid DCs through TLR8. Subsequently, activated myeloid DCs activate T cells and promote the production of cytokines by T cells, leading to psoriasis skin inflammation [195-197]. This theory is supported by the finding that extracellular DNA has been shown in the neutrophil extracellular traps (NETs) in the epidermis of psoriasis skin and that imiquimod (IMQ), a TLR7 agonist, has been reported to induce psoriasis-like skin inflammation [191, 198].

Psoriatic skin lesions result from dysregulated interactions of innate and adaptive immune systems with resident cutaneous cell types. In general, when individuals who are genetically susceptible to psoriasis encounter potential environmental triggers, the KCs in the affected skin are over activated, leading to the production of pro-inflammatory cytokines IL-1α/β and IL-18 via inflammasome activation and tumor necrosis factor (TNF). These pro-inflammatory cytokines activate other KCs and dermal dendritic cells (dDCs) and

epidermal Langerhans cells (LCs). Activated KCs enter terminal differentiation, which leads to the release of self-DNA and self-RNA into the extracellular compartment. pDCs are recruited to inflamed skin site in response to cytokine gradients, where they are stimulated by LL-37–self-DNA/RNA complexes or even by viral double-stranded DNA (dsDNA) and single-stranded RNA (ssRNA) in the infection setting. Stimulated pDCs produce interferon (IFN)-α, which in turn activates mDCs. Activated mDCS drive the differentiation of naïve CD4+ T cells into IFN-gamma producing Th1 cells through production of IL-12, and mDCS drive the differentiation of naïve CD4+ T cells into IL-17 producing cells through production of IL-23. IL-17 can be released from Th17, gamma delta T-cells, mast cells, innate lymphoid cells and possibly neutrophils. These IL-17 producing cells also produce, IL-22 and TNF-alpha. IL-17, IL-22 and TNF-alpha drive KCs to proliferate and release pro-inflammatory cytokines, chemokines and antimicrobial peptides to amplify inflammatory response. NK-T-cells and macrophages can also provide pro-inflammatory stimuli through the secretion of IFN-alpha, TNF- alpha, IL-1 and IL-6.
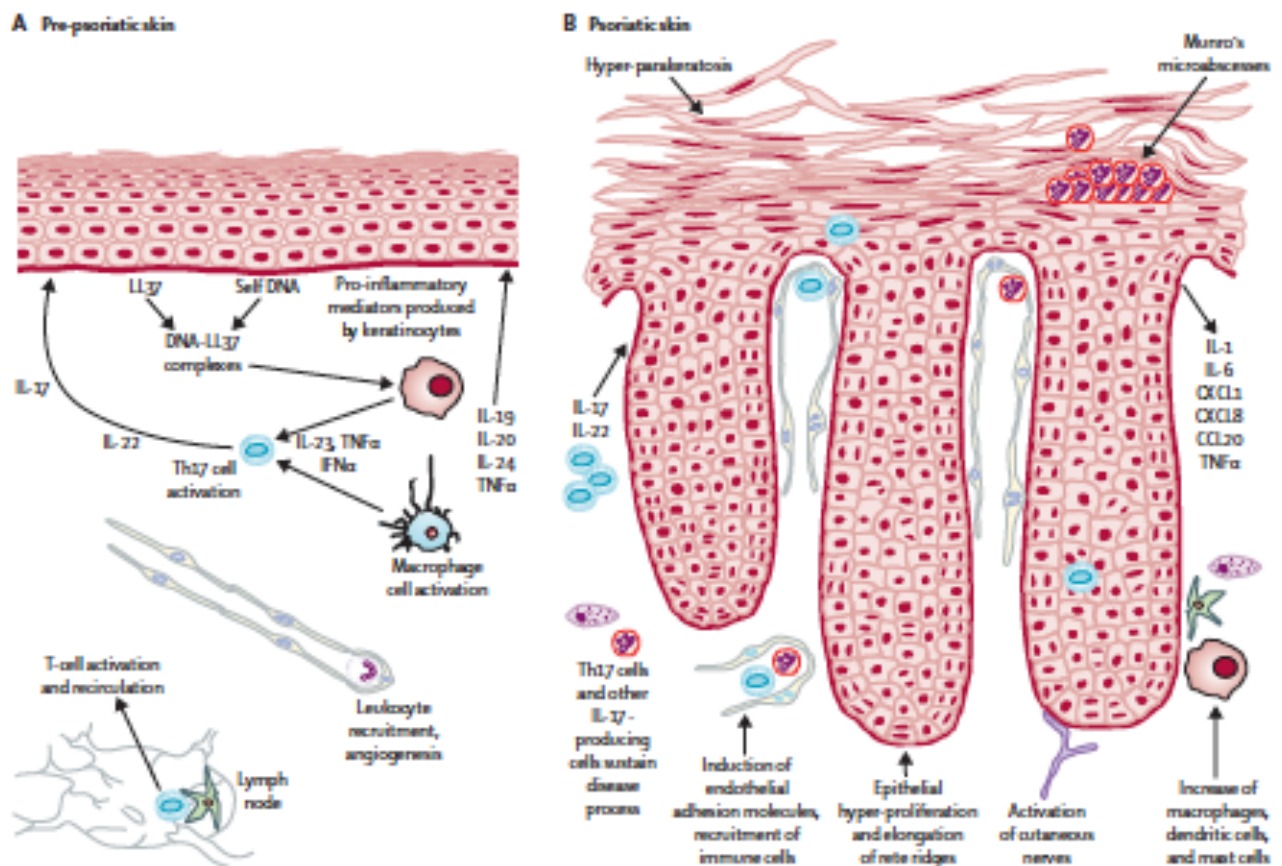

Figure 5. Immune pathogenesis of psoriasis. The complex interplay of cutaneous cell types, which is dependent on macrophages, dendritic cells, T cells, and other cells of the immune system, involves many cytokines and chemokines that orchestrate the pathological changes in pre-psoriatic skin.

Differentiation of Th1 and Th17 cells is stimulated by dendritic cells through IL-23 (A). Pathogenic cells of the adaptive (T cells) and innate immune system (macrophages, mast cells, granulocytes) produce several mediators that induce and maintain psoriatic hallmark features in both dermis (eg, endothelial cells) and epidermis (keratinocytes). In turn, the latter cell types facilitate the inflammatory response through their mediators (B). [**taken from (Wolf-Henning Boehncke et al. 2015)**]

3.4 Environmental risk factors

Psoriasis can be triggered or aggravated by environmental risk factors impairing the skin barrier function. Environmental triggers, such as mild trauma, sunburn, chemical irritants and infections, can provoke psoriasis. Drugs can exacerbate the disease, such as β-blockers, lithium, antimalarials, and non-steroidal anti-inflammatory agents. In addition, stress, smoking and alcohol also trigger psoriasis in patients with a genetic predisposition to developing psoriasis [199-205]. Psoriasis can occur in areas of the injured or traumatized skin, which is called the Koebner phenomenon. Injured or traumatized skin related to Koebner phenomenon is usually induced by vaccination, sunburn and scratch. Lithium is a drug for the treatment of manic depression and other psychiatric disorders. Lithium has been reported to aggravate psoriasis. Antimalarial agents such as plaquenil, quinacrine, chloroquine and hydroxychloroquine can induce a flare of psoriasis several weeks after the drugs are taken. Indomethacin is a non-steroidal anti-inflammatory drug for the treatment of arthritis. It has been reported to exacerbate some cases of psoriasis. Streptococcus infection (strep throat) usually leads to the onset of guttate psoriasis, which is common in children. Earaches, bronchitis, tonsillitis, and respiratory infection such as the flu, or pneumonia can flame psoriasis skin inflammation [199-204, 206].

3.5 Genetics of Psoriasis

Psoriasis is regarded as a multi-factorial genetic disorder, of which the initiation and severity of the psoriasis are associated with the patient's genetic background. Genome-wide scans for psoriasis- associated genes provide a mechanistic link between genetics and immunity. Genome-wide linkage analysis has identified several putative susceptible loci for psoriasis. The gene locus at 6p21 has been found to be associated with psoriasis development, which is called the psoriasis susceptibility region 1 (PSORS1). In

this locus, the Major Histocompatibility Complex class I gene, HLA-Cw6, is a chief susceptibility factor for psoriasis, accounting for up to 50% of disease heritability [207, 208]. The HLA-Cw6 gene has been found to be associated with early-onset chronic plaque type and guttate psoriasis [209]. A Genome-wide linkage analysis in European population revealed seven susceptible loci involved in IL-23 signaling pathway, NF-κB signaling pathway and Th2 mediated immune response [210], including *IL28RA*, *IFIH1*, *REL*, *ERAP1*, *TRAF3IP2*, *NFKBIA* and *TYK2*. *ERAP1* and *ZNF816A* were preferentially found to be associated with Type I psoriasis in Chinese population [211]. 15 psoriasis susceptibility loci have been identified in European population [212]. These genes inside these loci were found to be responsible for regulation of T-cell function and innate host defense mechanisms. For example, *RUNX3*, *TAGAP* and *STAT3* are responsible for regulation of T-cell function; *ZC3H12C* involve in macrophage activation; *CARD14* and *CARM1* involve in NF-κB signaling and *DDX58* involve in interferon-mediated antiviral response [212]. Variations in the genes that encode the interleukin-23 receptor are found to be associated in pathogenesis of psoriasis, suggesting a specific IL-23/Th-17 axis in the pathogenesis of psoriasis [213, 214]. Furthermore, about 40 additional loci are thought to be associated with psoriasis. These findings provide us a better understanding of the role for genetic determinants in the determination of immune-mediated inflammatory skin disorders.

3.6 Mouse models of psoriasis

There are five mouse models of psoriasis have been extensively used for studying psoriasis. They are K5-Tie2 mouse, K5-Stat3C mouse, K5-TGFb1 mouse, K14-AREG mouse, and IMQ-induced psoriasis mouse. In K5-Tie2 mouse, the endothelial-specific receptor tyrosine kinase is over-expressed in basal KCs [215]. In K14-AREG mouse, human amphiregulin is over-expressed in the basal epidermal layer [216]. In K5-Stat3C mouse, basal KC over-express a constitutively active mutant of signal transducer and activator of transcription 3 (K5-Stat3C) [217], and in K5-TGFb1mouse, basal KC over-express the latent form of transforming growth factor beta 1 [218]. IMQ-induced psoriasis mouse is achieved by topical application of the TLR agonist IMQ [219]. The phenotypes of these models recapitulate key features of human psoriasis such as epidermal hyperproliferation, thickening and altered differentiation of the epidermis, an inflammatory infiltrate that includes T- cells and altered vascularity, but differ with respect to

the initiating events. The K14-AREG and K5-Stat3C models directly perturb KC homeostasis, which leads to elevated cytokine or chemokine production and secondary inflammatory responses. The K5-Tie2 and K5-TGFb1phenotypes may also leads to direct perturbation of KC homeostasis, but in these models, growth factor release and certain concurrent processes (e.g., angiogenesis, oxidative stress accumulation, and/or basement membrane degradation), have also been postulated, contributing to KC proliferation and initiation of inflammatory cascades]. However, IMQ-induced psoriasis mouse develops a psoriasis-like inflammation that is arising from direct stimulation of the immune system. The phenotype IMQ is a TLR7 agonist that drives skin inflammation and immune cell infiltration, followed by KCs proliferation and enhanced dermal vascularity [215-219].   In IMQ-induced psoriasis mouse, the inflamed skin displays red, scaly skin similar to human psoriasis, and indeed, clinical observations have indicated that IMQ can exacerbate psoriasis in patients [219].

3.7 IL-23/IL-17/IL-22 axis and psoriasis

IL-23 is a member of the IL-12 heterodimeric cytokine family，it consists of a unique p19 subunit paired with a second subunit called p40 that is also a subunit of IL-12. The IL-23 receptor is also a heterodimer consisting of IL-12Rβ1 and IL-23R subunits. Initially, IL-23 was thought to have a similar role to IL-12 in promoting Th1-type responses, however, increasing evidence show that IL-23 drives naïve CD4+ T cell differentiate into Th17 cell population that is characterized by the production of the IL-17-related cytokines IL-17A and IL-17F [220, 221]. Antigen-presenting cells, such as dendritic cells and macrophages, produce IL-12 and IL-23 in response to environmental danger signals. Many toll-like receptor ligands, including lipopolysaccharide, CpG and PolyI: C can enhance the expression of the cytokine subunits p40 and p19, leading in the production of IL-23 [222-224]. In addition, epithelial and endothelial cells can also produce IL-23.

IL-23 is a key pro-inflammatory cytokine driving autoimmune diseases in both mouse and human. Mice lacking IL-23 can resist to the development of arthritis and multiple sclerosis [225, 226]. In humans, IL-23 is over-expressed in inflammatory sites of several immune-mediated diseases such as Crohn's disease, rheumatoid arthritis and multiple sclerosis. IL-23R is expressed on the surface of a variety of lymphoid cells, such as αβ and

γδ T cells, innate lymphoid cells, DCs, macrophages and monocytes [227]. IL-23 receptor signaling induces RORγ and RORα expression in naïve CD4+ T cells to establish Th17-specific cell differentiation [228, 229]. Initially psoriasis was regarded as a Th1-type and TNF- driven inflammatory disease, however this perception was changed after the discovery of the role of IL-23/Th17 axis in the pathogenesis of psoriasis. IL-23 was highly expressed in psoriasis lesional skins, which mainly produced by dendritic cells and KCs [230, 231]. Intradermal injection of IL-23 or over-expression of IL-12/23p40 in mouse KCs results in skin inflammation resembling psoriasis [232, 233]. Promising results have been obtained in the treatment of moderate to severe psoriasis using a human monoclonal antibody directed against IL12/ 23p40 [234, 235].

Consistent with the role of Th17 cells in a variety of autoimmune diseases, Th17 cells and Th17-related cytokines also play a major role of in psoriasis. Th17 cells have been detected in psoriasis lesional skin with Th17 cytokine expression at a high level. Th17 cell produce IL17 and IL-22. IL-17 can promote the production of pro-inflammatory cytokines, such as IL-6, IL-8, GM-CSF, IFN-γ and ICAM-1 in KCs, leading to epidermal hyperplasia, acanthosis, and hyperparakeratosis [236, 237]. IL-22 is also a key cytokine produced by Th17 cells. The role of IL-22 in the pathogenesis of psoriasis has been extensively examined. IL-22 has been found elevated in lesinal skin of a psoriasiform mouse model [238]. IL-23 injection in rodents induces IL-22-dependent dermal inflammation, KC hyperproliferation, and epidermal acanthosis [239]. Administration of IL-22-neutralizing Ab into a psoriasis mouse model leads to the downregulation of the cutaneous expression of S100A8, S100A9, defensin b1, and cathelicidins expression, which prevents the development of psoriatic skin lesions [240] These findings highlight that IL-17A and IL-22 are key mediators of psoriatic skin inflammation.
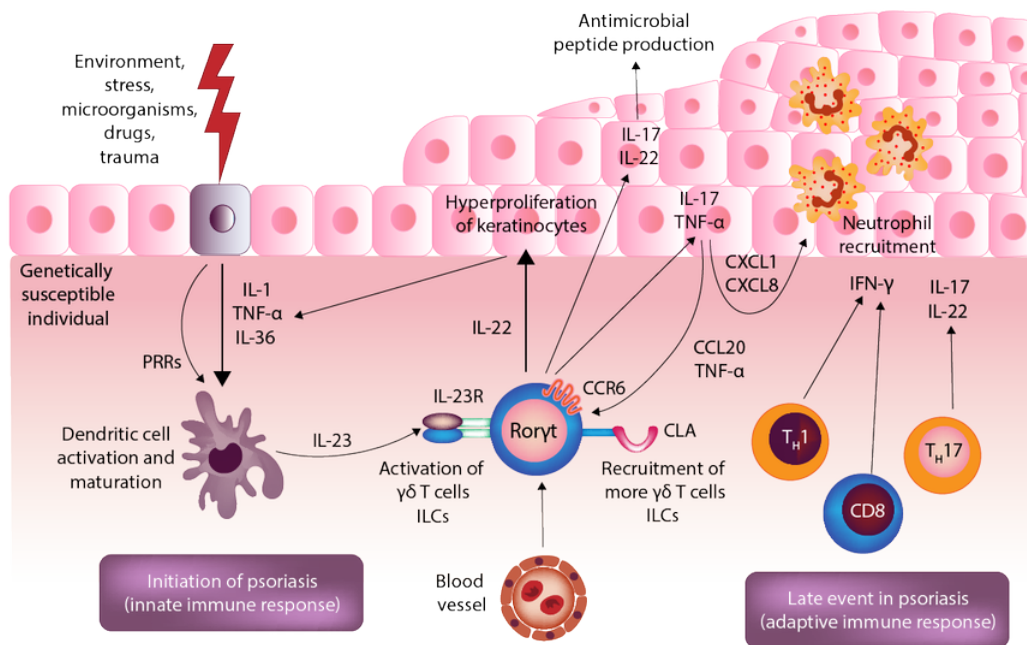
Figure 6. The IL-23/IL-17/IL-22 axis in the immunopathogenesis of psoriasis. In the 'IL-23/Th17 axis' model of psoriasis, T helper 17 (Th17) cells interact with skin-resident cells, contributing to the psoriatic disease phenotype, characterized by scaly plaques and thickened epidermis (acanthosis), with elongated rete ridges and hyperproliferative KCs retaining the nucleus in the stratum corneum (parakeratosis), as well as dermal inflammatory cell infiltrate. IL-23 secreted by dDCs is able to induce Th17 cell activation with production of pro-inflammatory cytokines such as IL-17A, IL-17F, IL-22 and IFN-g. These cytokines act on KCs, inducing epidermal hyperplasia and KC activation. Activated KCs produce pro-inflammatory mediators, including chemokines, members of the S100 family, pro-inflammatory cytokines and antimicrobial peptides. In particular, CCL20 is able to recruit more CCR6+ Th17 cells. Moreover, KCs might produce IL-23, which could mediate crosstalk with Th17 cells in synergy with IL-23 produced by dermal DCs, thus further sustaining and amplifying skin inflammation. [**taken from (Frank O Nestle et al. 2015)**]

Th17 cells provide host protective responses against invading pathogens, which rapidly recruit and activate granulocytes and macrophages that are capable of producing tissue-damaging reactive oxygen species. The normal Th17 response does not cause disease, however, Th17 response can cause severe local tissue injury if Th17 cells experience prolonged and dysregulated exposure to IL-1 and IL-23 [241, 242]. Skin-resident cells, such as keratinocytes, fibroblasts and endothelial cells, can respond to IL-17 and produce a variety of molecules that promote rapid cell division and neo-angiogenesis, strongly contributing to the development of psoriasis. For example, keratinocytes secret

antimicrobial peptides (β-defensins, lipocalins, LL-37 and S100A7) and chemokines (CXCL1, CXCL2, CXCL3, CXCL5, IL-18 and CCL20) inappropriately in response to constant IL-17 stimulation, which further recruits and activates mast cells, neutrophils and macrophages [243] to create amplification loops that drive epidermal hyperplasia and pro-inflammatory conditions of psoriasis.

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Results and discussion

Part I: Study of mechanisms underlying the type 2 immune response in atopic dermatitis pathogenesis

Summary

Atopic dermatitis (AD) is one of the most common inflammatory skin diseases, characterized by chronic cutaneous inflammation, hyper IgE and T helper type 2 (Th2) response. The prevalence of AD has increased, with the number of children suffering from AD tripled in industrialized nations in the past 30 years, now affecting 15-30% of the children and 2-10% of adult. Moreover, AD often progresses to other atopic diseases such as asthma. It has been thus urged to achieve a better understanding of AD and to develop effective prevention and treatment strategies.

It has been recognized that Th2 cell response is critically implicated in the pathogenesis of AD. The previous studies in my lab have established a central role of cytokine thymic stromal lymphopoietin (TSLP), which is expressed by epidermal keratinocytes, in promoting Th2 cell response and driving the pathogenesis AD. Recently, a new CD4+ helper cell subset, T follicular helper (Tfh) cell, has emerged to be a critical player in humoral immunity by providing help to B cells. Tfh cells together with B cells form germinal centers (GCs) at the site where high-affinity B cells are selected and differentiated into either memory B cells or long-lived plasma cells. However, the pathogenic role of Tfh cells in the context of AD and other atopic diseases remains unexplored.

The part I of my thesis aimed at studying the Tfh cell differentiation, function and regulation in AD pathogenesis. To this aim, I employed our previously established AD mouse model in which MC903 (a vitamin D analog) topical treatment on the skin induces TSLP production by keratinocytes, promotes Th2 cell response and drives the pathogenesis of AD. I first identified and characterized Tfh cells in the skin-draining lymph nodes (LNs) by flow cytometry analysis. Results showed that the frequency of Tfh cells in CD4+ T cells, as well as the Tfh cell number, increased with time. This is accompanied by GC formation, as showed by flow cytometry analysis and fluorescent immunohistochemical staining in the LNs. I then analyzed the expression of Th1/2/17 cytokines in Tfh cells using intracellular staining or/and cytokine reporter mice, showing that Tfh cells in the AD mice produced IL-4,

a key type 2 cytokine that regulates class switch recombination to IgE and IgG1 in humoral immune response. By performing kinetic analyses, I identified two stages of Tfh cell generation: an " initiation" stage (at D7) when Tfh cell lineage commitment has taken place, and an "expansion/maintenance" stage when the number of Tfh cells, GC B cells, IgG1$^+$ B cells and IgE$^+$ B cells all increases.

Next, I addressed whether TSLP is required for Tfh cell differentiation. Results showed that in MC903-treated TSLP$^{-/-}$ mice, Tfh and GC B cell numbers were all diminished at D11, in addition to the decreased Th2 cell response as previously reported. Interestingly, the generation of Tfh cells at the "initiation" stage was not affected, but IL-4 induction in Tfh cells was impaired.

In exploring the requirement of skin DCs in driving Tfh cell differentiation, I found that when Langerin+ DCs, including epidermal Langerhans cells, were depleted in Lang^{DEP} mice, the initial Tfh cell differentiation and the expansion/maintenance of Tfh cells, as well as GC formation, were all markedly impaired, suggesting a critical role of Langerin+ cells in promoting Tfh cell response.

Finally I examined the implication of OX40L signaling in Tfh cell differentiation, as OX40L has been reported to be a TSLP-downstream signaling but its role in type 2 response remains highly controversial. I showed that the blockade of OX40L signaling using OX40L neutralizing antibody led to a decrease in Tfh cell and GC B cell number, as well as a reduction of IL-4 expression in Tfh cells at D11. But similar to what was observed in TSLP$^{-/-}$ mice, blockade of OX40L signaling did not affect the initial commitment of naïve CD4+ T cell into Tfh lineage, suggesting that TSLP-OX40L signal may play an important role in the maintenance of Tfh cells rather than the initiation of Tfh cell differentiation.

Taken together, my thesis work investigated Tfh cell differentiation, its cytokine expression and germinal center formation using MC903-induced AD mouse model. By exploring the role of TSLP, Langerin+ DCs and OX40L signaling in Tfh cell differentiation and regulation, my study provides novel insights into the mechanisms underlying the type 2 immune response in AD pathogenesis.

Introduction

AD is one of the most common inflammatory skin diseases, characterized by chronic cutaneous inflammation, hyper IgE and T helper type 2 (Th2) response. The prevalence of AD has increased, with the number of children suffering from AD tripled in industrialized nations in the past 30 years, now affecting 15-30% of the children and 2-10% of adult [1]. Moreover, AD often progresses to other atopic diseases such as asthma. It has been thus urged to achieve a better understanding of AD and to develop effective prevention and treatment strategies.

The mechanisms underlying the pathogenesis of AD remain not completely understood. It is multifactorial process and involves a complex immunologic cascade, such as disruption of the epidermal barrier, IgE dysregulation and defects in the cutaneous cell-mediated immune response [2, 3]. It has been recognized that Th2 cell response is critically implicated in the pathogenesis of AD. The previous studies in our lab have established a central role of cytokine thymic stromal lymphopoietin (TSLP), which is expressed by epidermal keratinocytes, in promoting Th2 cell response and driving the pathogenesis AD [4-6]. In addition to Th2 cell response, humoral immune response is also a key feature of AD. Increased serum IgE and IgG1 levels have been found to be associated with AD [7, 8]. Our previous studies showed that elevated total IgE and total IgG levels were detected in sera either from TSLP-overexpressing mouse model [5] or MC903 tropical treatment mouse model [9]. A better understanding of humoral immune response is crucial for gaining insight into the complexity of the mechanism that cause AD and the treatment of patients with AD. Recently, a new CD4+ helper cell subset, T follicular helper (Tfh) cell, has emerged to be a critical player in humoral immunity by providing help to B cells. Tfh cells together with B cells form germinal centers (GCs) at the site where high-affinity B cells are selected and differentiated into either memory B cells or long-lived plasma cells [10]. The Tfh cell differentiation process is believed to begin with an initial dendritic cell (DC) priming of a naive CD4+ T cell, which undergoes a cell-fate decision with the acquisition of master transcription factor Bcl6 expression and chemokine receptor CXCR5 expressed on cell surface to become early Tfh cell, while the full differentiation and maintenance of Tfh cells implicate the Tfh cell-B cell interaction. Tfh cells are phenotypically defined by their high expression of chemokine receptor CXCR5 and programmed death 1 (PD-1), of which CXCR5 promotes their migration from T cell zone to the B cell follicles [11, 12]. Furthermore,

Tfh cells also express various receptors on the cell surface such as inducible T cell costimulator (ICOS), B and T lymphocyte attenuator (BTLA) and CD40L, which are important for their development and function [13]. It has been reported that Tfh cells produce cytokines such as IL-21, IL-4 and IFN-γ. IL-21 promotes B cell maturation, survival, isotype switching, and affinity maturation; IL-4 directs isotype class switching to the IgE and IgG1; and IFN-γ can dictate isotype class switching to the IgG2a [14, 15].

The role of Tfh cells in the context of AD and other atopic diseases remains to be explored. It is not clear during AD pathogenesis, how Tfh cell differentiation and GC response are initiated and maintained, what cytokine are expressed by Tfh cells and how they are regulated, and what is the relationship between Tfh and Th2 response. In this study, we employed MC903-induced AD mouse model [6, 9] to investigate the Tfh cell differentiation, cytokine expression and regulation, as well as GC formation in AD pathogenesis. Particularly, we identified the critical role of Langerin+ DCs in promoting Tfh cell differentiation, and uncovered the roles of TSLP and OX40L signaling in the maintenance of Tfh cells and IL-4 commitment.

Results

Skin topical MC903 treatment induces Tfh cell differentiation and germinal center formation in draining lymph nodes

To induce AD pathogenesis, Balb/c mouse ears were topically treated with MC903 as previously reported [9, 16](Fig. 1A), and ear-draining lymph nodes (EDLN) were analyzed at D11. In consistent with our previous findings, EDLNs from MC903-treated mice showed a significant increase in total cell number, as well as CD4+ T cell number (Fig. 1B). Intracellular cytokine staining showed that IL-4 but not IL-17 or IFN-γ expression was increased in CD4 T cells (Fig. 1C). Apart from Th2 cells, recent studies have identified that IL-4 can be alternatively produced by T follicular helper cells (Tfh), a specialized CD4+ T helper cell subset that is phenotypically and functionally distinct from other CD4+ T helper cell subsets [13, 17]. We thus performed surface staining of CXCR5 and PD-1. The percentage of CXCR5+PD-1+ cells in CD4+ T cells, as well as the total CXCR5+PD-1+CD4+

T cell number were both increased in EDLNs from Balb/c mice treated with MC903, compared with those from mice treated with ETOH (Fig. 1D). The $CXCR5^+PD-1^+CD4^+$ T cells expressed high level of ICOS and Bcl6, suggesting that they are bona fide Tfh cells located in germinal centers (GC) (Fig. 1E). These results indicate that MC903 treatment induces Tfh cell differentiation in skin-draining LNs.

We further performed intracellular cytokine staining of EDLN cells. Results showed that IL-4 expression, but not IL-17 or IFN-γ was increased in Tfh cells (Fig 1.F). To further validate this observation, we utilized $4C13R^{Tg/0}$ reporter mice, which have transgenic expression of AmCyan under *Il4* regulatory elements and destabilized dsRed (dsRed-DR) under *Il13* regulatory elements [18]. MC903-treated $4C13R^{Tg/0}$ mice exhibited an increase of AmCyan(IL-4) expression in $CD4^+$ T cells, as well as in Tfh cells in EDLNs (Fig. 1G). Moreover, calculation of cell number showed that about 50% of IL-4-producing $CD4^+$ T cells were $CXCR5^+PD-1^+$ Tfh cells (Fig. 1G). In contrast, while DesRed (IL-13) expression was also evident in $CD4^+$ T cells, very little of DesRed (IL-13) expression was seen in Tfh cells (Fig. 1H). This data suggests that IL-13 is mainly produced by Th2 cells but not Tfh cells, which is in agreement with a previous report [15].

Skin MC903 treatment induces GC formation in draining LNs

We then examined whether MC903 treatment led to GC formation and immunoglobulin isotype class switch in draining LNs. Total B cell number in EDLNs was increased upon the MC903 treatment (Fig. 2A), and more particularly, Fas^+GL7^+ GC B cells increased in both frequency and cell number (Fig. 2B). Moreover, we observed an increase in the number of $IgG1^+$ B cells (Fig. 2C), with most of them expressing GC B cell markers Fas and GL7, suggesting that GC B cells undergo class-switch to IgG1. In addition, IgE^+ B cells were also increased in both frequency and cell number (Fig. 2C), however only a few IgE^+ B cells expressed Fas and GL7. Confocal microscopy analyses on frozen sections of EDLNs revealed that the GC area was enlarged dramatically in MC903-treated Balb/c mice (see Fig. 4G). Taken together, these observations indicate that MC903 treatment leads to GC formation.

MC903-induced Tfh cell differentiation and GC formation are abolished in mice

lacking VDR

To test whether MC903-induced Tfh cell differentiation and GC formation are VDR dependent, we applied MC903 on the ear of VDR$^{-/-}$ mice and examined EDLNs at D11. As expected, MC903-treated VDR$^{-/-}$ mice did not exhibit any increase in total LN cell number or in CD4$^+$ T cell number (Fig. 3A). The frequency and total number of Tfh cells were comparable between MC903- and ETOH- treated VDR$^{-/-}$ mice (Fig. 3B). In addition, no increase of GC B cell was observed in MC903-treated VDR$^{-/-}$ mice (Fig. 3C). Further more, similar results were obtained in MC903-treated VDR$^{ep-/-}$ mice, in which VDR is ablated selectively in epidermal keratinocytes [9]. These data suggest that the activation of VDR signaling in keratinocytes leads to Tfh cell differentiation and GC formation.

MC903-induced Tfh cell differentiation and GC formation are diminished in mice lacking TSLP

Our previous studies have shown MC903 induces TSLP production by keratinocyte via VDR signaling, and TSLP plays a key role in triggering Th2 inflammation in AD [5, 6, 16]. We thus examined whether the MC903-trigged Tfh differentiation and GC formation are mediated by TSLP. MC903 was applied to the ears of TSLP$^{-/-}$ mice, and EDLNs were analyzed at D11. MC903-treated TSLP$^{-/-}$ mice showed an increase in total LN cell number and CD4$^+$ T cell number compared with ETOH-treated TSLP$^{-/-}$ mice, but such increase was much less than in TSLP$^{+/+}$ mice (Fig. 4 A). The percentage of Tfh cells in CD4$^+$ cells and the total Tfh cell number were both lower in the EDLNs of MC903-treated TSLP$^{-/-}$ mice, compared with MC903-treated TSLP$^{+/+}$ mice (Fig. 4B). We then examined IL-4 expression by intracellular staining. As shown in Fig. 4C, MC903-induced IL-4 expression in CD4$^+$ T cells, which could be sourced from Th2 and Tfh cells, was much lower in TSLP$^{-/-}$ than in TSLP$^{+/+}$ mice. Particularly, IL-4 expression in Tfh cells was highly reduced in MC903-treated TSLP$^{-/-}$ mice (Fig. 4C). Therefore, upon MC903 treatment, TSLP$^{-/-}$ mice not only exhibited lower number of Tfh cells, but also decreased expression of IL-4. The reduction of IL-4 expression by Tfh cells was further confirmed by examining AmCyan (IL-4) reporter expression in TSLP$^{-/-}$/4C13R$^{Tg/0}$ mice and TSLP$^{+/+}$ /4C13R$^{Tg/0}$ mice (Fig. 4D). In addition, comparison of IL-13 intracellular staining or DsRed(IL-13) reporter levels showed

that MC903-induced IL-13 expression in CD4$^+$ T cells was also highly reduced in TSLP$^{-/-}$ mice (Fig. 4E), indicating a decreased Th2 cell differentiation.

Along with impaired Tfh cell generation and IL-4 production by Tfh cells, decreased number of GC B cells was observed in MC903-treated TSLP$^{-/-}$ mice (Fig. 4F). Immunofluorescent staining of EDLN sections, which identifies GCs with PNA, anti-IgD and anti-CD4, showed that GC area in MC903-treated TSLP$^{-/-}$ EDLNs was significantly smaller than in MC903-treated TSLP$^{+/+}$ EDLNs (Fig. 4G). Besides, the number of IgG1$^+$ B cells and IgE$^+$ B cells was also reduced (Fig. 4H&I).

Taken together, these data indicate that the ablation of TSLP leads to an impaired Tfh and GC response at D11 in MC903-induced AD.

TSLP is not required for the initial generation of Tfh cells but is crucial for their IL-4 expression

As MC903-treated TSLP$^{-/-}$ mice exhibited diminished but not abolished generation of Tfh cells in EDLNs at D11, we sought to know whether the initiation of Tfh cell differentiation occurred normally in TSLP$^{-/-}$ mice. To this aim, we first performed kinetic analyses of Tfh and GC B cells in wildtype Balb/c mice at different time points upon MC903 treatment (Fig. 5A). Results showed that the frequency of CXCR5$^+$PD-1$^+$ Tfh cells in CD4$^+$ T cells increased significantly at D7 but not at D5 (Fig. 5B), indicating an initial stage of Tfh cell generation at D7. The frequency of Tfh cells in CD4$^+$ T cells stayed at a similar level at D11 compared with D7, while the Tfh cell number further increased (Fig. 5B). In addition, the frequency of GC B cells in EDLN cells was significantly increased at D11 but not at D7 (Fig. 5C), and IgG1$^+$ or IgE$^+$ B cell number also increased at D11 (Fig. 5D&E). These data thus suggest D7 as an "initiation" stage while D11 as an "expansion or maintenance" stage for Tfh cell generation in EDLNs upon MC903 treatment.

We then analyzed the generation of Tfh cell and IL-4 expression in MC903-treated TSLP$^{-/-}$/4C13R$^{Tg/0}$ at D7. Interestingly, while total EDLN cells number and CD4$^+$ T cell number decreased or tended to decrease in these mice (Fig. 6A), the frequency of CXCR5$^+$PD-1$^+$ Tfh cells in CD4$^+$ T cells, or CXCR5$^+$PD-1$^+$ Tfh cell number, did not exhibit significant difference between TSLP$^{-/-}$/4C13R$^{Tg/0}$ and TSLP$^{+/+}$/4C13R$^{Tg/0}$ mice (Fig. 6B). However, AmCyan(IL-4) expression in CD4$^+$ T cells (Fig. 6C upper panel) and in Tfh cells (Fig. 6C

lower panel) were both much lower in TSLP$^{-/-}$/4C13R$^{Tg/0}$ mice. These results indicate that despite of a defective Tfh and GC response in MC903-treated TSLP$^{-/-}$ mice at D11 (Fig. 4), TSLP is dispensable for the initial generation of Tfh cells (i.e. at D7). However, TSLP plays a critical role for IL-4 commitment in Tfh cells at the initial stage. As expected, we also observed a significant decrease of DsRed(IL-13) expression in CD4$^+$ T cells in EDLNs of MC903-treated TSLP$^{-/-}$/4C13R$^{Tg/0}$ mice at D7 (Fig. 6D), indicating a diminished Th2 response in these mice.

MC903-induced Tfh cell differentiation implicates skin antigen

The interaction between antigens and T cell receptor (TCR) is generally required for Tfh cell differentiation. However, there was no experimental antigen administrated in our MC903-triggered AD model. Moreover, MC903 does not appear to act as a hapten or allergen, as its activiation of VDR signaling is absolutely required for mediating the effect of MC903 in triggering the Tfh cell differentiation (Fig. 3). We thus wondered whether the increased number of Tfh cells in EDLN upon MC903 treatment may result from the expansion of pre-existing Tfh cells or from the differentiation of Tfh cells induced by "unknown" antigens present on/in the skin. To test the implication of skin antigens in MC903-induced Tfh cell generation, we took use of DO11.10$^{Tg/Tg}$ mice, which carry the MHC class II restricted rearranged T cell receptor (TCR) transgene and react to ovalbumin (OVA) peptide antigen [19]. In DO11.10$^{Tg/Tg}$ mice, about 70% of CD4+ T cells expressed OVA-specific TCR (Fig. 7A). We reasoned that if MC903-triggered Tfh cell accumulation in EDLN does not implicate any antigen, MC903 treatment would lead to similar Tfh cell number in DO11.10$^{Tg/Tg}$ mice as in WT mice; however, if it implicates "unknown" antigens present on/in the skin, MC903 treatment would lead to a lower Tfh cell number in DO11.10$^{Tg/Tg}$ mice, because only 30% of CD4 T cells in the EDLN of DO11.10$^{Tg/Tg}$ can respond to these "unknown" antigens (Fig. 7B). DO11.10$^{Tg/Tg}$ mice and Balb/c WT mice were then treated with MC903 and EDLNs were analyzed at D11. Total CD4$^+$ T cell expansion in EDLNs was comparable in DO11.10$^{Tg/Tg}$ mice as in WT mice (Fig. 7C), but the percentage of Tfh cells in CD4 T cells and the total Tfh cell number were significantly lower in DO11.10$^{Tg/Tg}$ than in WT mice (Fig. 7D). In line with the diminished Tfh cell generation, GC B cell response was also lower, showing less GC B cell number in MC903-treated

DO11.10$^{Tg/Tg}$ mice (Fig. 7E). These data imply that the generation of Tfh cells in EDLN of MC903-induced AD mice implicates antigens, although the nature of antigens remains unidentified.

Role of Langerin+ DCs in Tfh cell differentiation, expansion and IL-4 production

We then asked which DCs in the skin are responsible for inducing Tfh cell differentiation in MC903-induced AD mice. Langerhans cells (LCs) are located in the epidermis, with an early accessibility to skin pathogens, commensal organisms and epidermal self-antigens. To test the potential role of LCs, Lang-DTREGFP$^{+/KI}$ mice [20] were injected with diphtheria toxin (DT) (called hereafter Lang^{DEP}) every 4 days started from D-2. MC903 or ETOH was topical applied to ears every 2 days starting from D0, and mice were analyzed at D7 and D11 (Fig. 8A). RT-qPCR analyses of ears showed that the Langerin mRNA was barely detected in ETOH- or MC903- treated Lang^{DEP} mice skin, suggesting an efficient depletion of Langerin$^+$ cells (including LCs and Langerin$^+$ dermal DCs) in these mice [21] (Fig. 8B). FACS analysis confirmed that LCs (CD45$^+$CD24$^+$MHCII$^+$) in the epidermis of ear skin from Lang^{DEP} mice, either treated with ETOH or MC903, were depleted at D7 and D11 (Fig. 8C). In the dermis of ETOH-treated Lang^{DEP} mice, CD45$^+$MHCII$^+$CD24$^+$ population containing mainly Langerin$^+$ dermal DCs (CD11b$^-$CD24$^+$) and migrating LC (CD11b^{int}CD24$^+$) was also depleted. The CD45$^+$MHCII$^+$CD24$^+$ population in the dermis from MC903-treated Lang^{DEP} skin showed a partial decrease, possibly due to the existence of other Langerin$^-$ cells inside this population upon MC903 treatment (Fig. 8C).

Upon MC903 treatment, the expansion of CD4$^+$ T cells in EDLNs was comparable between CT and Lang^{DEP} at D7 and D11 (Fig.8D). However, the differentiation of Tfh cells was markedly impaired in Lang^{DEP} mice, showing the reduced Tfh cell frequency and total cell number, both at the initiation stage (D7) and at the expansion/maintenance stage (D11) (Fig. 8E). Moreover, the IL-4 production in Tfh cells was substantially impaired in Lang^{DEP} mice at D7 and D11 (Fig. 8F). In contrast, the depletion of Langerin$^+$ DCs did not appear to have a major impact on IL-13 expression by CD4$^+$ T cells (Fig. 8G), which was different from TSLP$^{-/-}$ mice where IL-13 expression was largely reduced (Fig. 4E). These results suggest that Langerin$^+$ DCs play a crucial role not only in the initiation and expansion of Tfh cells, but also in IL-4 expression in Tfh cells. Along with the impaired Tfh cell differentiation,

GC B cell number was also largely reduced in MC903-treated Lang^{DEP} mice at D11 (Fig. 8H). Moreover, IgG1^{+} (but not IgE^{+}) B cell number was also significantly reduced (Fig. 8I&J).

Role of OX40L co-stimulatory signaling in Tfh cell differentiation, expansion and IL-4 production

The co-stimulatory signal of OX40L/OX40 in promoting Th and Tfh differentiation remains highly controversial [22-30]. Nevertheless, OX40L has been recognized to be a downstream event of TSLP-TSLPR signaling [31, 32]. We next examined the implication of OX40L signaling in Tfh cell differentiation and GC response in MC903-trigged AD. Balb/c WT mice were i.p injected with anti-OX40L neutralizing antibody or isotype antibody every three days starting from D-1, and MC903 was topically applied to ears every two days from D0. EDLNs were analyzed at D7 and D11 (Fig. 9A). Blockade of OX40L led to a decreased CD4^{+} T cell expansion at both D7 and D11 (Fig. 9B). Interestingly, Tfh cell differentiation did not seem to be affected at D7, as the percentage of Tfh rather tended to increase and Tfh cell number was not different from that in isotype-injected mice (Fig. 9C). However, at D11, OX40L blocking led to a significant decrease in Tfh cell frequency in CD4^{+} T cells, as well as in Tfh cell number (Fig. 9C). These results are reminiscent of the results from TSLP$^{-/-}$ mice (Fig. 6B & Fig. 4B), suggesting that OX40L signaling is dispensable for the initial differentiation of naïve CD4+ T cells into CXCR5^{+}PD-1^{+} Tfh cells, but is required for the expansion/maintenance of Tfh cells at the later stage.

Further, we examined IL-4 and IL-13 expression in Tfh and in CD4^{+} T cells either by intracellular staining or by performing OX40L blocking experiment in 4C13R$^{Tg/0}$ reporter mice. Results showed that IL-4 expression in Tfh cells was decreased at both D7 and D11, indicating an important role of OX40L for IL-4 commitment in Tfh cells (Fig. 9D&E). In contrast, IL-13 expression in CD4^{+} T was not markedly impaired at D7 or D11 (Fig. 9F&G). Actually, DsRed(IL-13) exression in OX40L-blocking mice rather exhibited an increase at D7 (Fig. 9G). These results suggest that OX40L signaling may not be primarily required for IL-13 expression by Th2 cells. In accompany with the decreased Tfh cell number, GC B cell number was also reduced in OX40L-blocking mice at D11 (Fig. 9H). In addition, the increase of IgG1^{+} B cells and IgE^{+} B cells was also impaired (Fig. 9I).

Taken together, our data indicate that while OX40L signaling is dispensable for the initial differentiation of Tfh cells, it is required for the expansion/maintenance of Tfh cell and GC response at a later stage. Also importantly, OX40L signaling is crucial for IL-4 commitment in Tfh cells.

Discussion and perspectives

By employing a MC903-induced AD model, we investigated in this study the Tfh cell differentiation and GC response in skin-draining lymph nodes. We showed that the skin topical treatment of MC903 led to the generation of bona fide Tfh cells (Fig. 10). By performing kinetic analyses we identified two stages of Tfh cell generation: an " initiation" stage (at D7) when Tfh cell lineage commitment has taken place, and an "expansion/maintenance" stage when the number of Tfh cells, GC B cells, IgG1+ B cells and IgE+ B cells all increases (Fig. 10). Our analyses also reveal an induction of IL-4 expression in Tfh cells at the initiation stage (D7), which is maintained at D11.

Based on these characterizations, we have focused on exploring the roles of Langerin+ DCs, TSLP and OX40L signaling in the initiation and expansion/maintenance stages of Tfh cell generation, as well as in the IL-4 commitment of Tfh cells (Fig. 10). Our findings are discussed below:

1) Initial generation of Tfh cells

We have identified that in MC903-induced AD, Langerin+ DCs play a crucial role in promoting Tfh cell differentiation, as the generation of Tfh cells at D7 was nearly abolished in Lang^DEP mice (Fig. 8E). This finding is in agreement with the implication of skin antigens in the Tfh cell generation in MC903-induced AD model, suggesting that Langerin+ DCs are the major antigen presenting cells (APCs) sensing and presenting skin antigens to prime Tfh cell differentiation. We have ongoing study to further define which Langerin+ DCs (epidermal LCs or Langerin+ dermal DCs) could be responsible for Tfh cell linage commitment, by taking use of the different recovery time for LCs and Langerin+ dDCs after DT-mediated depletion, as previous reported [21].

We have previously demonstrated that TSLP is required for MC903-induced AD [6, 9], and it has been recognized that TSLP induces Th2 cell differentiation through OX40L signaling [31, 32]. To our surprise, we find in this study that TSLP or OX40L signaling is dispensable for the initial generation of CXCR5^{+}PD-1^{+} Tfh cells, as ablation of TSLP or blockade of OX40L signaling does not lead to any reduction in Tfh cell number at D7, in contrast to the result from Lang[DEP] mice. This is also different from VDR^{-/-} or VDR[ep-/-] mice, where MC903-triggered Tfh cell generation is completely abolished. This suggests that MC903 activates certain factor(s) via VDR signaling in keratinocytes (KCs) which may act as an initial trigger for Tfh cell differentiation. It may be KC-derived molecules that participate in triggering skin inflammation thus facilitating the accessibility of foreign antigens, or self-antigens presented by Langerin[+] DCs for the Tfh cell lineage commitment.

2) IL-4 commitment in Tfh cells

Using intracellular cytokine staining and 4C13R[Tg/0] reporter mice, we showed that IL-4 expression was induced in Tfh cells as early as at D7. This is in consistent with previous reports that both Th2 and Tfh cells produce IL-4 [15, 17]. Interestingly, we found that although TLSP or OX40L signaling was not required for the initial generation of CXCR5^{+}PD-1^{+} Tfh cells, both of them are crucial for IL-4 induction in Tfh cells, thus suggesting that TSLP-OX40L signaling may play a crucial role in IL-4 commitment in Tfh cells. Moreover, as IL-4 induction in Tfh cells is also impaired in Lang[DEP] mice, this suggest that TSLP may activate LCs or Langerin[+] dermal DCs to carry out its role in the induction of IL-4 expression in Tfh cell, probably through OX40L signaling. However, the exact mechanism still needs to be determined. It has been reported that IL-4 expression in Tfh cells and Th2 cells is differentially regulated [33]. For example, the basic leucine zipper transcription factor ATF-like (Batf) was shown to be important for IL-4 expression in Tfh cells rather than in canonical Th2 cells [34]. It remains to be studied whether the regulation of IL-4 expression in Tfh cells by TSLP or OX40L involves Batf.

3）Expansion/ maintenance of Tfh cells and GC responses

Our analyses at different time points have revealed that although TSLP and OX40L

signaling are not required for the initial Tfh cell generation, they play an important role for the expansion/maintenance of Tfh cells. Indeed, Tfh cell number and GC B cell number are largely reduced at the later stage (i.e. D11) in mice with TSLP ablation or OX40L blockade (Fig. 4F&G, Fig. 9H).

So how does this happen? It could be due to the impaired IL-4 expression by Tfh cells in these mice. IL-4 was originally identified as a B cell-stimulating factor [35, 36], and numerous *in vivo* infection models have shown that IL-4 is critical for B cell expansion, B cell maturation and the isotype switch of B cells to IgE and IgG1 [33, 37-39]. Therefore, Tfh-derived IL-4 may promote GC B cell development, and the reciprocal interaction between Tfh cells and B cells in GC maintains the stability and survival of Tfh cells. Another possibility could the anti-apoptotic function of OX40L/OX40 interaction, as OX40L signaling has been reported to prolong the survival of activated T cells by promoting the expression of anti-apoptotic genes, such as Bcl2, Bcl-xL and BCL2A1, and reducing the expression of pro-apoptotic genes like FAS expression [40, 41]. A third possibility could be that OX40L expressed by B cells or GC B cells[42, 43] is implicated in the expansion/maintenance of Tfh cells. However, it is not clear whether there is a link between TSLP and OX40L signaling in B cells in draining lymph nodes.

4) Tfh and Th2

In addition to the regulation of IL-4 production by Tfh cells, TSLP also plays a key role in promoting Th2 response, as IL-13 expression in CD4^{+} T cells in MC903-treated TSLP^{-/-} mice is largely diminished at both D7 (Fig. 6D) and D11 (Fig. 4E). In contrast, IL-13 induction in CD4^{+} T cells is not apparently affected in mice either with OX40L blocking (Fig. 9G) or depletion of Langerin^{+} DCs (Fig. 8G). This may suggest that the role of TSLP in promoting Th2 differentiation is not mediated by Langerin[+] DCs or OX40L signaling. It remains to be seen which skin DCs are implicated in TSLP-induced Th2 differentiation and what are the signaling axis involved.

Taken together, we have used in this study the MC903-induced AD model to investigate the Tfh cell differentiation and regulation. Our findings provide insight into the mechanisms underlying the type 2 immune response in AD pathogenesis. It will be interesting to validate our findings in mice with pathophysiological relevance to human AD, and to further explore

the pathogenic role of Tfh cells in AD.

Methods and Materials

Mice

All mice used were on the Balb/c background TSLP-/- [44], VDR$^{-/-}$ [45], VDR$^{ep-/-}$ [9] have been previously described. DO11.10$^{Tg/Tg}$ mice were from Jackson Laboratory [19]. 4C13R$^{Tg/0}$ mice were kindly provided by Dr. W. Paul (NIH) [18]. Lang-DTREGFP$^{+/KI}$ mice were kindly provided by Dr. B. Malissen (CIML) [20]. Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the Animal Care and Use Committee of the Institut de Génétique et de Biologie Moléculaire et Cellulaire.

MC903 Topical Application

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

Depletion of Langerin+ DCs *in vivo*

For depletion of Langerin+ DCs, 1µg of DT (Sigma) in PBS was i.p injected into Lang-DTREGFP$^{+/KI}$ mice every 4 days started from D-2 (Fig. 5A)，DT-injected wildtype littermatte mice were used as control.

In Vivo Blockade of OX40L

To block OX40L-OX40 interaction, mice were i.p injected with 300µg rat-anti-mouse-OX40L neutralizing mAB (clone RM134L, BioXCell) very three days from D-1 to D11, Rat IgG2b was used as an isotype control.

Preparation of dermal cell suspension

Mouse ears were taken and were split into ventral and dorsal halves with fine forceps. The ear halves were incubated with 2.5 mg/mL Dispase (Gibco) in PBS for 1h at 37°C. After separation from the epidermis, the dermis was chopped and incubated in PBS with 1

mg/mL collagenase D (Roche), 0.25 mg/mL DNase I (Sigma) and 2.5 % fetal calf serum at 37°C for 1h. Dermal cells were filtered with 70 µm strainer (BD Biosciences) and cell number was counted with a hemocytometer (Neubauer improved, Marienfield).

Preparation of epidermal cell suspension

After separation from the dermis, the epidermis was chopped and incubated in PBS with 1 mg/mL collagenase D (Roche), 0.25 mg/mL DNase I (Sigma) and 2.5 % fetal calf serum at 37°C for 30 min. Epidermal cells were filtered with 70 µm strainer (BD Biosciences) and cell number was counted.

Antibodies and flow cytometry

The following antibodies were used:

antibody	Fluorochrome	Clone	Company
Bcl6	Alexa Fluor 647	K112-91	BD
CD3	FITC	145-2C11	eBioscience
CD3	V500	500A2	BD
CD4	BV421	GK1.5	Biolegend
CD4	Pacific Blue	RM4.5	Biolegend
CD11b	Pacific Blue	M1/70	Biolegend
CD11c	Biotin	HL3	BD
CD16/CD32		2.4G2	BD
CD19	FITC	1D3	BD
CD19	PerCP-Cy5.5	eBio1D3	eBioscience
CD24	APC	M1/69	BD
CD24	BV650	M1/69	BD
CD45	APC	30-F11	eBioscience
CD45	APC-eFluor 780	30-F11	eBioscience
CD45R/B220	APC	RA3-6B2	eBioscience
CD45R/B220	FITC	RA3-6B2	Biolegend
CD45R/B220	PE-Cy7	RA3-6B2	Biolegend

CD49b	Biotin	DX5	eBioscience
CD8a	PerCP-Cy5.5	53-6.7	eBioscience
CD95 (Fas)	PE-Cy7	Jo2	BD
CXCR5	Biotin	2G8	BD
DO11.10	FITC	KJ1-26	eBioscience
GL7	Pacific Blue	GL-7	Biolegend
GL7	PE	GL-7	eBioscience
IFN-g	APC	XMG1.2	eBioscience
IgD	FITC	11-26c.2a	BD
IgE	Biotin	R35-72	eBioscience
IgG1	PerCP-Cy5.5	RMG1-1	Biolegend
IL-13	eFluor 660	eBio13A	eBioscience
IL-17a	PE	TC11-18H10	BD
IL-21	PE	FFA21	eBioscience
IL-3	PE	MP2-8F8	Biolegend
IL-4	Alexa Fluor 488	11B11	BD
MHCII	APC	M5/114.15.2	eBioscience
MHCII	FITC	M5/114.15.2	eBioscience
OX40	APC	OX86	eBioscience
PD-1	APC	RMP1-30	Biolegend
PD-1	PE-Cy7	RMP1-30	Biolegend
Streptavidin	BV605		BD
TCR Beta	PerCP-Cy5.5	H57-597	eBioscience

For surface marker staining, 1×10^6 cells were incubated with CD16/CD32 antibody on ice for 10 minutes to block Fc receptors, washed and incubated with biotinylated antibody, and then washed and incubated with a mix of antibodies on ice for 10 minutes. Finally, cells were washed and analyzed. For Tfh cell surface staining, 1.5×10^6 EDLN cells were incubated with CD16/CD32 antibody on ice for 10 minutes to block Fc receptors, washed and incubated with CXCR5 antibody on ice for 45 minutes. Cells were then washed and incubated with BV605-conjugated streptavidin and other surface markers on ice for 10 minutes. Finally cells were washed and analyzed.

For cytokine intracellular staining of EDLN cells, 1.5×10^6 EDLN cells were incubated with FcRIII/II antibody on ice for 10 minutes to block Fc receptors, washed and incubated with CXCR5 antibody on ice for 45 minutes. Cells were then washed and incubated with BV605-conjugated streptavidin on ice for 20 minutes. After wash, EDLN cells were stimulated for 4 hours at 5×10^6 cells/ml in RPMI 1640 medium with Cell Stimulation Cocktail (PMA: 50 ng/ml, ionomycin, 500 ng/ml; eBioscience) and BD golgi stop (BD Biosciences). After washing, cells were incubated with surface markers. Cells were then fixed and permeabilized using BD Cytofix/CytopermTM Kit (BD Biosciences) and stained with anti-IL-4 antibody, anti-IL-3 antibody, anti-IL-13 antibody, anti-IL-17 antibody, anti-IL-21 antibody or anti-IFN-γ antibody. Cells were analyzed on a LSRII flow cytometer (BD Biosciences). Results were analyzed using FlowJo (Treestar).

For Bcl6 intracellular staining of EDLN cells, 1.5×10^6 EDLN cells were incubated with FcRIII/II antibody on ice for 10 minutes to block Fc receptors, washed and incubated with CXCR5 antibody on ice for 45 minutes. Cells were then washed and incubated with surface markers. Cells were then fixed and permeabilized using BD Cytofix/CytopermTM Kit (BD Biosciences) and stained with anti-Bcl6 antibody. Cells were analyzed on a LSRII flow cytometer (BD Biosciences). Results were analyzed using FlowJo (Treestar).

For intracellular staining of dermal cells, 1.5×10^6 cells were cultured in a medium (RPMI 1640 with 10% FCS) with Cell Stimulation Cocktail (PMA: 50 ng/ml, ionomycin, 500 ng/ml; eBioscience) and BD GolgiStop (BD Biosciences) for 2 h. After washing, cells were blocked with anti-FcγRIII/II, followed by surface staining of anti-CD45, anti-CD3, anti-CD49b, anti-TCRβ. Cells were then fixed and permeabilized using BD Cytofix/CytopermTM Kit (BD Biosciences), and stained with anti-IL-4, anti-IL-3 antibodies. Stained cells were finally analyzed on a LSRII flow cytometer (BD Biosciences). Results were analyzed using FlowJo (Treestar).

RNA extraction

Whole ear tissue was homogenized with ultraturax in 1mL Trizol reagent (Sigma-Aldrich), and incubated 5 min at room temperature. 200µL chloroforme was added into homogenised tissue solution, and then vortexed for 15 second, incubated 5 min at room temperature, and then centrifuged at 12000 rpm for 15 minutes at 4°C. The aqueous at the

upper phase was taken to a new tube, added with 500µL isopropanol, incubated for 10 minutes at room temperature, and was centrifuged at 12000rpm for 10 minutes at 4°C. Supernatant was then removed, and precipitation was washed with 1mL 75% EtOH and was centrifuged at 7500 rpm for 5 minutes at 4°C. Pellet was dried and dissolved in RNase-free H_2O.

Quantitative PCR

mRNA was reverse-transcribed using random oligonucleotide hexamers and amplified by quantitative PCR with a Ligthcycler 480 (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturers instructions. Relative RNA levels were calculated using hypoxanthine phosphoribosyltransferase (HPRT) as internal control.

Microscopy

After harvesting, lymph nodes were fixed in 4% PFA overnight at +4°C. After 2 washing of 30 minutes with PBS, the samples were included in 4% low melting point agarose in PBS. The sections of 100 um, obtained using a vibratome (Leica VT1000S), were blocked with 5% NDS, 0,1% Triton X-100 in 1x PBS and then stained overnight at +4°C with anti CD4-AlexaFluor 647 (RM4-5, Biolegend, d=1/100), anti IgD-FITC (11-26c.2a, BD Biosciences, d-1/50) and biotinylated PNA (ref. B-1075, Vectorlabs, d=1/250) diluted in 5% NDS, 0,1% Triton X-100 in 1x PBS. After 2 washing of 30 minutes with PBS, sections were incubated 1 hour with Neutravidin-DyLight 550 (ref. 84606, ThermoFischer, d=1/200) diluted in PBS. After 2 washing of 30 minutes with PBS, sections were kept in PBS containing 1 ug/mL Hoechst 33342 (ref. B-2261, Sigma Aldrich) and images were acquired using Leica LSI confocal macroscope.

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A

1 nmol MC903 in ETOH or ETOH on ear:

D0   D2   D4   D6   D8   D10

Analyses: D11

B

EDLN (D11)

Total lymph node cell number ($\times 10^6$)

P=0.008

ETOH   MC903

EDLN (D11)

CD4+ T cells in EDLN cells(%)

ETOH   MC903

EDLN (D11)

CD4+ T cell number ($\times 10^6$)

P=0.008

ETOH   MC903

C

EDLN (D11) ICS
gated on CD3+CD4+ cells

SSC-A

0.364   0.303   2.36   ETOH

1.08   0.282   2.62   MC903

IL-4   IL-17   IFN-g

Percentage in total CD3+ CD4+ cell(%)

□ EtOH
■ MC903

P=0.08   P=0.62   P=0.56

IL-4+   IL-17+   IFN-g+

D

EDLN (D11)
gated on CD3+CD4+ cells

ETOH   MC903

PD-1

0.665   4.52

CXCR5

EDLN (D11)

CXCR5+PD-1+ cell in CD4+ T cells (%)

P=0.003

ETOH   MC903

EDLN (D11)

CXCR5+PD-1+ cell number ($\times 10^5$)

P=0.007

ETOH   MC903

E

EDLN
gated on CD3+CD4+CXCR5+
PD-1+ cells

■ CXCR5-PD-1-
□ CXCR5+PD-1+

MFI: 32.2   510
1515   14505

D0   D11

Events (% of Max)

ICOS

EDLN
gated on CD3+CD4+CXCR5+
PD-1+ cells

■ CXCR5-PD-1-
□ CXCR5+PD-1+

MFI: 172   261
262   792

D0   D11

Events (% of Max)

Bcl6

Figure1 I

88

F

EDLN (D11) ICS

gated on CD3+CD4+CXCR5+PD-1+ cells

G

EDLN (D11)

gated on CD3+CD4+ cells

EDLN (D11)

gated on CD3+CD4+CXCR5+PD-1+ cells

AmCyan(IL-4)+ cells in EDLN (D11)

H

EDLN (D11)

gated on CD3+CD4+ cells

EDLN (D11)

gated on CD3+CD4+CXCR5+PD-1+ cells

DsRed(IL-13)+ cells in EDLN (D11)

Figure 1 II

89

Figure 1. Skin MC903 treatment promotes Tfh cell differentiation in draining lymph nodes. (A) Experimental protocol. Ears of Balb/c mice were topically treated with MC903 or ethanol (ETOH; as vehicle control) every other day from day (D) 0 to D11 and analyzed at D11. (B) Total cell number and CD4 T cell number in the ear-draining lymph nodes (EDLN) of ETOH- or MC903-treated mice. (C) Comparison of percentages of IL-4-, IL-17- or IFN-γ-producing cells in $CD3^+CD4^+$ T cells, examined by intracellular staining (ICS). (D) Comparison of $CXCR5^+PD-1^+$ cells in the EDLNs of ETOH- or MC903-treated mice. (E) $CXCR5^+PD-1^+$ $CD4^+$ T cells express ICOS and Bcl6, indicating they are bona-fide Tfh cells. (F) Comparison of percentages of IL-4-, IL-17- or IFN-γ-producing cells in $CXCR5^+PD-1^+$ Tfh cells, examined by ICS. (G) Comparison of Amcyan(IL-4)$^+$ cells in $CD4^+$ T cells, and in Tfh cells from EDLNs of ETOH- and MC903-treated 4C13R$^{tg/0}$ (IL-4 and IL-13 dual reporter) mice. (H) Comparison of DsRed(IL-13)$^+$ cells in $CD4^+$ T cells and in Tfh cells from EDLNs of ETOH- and MC903-treated 4C13R$^{tg/0}$ mice.
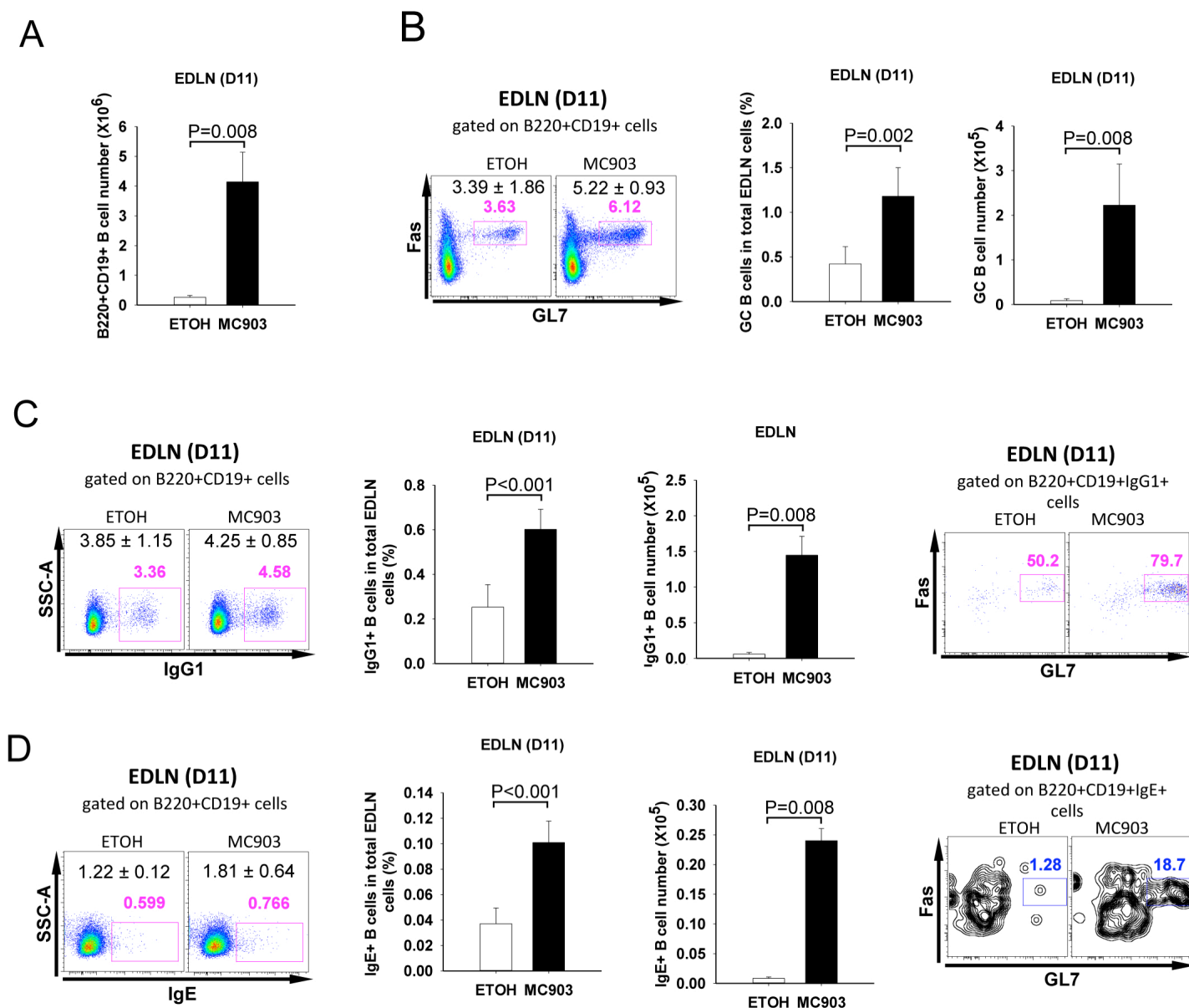
Figure 2. Skin MC903 treatment induces germinal center (GC) formation in draining lymph nodes. (A) Total B cell number in EDLNs from ETOH- or MC903-treated Balb/c mice at D11. (B) Percentage and cell number of GC B (B220$^+$CD19$^+$Fas$^+$GL7$^+$) cells. (C) Percentage and cell number of IgG1$^+$ B cells. (D) Percentage and cell number of IgE$^+$ B cells. Values are mean±s.e.m. (n>=4) (Student's t-test).
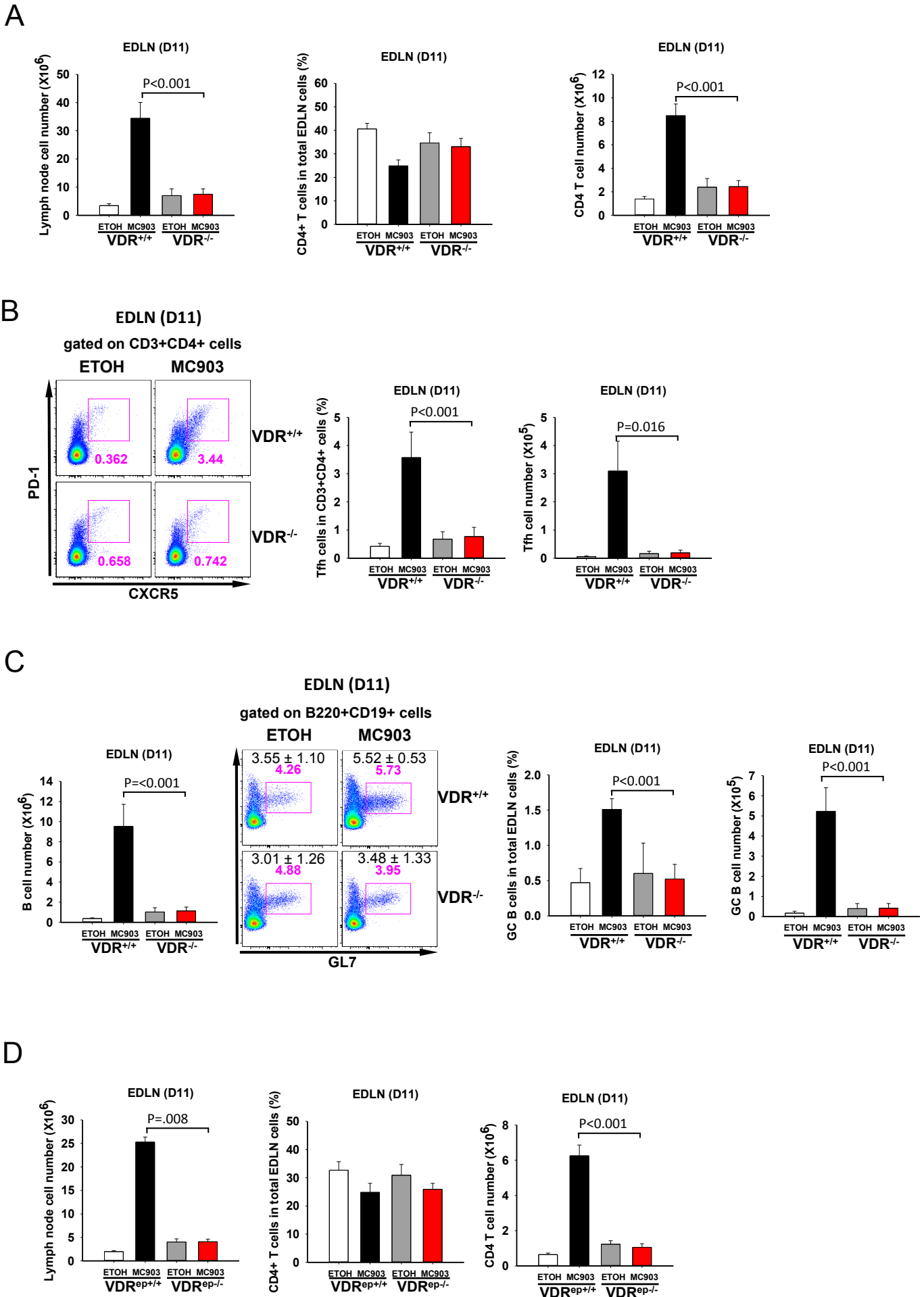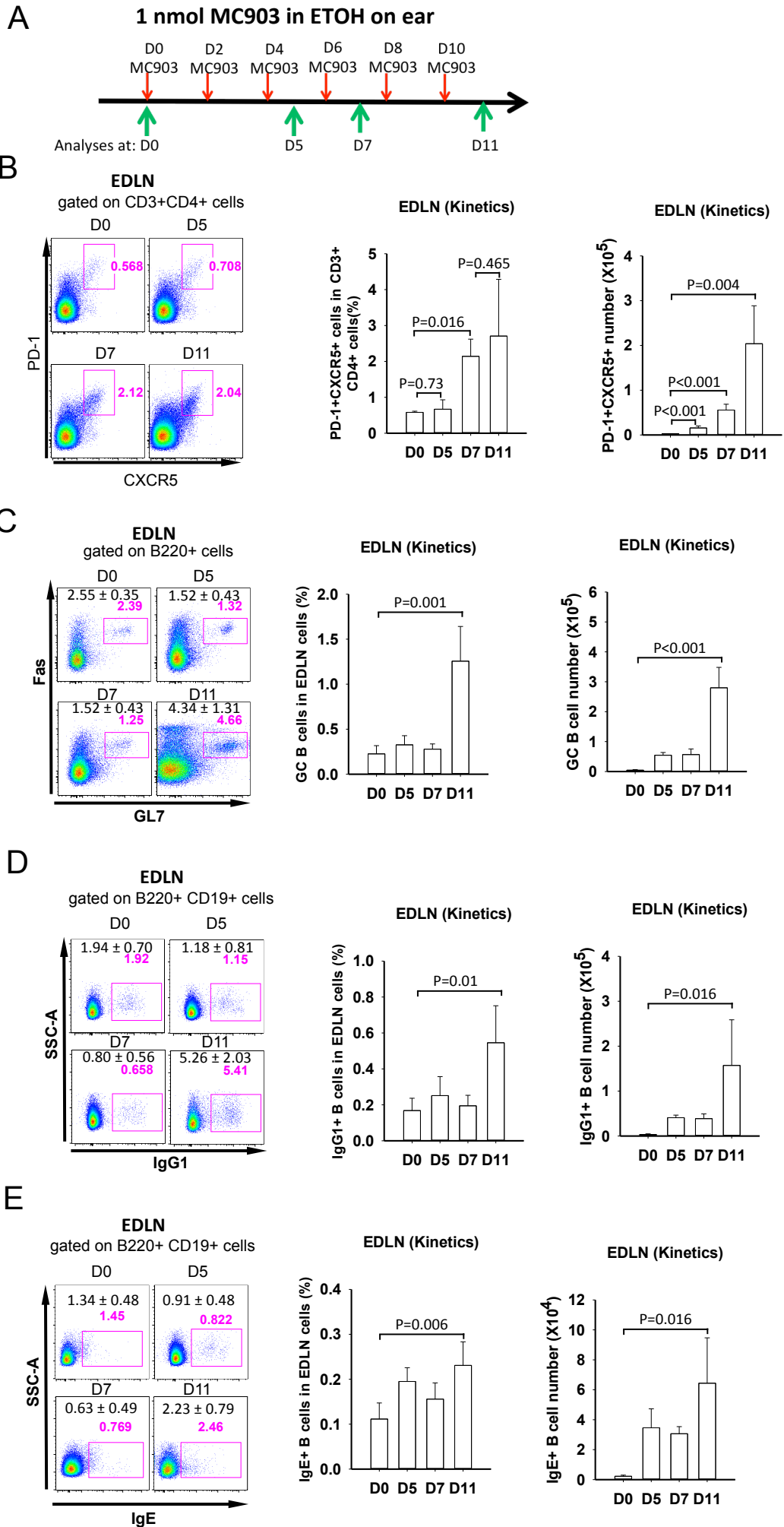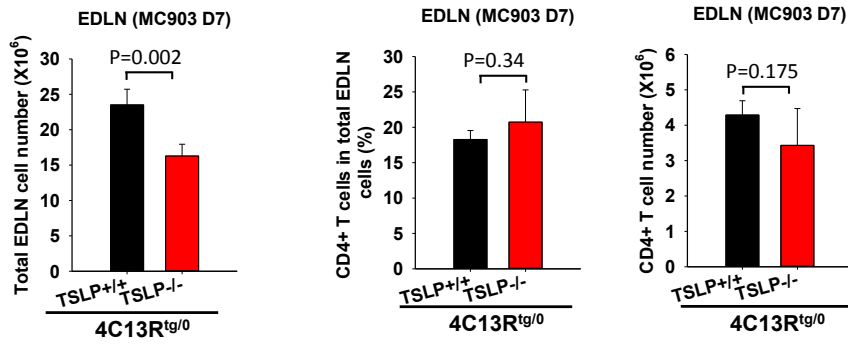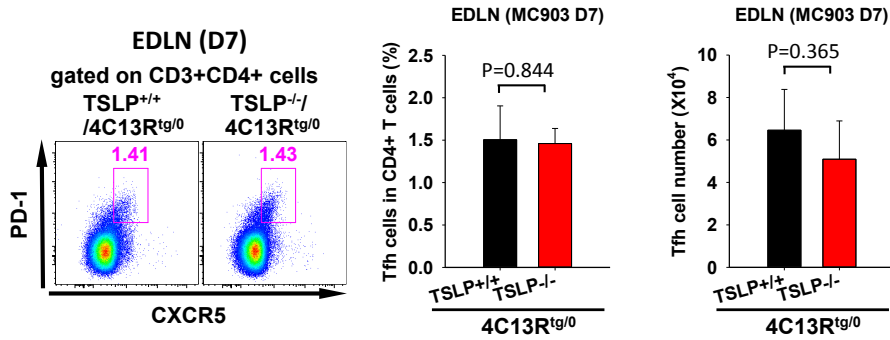
Figure 3 I
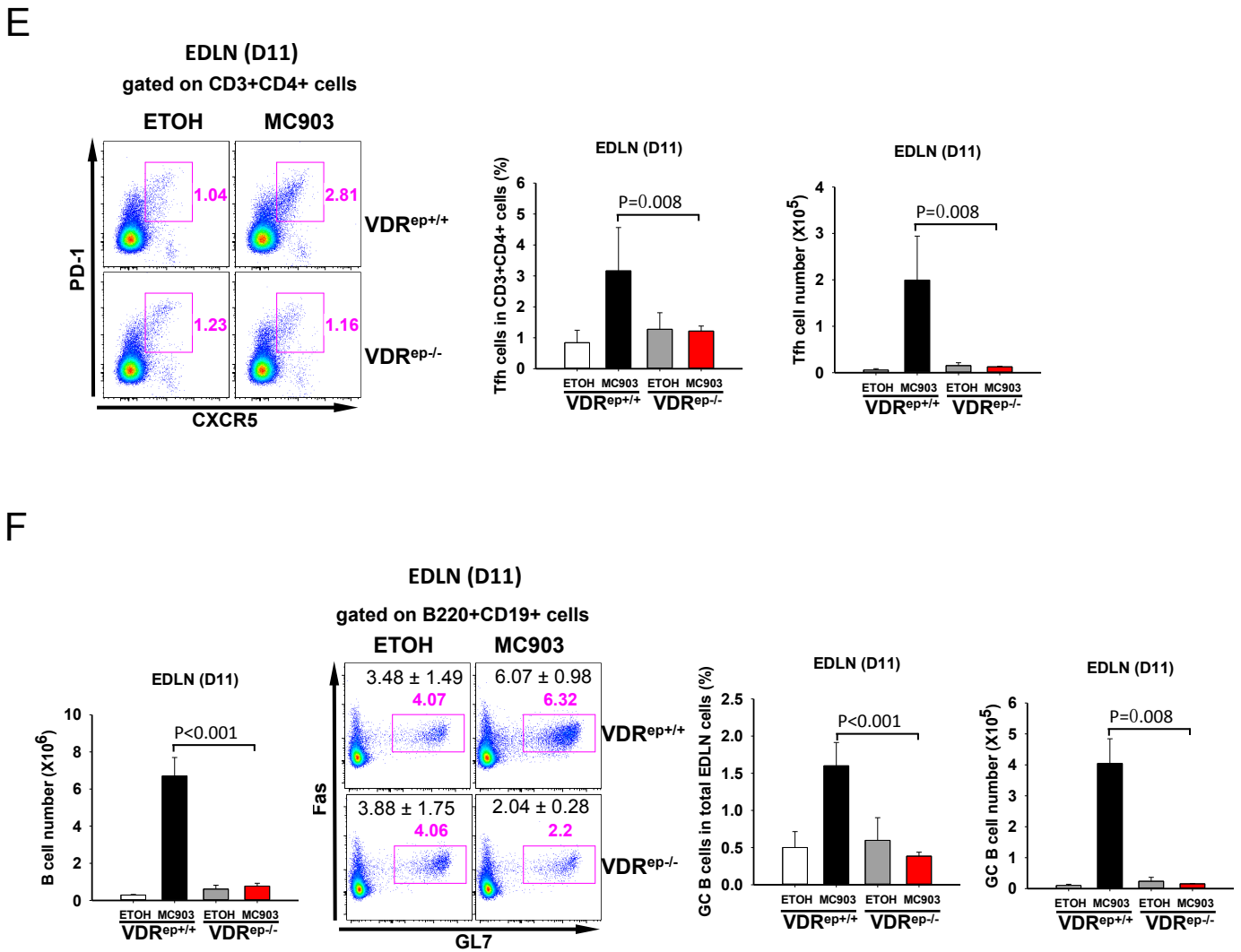
Figure 3 II

Figure 3. MC903-induced Tfh cell differentiation and GC formation are abolished in mice lacking VDR. (A) Comparison of total cell number and CD4 T cell number in EDLNs of ETOH- or MC903-treated VDR$^{-/-}$ mice and WT mice at D11. (B) Percentage and total number of Tfh (CD3$^+$CD4$^+$CXCR5$^+$PD-1$^+$) cells. (C) Percentage and total number of GC B (Fas$^+$GL7$^+$B220$^+$CD19$^+$) cells. (D) Comparison of total cell number and CD4$^+$ T cell number in EDLNs of ETOH- or MC903-treated VDR$^{ep-/-}$ mice and WT mice at D11. (E) Percentage and total number of Tfh cells. (F) Percentage and total number of GC B cells. Values are mean±s.e.m. (n>=4). (Student's t-test).
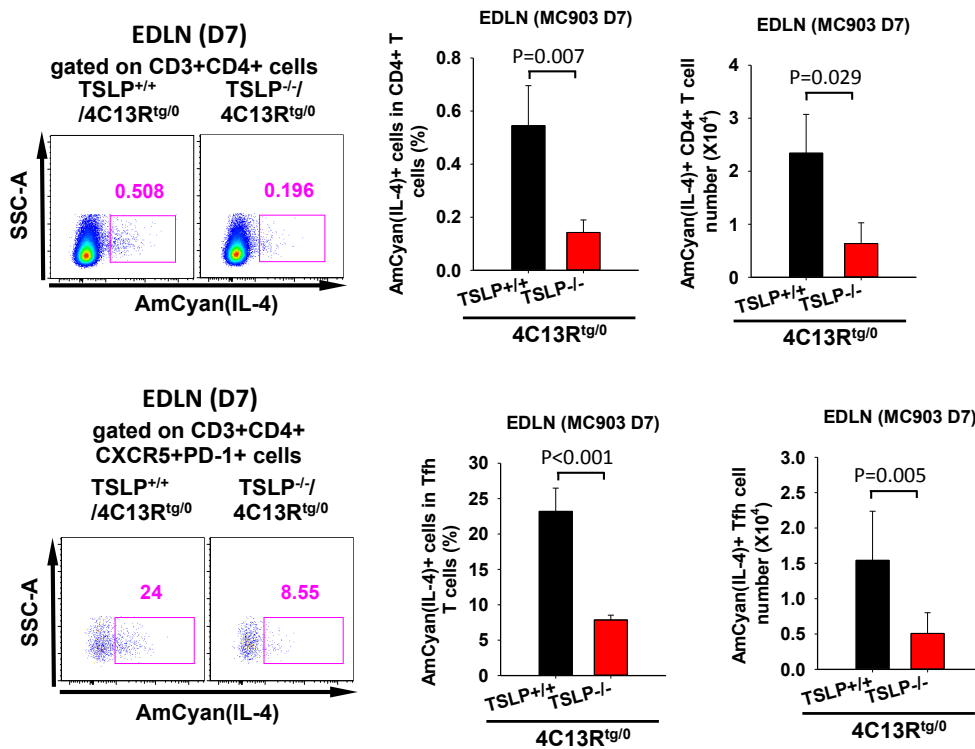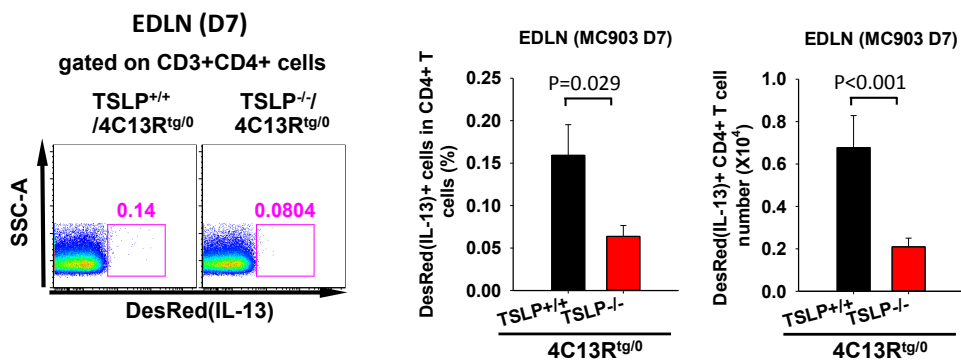
Figure 4 I

D

EDLN (D11)
gated on CD3+CD4+ cells
ETOH   MC903

TSLP+/+/4C13R^tg/0

TSLP-/-/4C13R^tg/0

EDLN (D11)
gated on CD3+CD4+CXCR5+PD-1+ cells
ETOH   MC903

TSLP+/+/4C13R^tg/0

TSLP-/-/4C13R^tg/0

E

EDLN (D11) ICS
gated on CD3+CD4+ cells
ETOH   MC903

TSLP+/+

TSLP-/-

EDLN (D11)
gated on CD3+CD4+ cells
ETOH   MC903

TSLP+/+/4C13R^tg/0

TSLP-/-/4C13R^tg/0

Figure 4 II

95

F

EDLN (D11)

B cell number (X10⁶) — P<0.001

TSLP+/+ ETOH, TSLP+/+ MC903, TSLP-/- ETOH, TSLP-/- MC903

EDLN (D11)
gated on B220+CD19+ cells

ETOH   MC903

4.48 ± 0.93   5.59 ± 0.71   TSLP⁺/⁺
3.82   6.14

4.39 ± 0.63   6.33 ± 2.27   TSLP⁻/⁻
4.32   7.88

Fas / GL7

EDLN (D11)

GC B cells in total EDLN cells(%) — P=1

EDLN (D11)

Total GC B cell number (X10⁵) — P=0.001, P=0.014

G

EDLN (D11)

TSLP⁺/⁺   TSLP⁻/⁻

IgD-FITC
CD4-Alexia 647
PNA-biotin-s
Dylight 550

ETOH

MC903

EDLN (D11)

GC number in EDLN

EDLN (D11)

GC area in EDLN (X10⁶ um²) — P=0.003

H

EDLN (D11)
gated on B220+CD19+ cells

TSLP+/+   TSLP-/-

SSC-A

2.53 ± 0.26   3.55 ± 0.70
2.69   3.21

IgG1

EDLN (D11)

IgG1+ B cells in total EDLN cells (%) — P=0.82

EDLN (D11)

IgG1+ B cell number (X10⁵) — P<0.001

I

EDLN (D11)
gated on B220+CD19+ cells

TSLP+/+   TSLP-/-

SSC-A

0.34 ± 0.05   0.18 ± 0.03
0.352   0.188

IgE

EDLN (D11)

IgE+ B cells in total EDLN cells(%) — P<0.001

EDLN (D11)

IgE+ B cell number (X10⁵) — P=0.008

Figure 4 III

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Figure 4. Mice lacking TSLP exhibit decreased number and IL-4 expression of Tfh cells, as well as diminished GC formation upon MC903 treatment. (A) Comparison of total cell number and CD4 T cell number in EDLNs of ETOH- or MC903-treated TSLP$^{-/-}$ mice and WT mice at D11. (B) Percentage and total number of Tfh (CD3$^+$CD4$^+$CXCR5$^+$PD-1$^+$) cells. (C) Intracellular staining of IL-4 in CD4$^+$ T cells (upper panel), and in Tfh cells (lower panel). (D) Amcyan(IL-4)$^+$ cells in CD4+ T cells (upper panel) and in Tfh cells (lower panel) in EDLNs from ETOH- or MC903-treated TSLP$^{+/+}$4C13R$^{tg/0}$ and TSLP$^{-/-}$4C13R$^{tg/0}$ mice at D11. (E) Comparison of IL-13 expression in CD4$^+$ T cells, shown by intracellular staining of EDLN cells from ETOH- or MC903-treated TSLP$^{+/+}$ and TSLP$^{-/-}$ mice (upper panel), or by DsRed(IL-13)+ cells in EDLNs from ETOH- or MC903-treated TSLP$^{+/+}$4C13R$^{tg/0}$ and TSLP$^{-/-}$4C13R$^{tg/0}$ mice (lower panel). (F) Percentage and total number of GC B (Fas$^+$GL7$^+$B220$^+$CD19$^+$) cells in EDLNs from ETOH- or MC903-treated TSLP$^{-/-}$ and WT mice. (G) EDLN sections stained with CD4 (blue), IgD (green) and peanut agglutinin (PNA, red), and comparison of GC number and GC areas in EDLNs (H) Percentage and total number of IgG1$^+$ B cells in EDLN. (I) Percentage and total number of IgE$^+$ B cells in EDLN. Values are mean±s.e.m. (n=4) (Student's t-test).

Figure 5. Kinetics of Tfh and GC B cells in EDLN upon MC903 treatment. (A) Experimental protocol. Ears of Balb/c mice were topically treated with MC903 every other day from day (D) 0, and EDLN were analyzed at D0, 5, 7 and 11. (B) Percentage and total number of Tfh (CD3$^+$CD4$^+$CXCR5$^+$PD-1$^+$) cells. (C) Percentage and total number of GC B (Fas$^+$GL7$^+$B220$^+$CD19$^+$) cells. (D) Percentage and total number of IgG1$^+$ B cells. (E) Percentage and total number of IgE$^+$ B cells. Values are mean±s.e.m. (n>=4). (Student's t-test).

Figure 6. TSLP is not required for the initial generation of Tfh cells, but is crucial for their IL-4 expression. TSLP$^{+/+}$4C13R$^{tg/0}$ and TSLP$^{-/-}$4C13R$^{tg/0}$ mice were treated with MC903, and EDLNs were analyzed at D7. (A) Total cell number and CD4^{+} T cell number in EDLNs. (B) Percentage and total number of Tfh (CD3^{+}CD4^{+}CXCR5^{+}PD-1^{+}) cells. (C) Amcyan(IL-4)$^{+}$ cells in CD4^{+} T cells (upper panel) and in Tfh cells (lower panel). (D) DsRed(IL-13)$^{+}$ cells in CD4^{+} T cells. Values are mean±s.e.m. (n>=4). (Student's t-test).

A EDLN (D11)
gated on CD3+CD4+ cells

B

C EDLN (D11)

D EDLN (D11)
gated on CD3+CD4+ cells

E EDLN (D11)
gated on B220+CD19+ cells
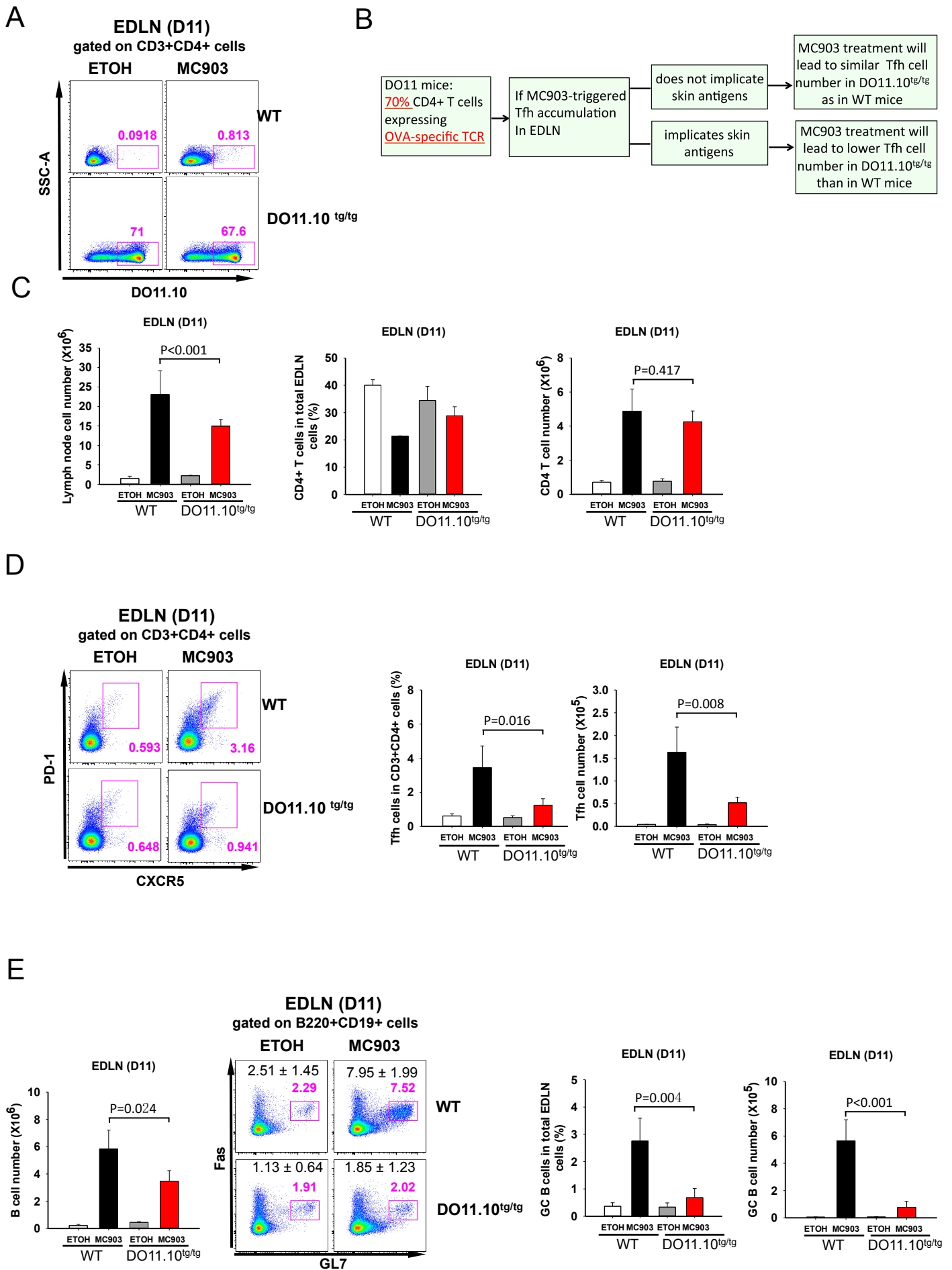
Figure 7. MC903-induced Tfh cell differentiation implicates skin antigens. (A) Staining of EDLN cells with anti-DO11.10 TCR antibody, showing about 70% of CD4$^+$ cells expressing OVA-specific TCR in DO11.10$^{tg/tg}$ mice. (B) Test for the implication of skin antigens in MC903-induced Tfh differentiation using DO11.10$^{tg/tg}$ mice. (C) Total cell number and CD4$^+$ T cell number in EDLNs of ETOH- or MC903-treated Balb/c WT and DO11.10$^{tg/tg}$ mice, at D11. (D) Percentage and total number of Tfh (CD3$^+$CD4$^+$CXCR5$^+$PD-1$^+$) cells. (E) Percentage and total number of GC B (Fas$^+$GL7$^+$B220$^+$CD19$^+$) cells. Values are mean±s.e.m. (n=4) (Student's t-test).

A

1 nmol MC903 in ETOH or ETOH on ear:

D0   D2   D4   D6   D8   D10

D-2   D2   D6   D10

DT: 1μg /mouse/ injection

Analyses: D7   D11

CT: DT-injected Lang-DTREGFP⁺/⁺
Lang^DEP: DT-injected Lang-DTREGFP⁺/KI

B

Langerin (D7)

Langerin (D11)

C

Epidermis D7
Gated on CD45⁺ cells

Dermis D7
Gated on CD45⁺ cells

Dermis D7
Gated on CD45⁺ MHCII⁺ cells

Epidermis D11
Gated on CD45⁺ cells

Dermis D11
Gated on CD45⁺ cells

D

EDLN   EDLN   EDLN

E

EDLN (D7)
gated on CD3+CD4+ cells

EDLN (D11)
gated on CD3+CD4+ cells

EDLN   EDLN

Figure 8 I

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F

EDLN (MC903) ICS
gated on CD3+CD4+ cells

EDLN (MC903) ICS
gated on CD3+CD4+CXCR5+PD-1+cells

G

EDLN (MC903 D11)
gated on CD3+CD4+ cells

Figure 8 II

Figure 8 III

Figure 8. Role of Langerin$^+$ DCs in the generation and maintenance of Tfh cells, in the expression of IL-4 by Tfh cells, and in GC formation. (A) Experimental protocol. Lang-DTREGFP$^{+/KI}$ and littermate wildtype control (Lang-DTREGFP$^{+/+}$) mice were injected with DT every 4 days started from D-2, and MC903 or ETOH was topical applied to ears every 2 days from D0. CT, DT-injected Lang-DTREGFP$^{+/+}$ mice; Lang^{DEP}, DT-injected Lang-DTREGFP$^{+/KI}$ mice. EDLNs were analyzed at D7 and D11. (B) RT-qPCR detection of Langerin gene in ears at D7 and D11. (C) Epidermal and dermal cells were analyzed by FACS. In epidermis, Langerhans cells are gated as CD45$^+$MHC II$^+$CD24$^+$. In dermis, the gated CD45$^+$MHC II$^+$CD24$^+$ cells contain migrating LCs (CD11b^{int}CD24$^+$) and dermal Langerin$^+$ DCs (CD11b^{low}CD24$^+$). (D) Total cell number and CD4$^+$ T cell number in EDLNs of ETOH- or MC903-treated Lang^{DEP} mice and CT mice at D7 and D11. (E) Percentage and total number of Tfh (CD3$^+$CD4$^+$CXCR5$^+$PD-1$^+$) cells. (F) Intracellular staining of IL-4 in CD4+ T cells (upper panel), and in Tfh cells (lower panel), at D7 and D11. (G) Intracellular staining of IL-13 in CD4$^+$ T cells at D11. (H) Percentage and total number of GC B (Fas$^+$GL7$^+$B220$^+$CD19$^+$) cells at D11. (I) Percentage and total number of IgG1$^+$ B cells at D11. (J) Percentage and total number of IgE$^+$ B cells at D11. Values are mean±s.e.m. (n=4) (Student's t-test).
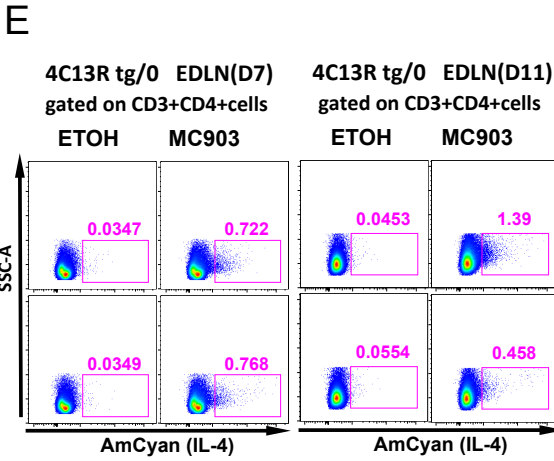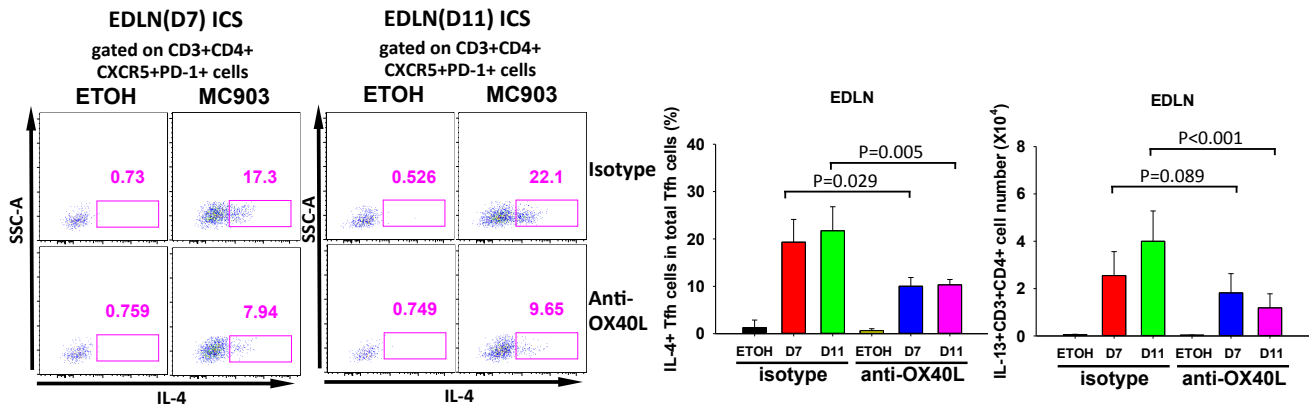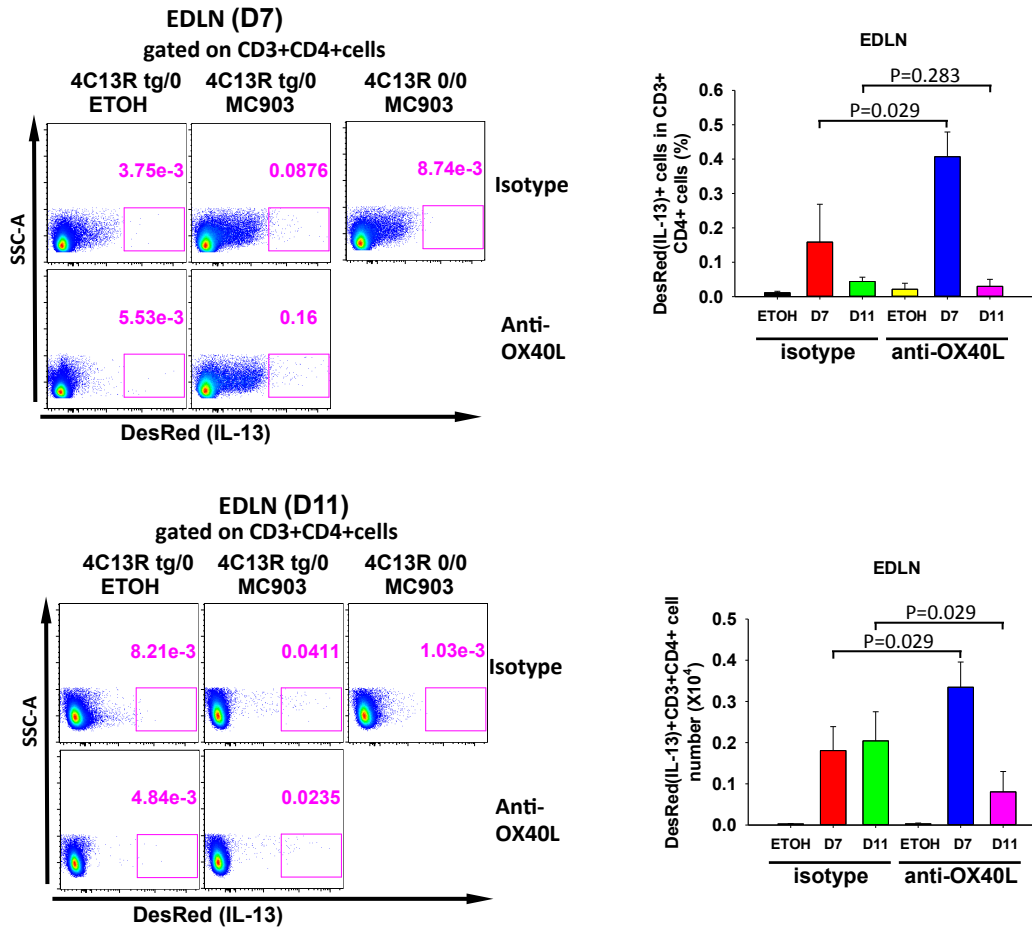
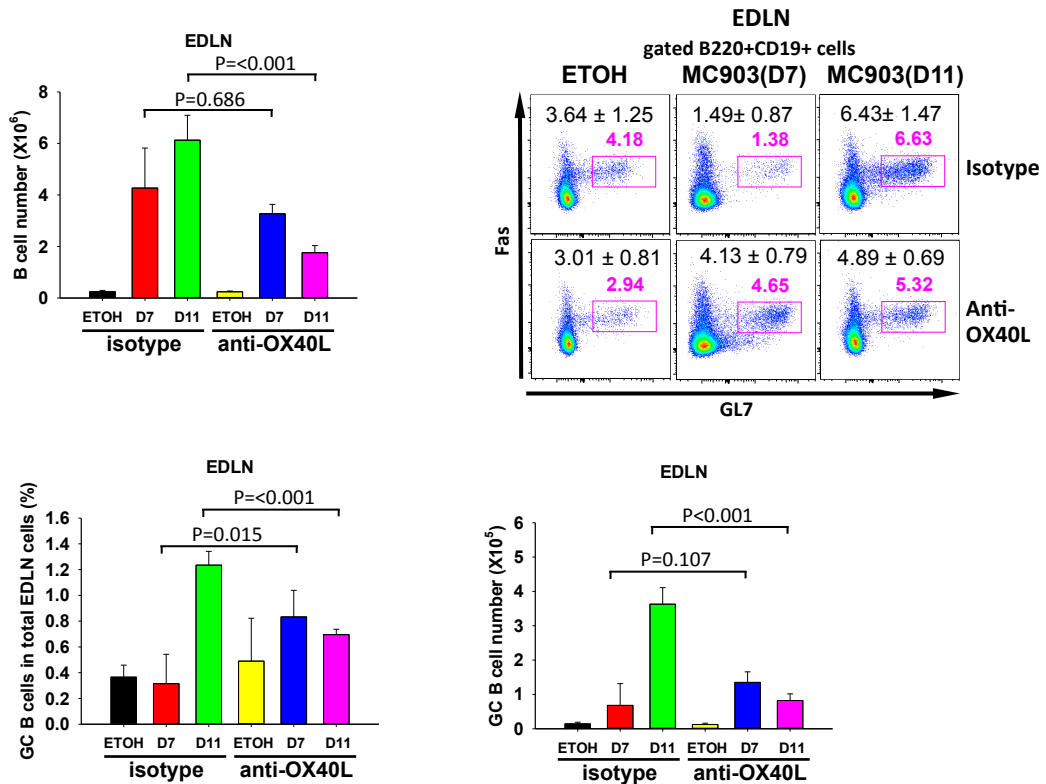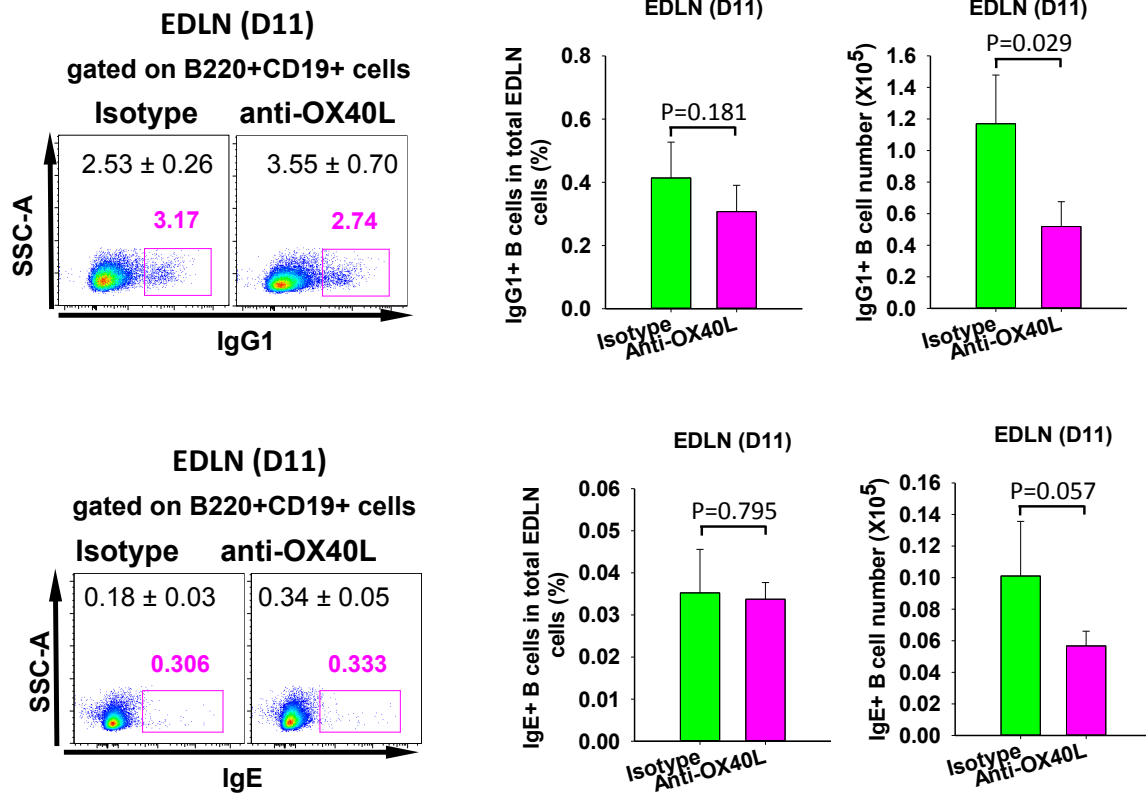.

Figure 9 I

E

F
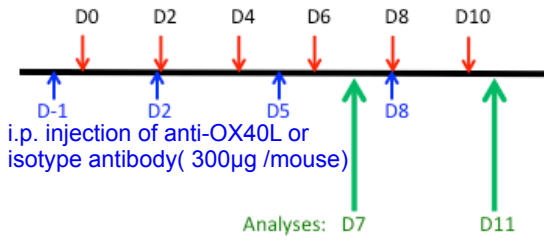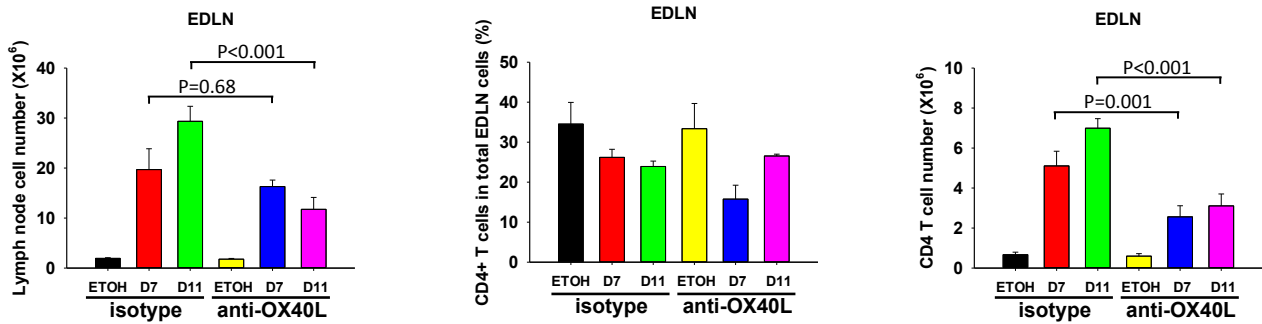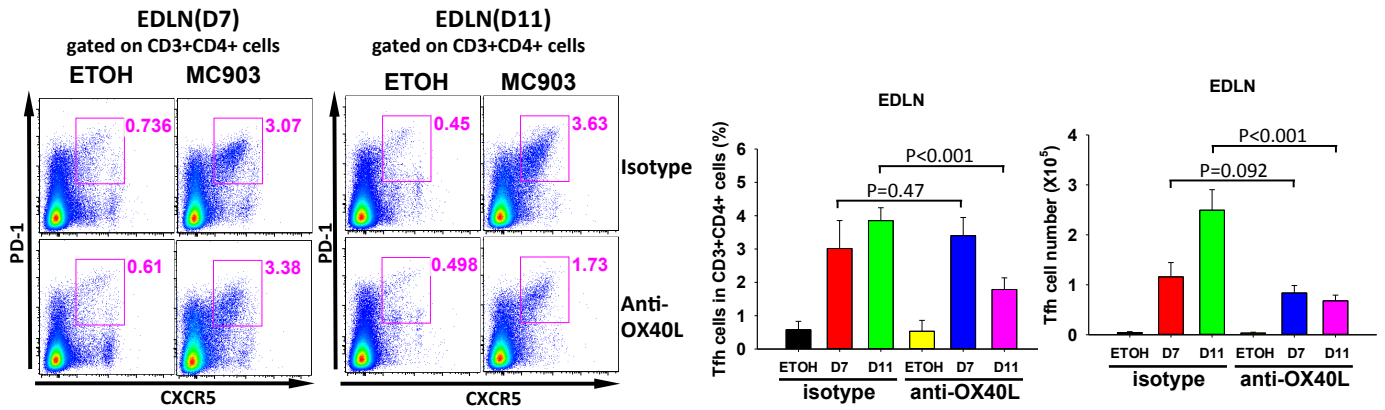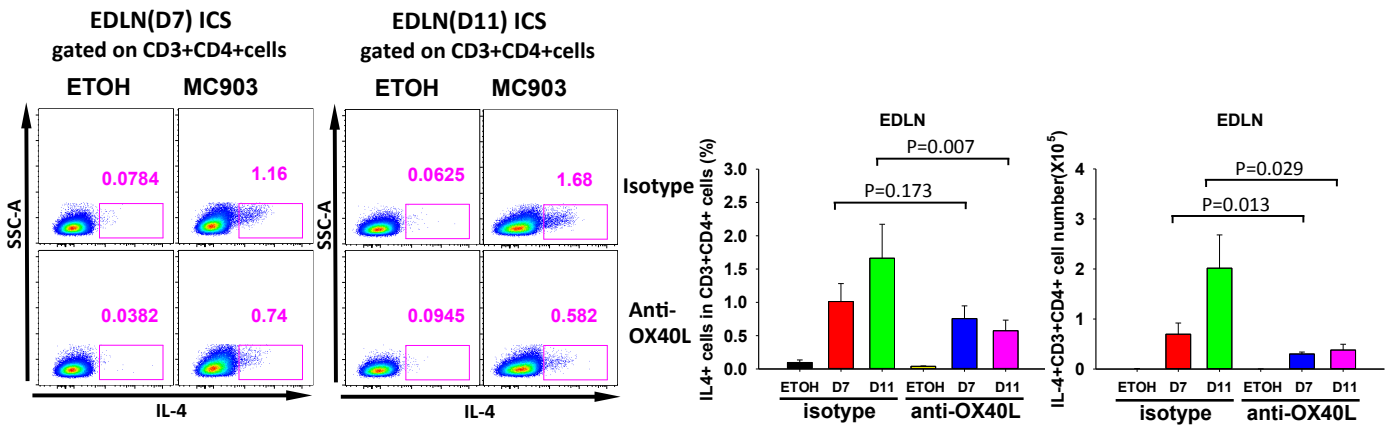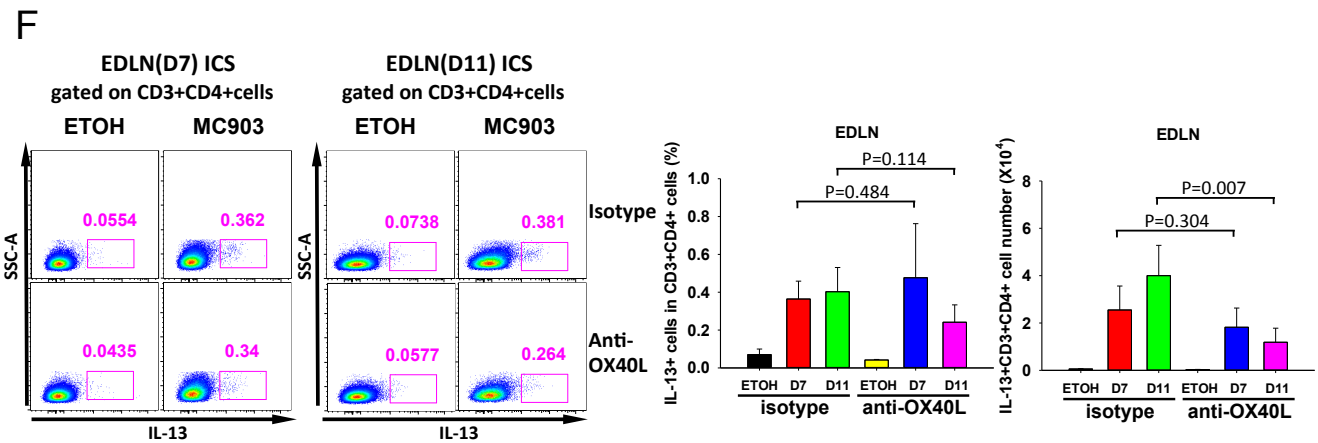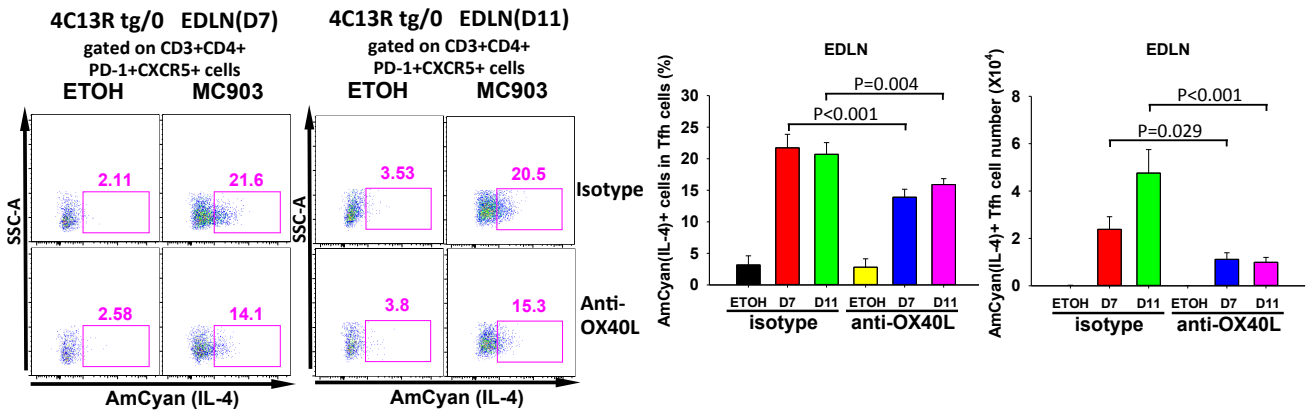
Figure 9

Figure 9 II

Figure 9 III

Figure 9 IV

Figure 9. Role of OX40L signaling in the generation and maintenance of Tfh cells, in the expression of IL-4 by Tfh cells, and in GC formation. (A) Experimental protocol. Mice (Balb/c or 4C13$^{tg/0}$, as indicated) were i.p injected with anti-OX40L neutralizing antibody or isotype antibody every three days from D -1, and MC903 was topically applied to mouse ears every two days from D0. EDLNs were analyzed at D7 and D11. (B) Total cell number and CD4 T cell number in EDLNs from treated Balb/c mice. (C) Percentage and total number of Tfh (CD3$^+$CD4$^+$CXCR5$^+$PD-1$^+$) cells in EDLNs from treated Balb/c mice. (D) Intracellular staining (ICS) of IL-4 in CD4$^+$ T cells (upper panel), and in Tfh cells (lower panel), in EDLNs from treated Balb/c mice at D7 and D11. (E) Amcyan(IL-4)$^+$ cells in CD4$^+$ T cells and in Tfh cells in EDLNs from treated 4C13R$^{tg/0}$ mice at D7 and D11. (F) Intracellular staining of IL-13 in CD4+ T cells in EDLNs of treated Balb/c mice, at D7 and D11, (G) DsRed(IL-13)$^+$ cells in CD4$^+$ T cells in EDLNs from treated 4C13R$^{tg/0}$ mice at D7 and D11. (H) Percentage and total number of GC B (Fas$^+$GL7$^+$B220$^+$CD19$^+$) cells in treated Balb/c mice at D7 and D11. (I) Percentage and total number of IgG1$^+$ B cells and IgE$^+$ B cells in treated Balb/c mice at D11. Values are mean±s.e.m. (n=4) (Student's t-test).
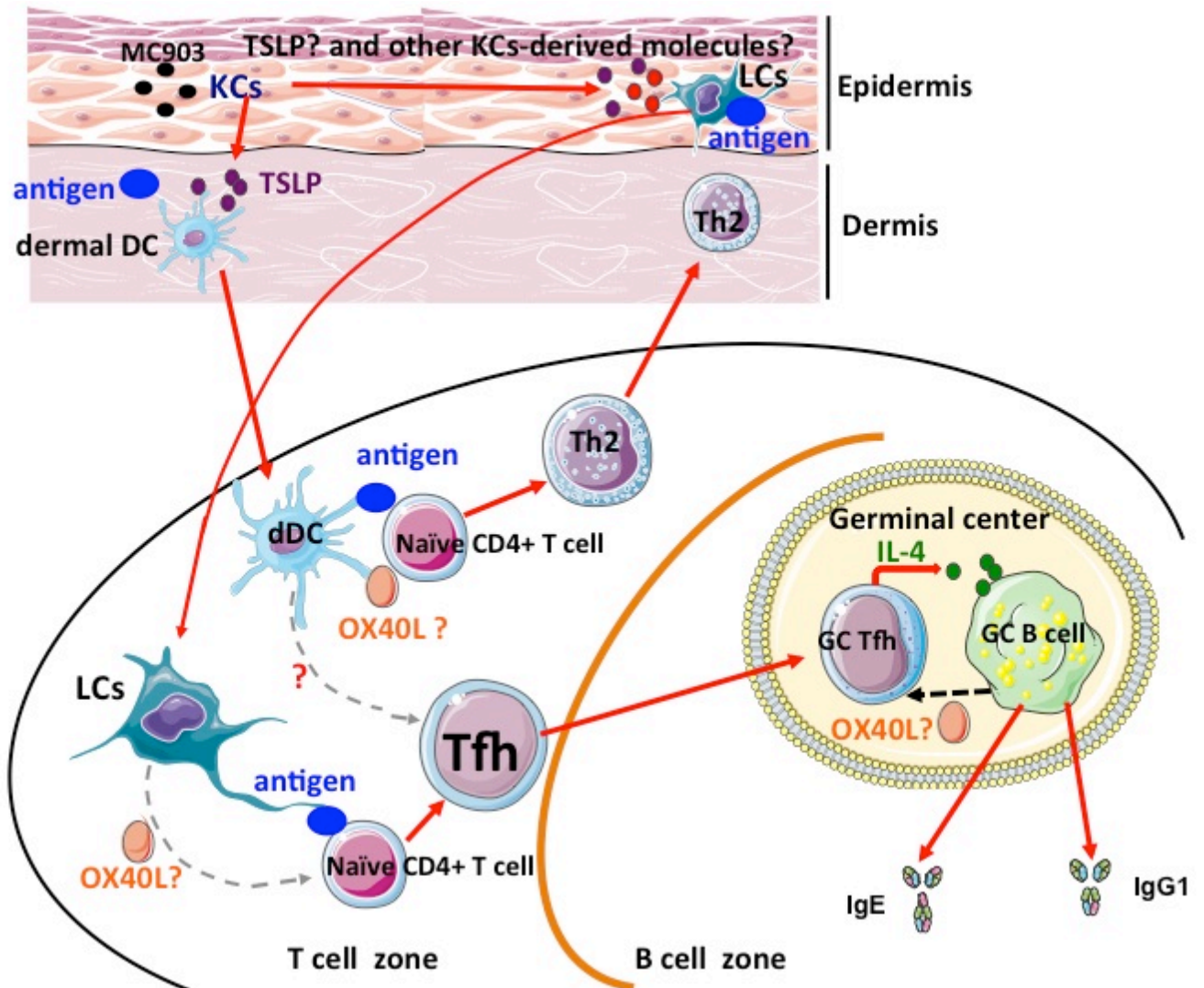
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Figure 10. Induction and regulation of Tfh cell differentiation and GC responses in AD mice. Topical MC903 treatment on skin activates VDR signaling pathway in KCs, activated KCs secrete molecules to generate proinflammatory environment in local skin, which induces Tfh cell differentiation in skin-draining LNs and promotes Tfh cells to produce IL-4. Along with induction of Tfh cell differentiation, MC903 treatment leads to GC formation, in which GC B cells may undergo immunoglobulin isotype class-switch to IgG1. In this process, Langerin+ DCs play a crucial role in Tfh cell differentiation and IL-4 commitment. In addition, TSLP and OX40L also involve in the regulation of Tfh cell differentiation and IL-4 commitment. TSLP and OX40L are dispensable for the initial generation of Tfh cells, but they are crucial for the expansion/maintenance of Tfh cells. Moreover TSLP and OX40L play a critical role for IL-4 commitment in Tfh cells.

Part II: MC903 (Calcipotrol) inhibits the IL-23/IL-17/IL-22 in mouse psoriatic inflammation

Abstract

Psoriasis is a common skin disorder affecting 2% of the worldwide population. It is characterized by hyperplasia of the epidermis, hyperproliferation and aberrant differentiation of keratinocytes, infiltration of leukocytes (such as T cells, macrophages and neutrophils). A critical role of the IL-23/IL-17/IL-22 axis has been recognized in the pathogenesis of psoriasis. Interestingly, MC903, also called calcipotriol, has been used in the treatment of mild to moderate plaque psoriasis. Although it has been suggested that MC903 inhibits keratinocytes proliferation and induces the terminal differentiation of keratinocytes, the exact mechanism of MC903 in regulating psoriasis inflammation is not clear. As MC903 triggers keratinocytes to produce TSLP that can induce Th2 cell response, our initial question was whether MC903 could inhibit IL-23/IL-17/IL-22 axis, and if yes, whether it is through TSLP.

To address this question, we used a psoriasis mouse model induced by topical application of Imiquimod (IMQ), a TLR7/8 ligand. It has been shown that IMQ-induced psoriasis is mediated via the IL-23/IL-17/IL-22 axis. We found that MC903 inhibited the IMQ-induced IL-23/IL-17/IL-22 axis in a dose-dependent manner. This led to an attenuated neutrophil infiltrate in the skin. Unexpectedly, this inhibiting role of MC903 was not mediated by TSLP, as IMQ-treated TSLP$^{-/-}$ mice exhibited similar inhibitory effect of L-23/IL-17/IL-22 axis by MC903, as shown in IMQ-treated wildtype mice. However, MC903-induced inhibition of L-23/IL-17/IL-22 axis was abolished in VDR$^{-/-}$ mice, indicating that it is VDR-dependent. Further, by using keratinocyte-selective VDR knockout mice (VDR$^{ep-/-}$), we found that the inhibition of IL-23/IL-17/IL-22 axis by MC903 was abolished in IMQ-treated VDR$^{ep-/-}$ mice, suggesting that epidermal keratinocyte-derived factor(s) is(are) implicated in MC903-induced inhibition of L-23/IL-17/IL-22 axis.

Introduction

Psoriasis is a common chronic inflammatory skin disease characterized by hyperplasia of the epidermis, hyperproliferation and aberrant differentiation of keratinocytes, infiltration of

leukocytes into dermis and epidermis, dilation and growth of blood vessels. The underlying pathogenesis of psoriasis is still poorly known, however, the critical role for the IL-23/IL-17/IL-22 axis has been postulated in the pathogenesis of psoriasis. IL-23, a master regulatory cytokine of IL-23/IL-17/IL-22 axis, drives the development of IL-17 and IL-22-producing Th17 cells, γδ T, CD4+ T cells and innate like lymphoid cells, which leads to neutrophil skin infiltration in psoriasis. Indeed, expressions of IL-23, IL-17, and IL-22 were found to increase in psoriasis lesional skin, as well as the number of Th17 cells [1-3]. In addition, intradermal injection of IL-23 into mouse skin resulted in psoriasis-like inflammation, including leukocyte infiltrates and epidermal hyperplasia, which is mediated via the activation and recruitment of Th17 cells and γδ T cells to the affected skin [4, 5]. Furthermore, Genome-wide scan revealed the association of psoriasis susceptibility with IL-23 and NF-κB pathways [6-8].

An imiquimod (IMQ)-induced model of psoriasis was first described in 2009 [9], and has been since then used to better understand the inflammatory mechanisms of psoriasis [10]. This model uses the Aldara™ cream containing the active ingredient IMQ, which is applied in clinic to treat actinic keratoses and superficial basal cell carcinomas. Being a TLR7 and TLR8 ligand, IMQ functions as a potent immune modulator that is capable of inducing local production of cytokines, especially inducing the production of interferon alpha (IFN-α) and tumor necrosis factor alpha (TNF-α) [11-14]. Interestingly, one side effect of IMQ is the induction and exacerbation of psoriasis-like skin inflammation in human, which occurs at both the treated area and distant skin sites [15-17]. Such clinical incidence of psoriasis induction following the use of Aldara indicates the human relevance of the IMQ-induced psoriasis mouse model [10]. In this mouse model, the application of Aldara cream to mouse skin rapidly results in skin inflammation strikingly resembling human psoriasis, including epidermal thickness, erythema, and inflammation. Recent studies have implicated a crucial role of IL-23/IL-17/IL-22 axis in IMQ-induced psoriasis-like inflammation in mice [9, 10]. IL-23 has been shown to be a major inducer of IL-17 and IL-22 cytokines expressed by γδ T cells and Th17 cells, and plays a crucial role for neutrophil recruitment in IMQ-reated skin. Based on currently available data, topical treatment of mouse skin with IMQ has been well accepted as a powerful model to study psoriasis-like skin inflammation [10].

Calcipotriene cream and ointment, comprising MC903 (also called Calcipotriol), have been used to treat mild to moderate plaque psoriasis for decades [18-22]. More recently,

combination of MC903 and betamethasone has been approved for achieving better therapeutic effects, and additional drugs are in development targeting different immune mediators (e.g. IL-12/23p40, IL-23p19 and IL-17A) [23]. However, why and how MC903 is effective (or ineffective) in regulating psoriatic inflammation remain still unclear, despite the initial thought that MC903 inhibits keratinocyte proliferation and induces terminal differentiation of keratinocytes, thus reversing the abnormality of keratinocyte in psoriasis [18-22], a better understanding of the mechanisms underlying therapeutic effects of MC903 in the treatment of psoriasis will provide us insights into the development of improved or new interventions.

Our previous studies have shown that topical MC903-treatment induces thymic stromal lymphopoietin (TSLP) expression in mouse keratinocytes and triggers an atopic dermatitis-like skin inflammation, associated with an inflammatory infiltrate mainly composed of CD4$^+$ T helper (Th) type 2 cells, eosinophils, mast cells and basophils [24, 25]. TSLP is a master initiator for Th2 differentiation in both mice [26] and human [27, 28], and overexpression of TSLP in mouse skin leads to the pathogenesis of atopic dermatitis [24, 25, 29].

More recently, our lab reported that TSLP-type 2 axis counter regulates IL-23-type 17 axis in a mouse model bearing skin barrier defects [30]. In addition, it was previously shown that TSLP produced by dendritic cells (DCs) limits the differentiation of Th17 cells [31]. These findings thus raise the possibility that MC903 may inhibit IL-23/IL-17/IL-22 axis in psoriasis by skewing the T cell response from type 17 towards type 2.

In this study, we examined the role of MC903 in regulating the psoriatic inflammation using Aldara-induced psoriasis model. We showed that MC903 inhibited IL-23/IL-17/IL-22 axis in mouse psoriasis. Moreover, this inhibition exhibited a dose-dependent manner. We further explored the role of TSLP and VDR in mediating such effect of MC903.

Results

MC903 inhibits the IL-23/IL-17/IL-22 axis and neutrophil infiltrate in mouse psoriatic skin

Balb/c wildtype mouse ears were treated daily with Aldara from day (D) 0 to D4 to generate psoriasis-like skin inflammation, and were combined with MC903 or ETOH treatment (Fig.1A). In agreement with previous reports, Aldara treatment induced the expression of IL-23p19, IL-23/IL-12p40, IL-17A and IL-22, as well as the calcium binding proteins S100A7A and S100A8, which are characterized for psoriatic inflammation (Fig. 1B). Strikingly, the induction of those genes was all abolished upon MC903 treatment (Fig. 1B). We then perform histological and immunohistochemical (IHC) analyses of ear. H&E-stained sections from the Aldara-treated ears showed an increased epidermal thickening and a heavy dermal cell infiltrate (Fig. 1C). In contrast, ears treated with Aldara plus MC903 showed an attenuated dermal infiltrate, although epidermal thickness was not apparently reduced (Fig. 1C). IHC staining with an antibody against IL-23p19 showed that few IL-23$^+$ cells were detected in ETOH-treated dermis (Fig. 1C and data not shown). MC903 alone did not change IL-23$^+$ cell number in dermis. In contrast, massive IL-23$^+$ cells were observed in skin treated with Aldara, which was abolished in Aldara+MC903-treated skin (Fig. 1C). The IL-23$^+$ positive cells were mainly located in dermis, most of which could be DCs and monocytes/macrophages as previously reported [32]. Neutrophilia, one of manifestation in psoriasis skin inflammation, was observed in Aldara-treated skin dermis (shown by NIMP-R14 staining, Fig.1C). In Aldara+MC903 skin, infiltrate of neutrophils was largely diminished, indicating that MC903 treatment suppresses the neutrophil infiltration to skin, in agreement with the inhibition of IL-23/IL-17/IL-22 axis in Aldara+MC903 skin (Fig. 1B). Together, these data indicate that MC903 inhibits IL-23/IL-17/IL-22 axis and neutrophil infiltrate in mouse psoriatic skin.

The inhibition of IL-23/IL-17/IL-22 axis by MC903 is dose dependent

To examine whether the inhibition of IL-23/IL-17/IL-22 axis by MC903 is in a dose dependent manner, we designed experiments in two strategies. In the first one, 4 times (4X), 3X or 2X MC903 treatment was combined with Aldara treatment (Fig. 2A). In the second strategy, Aldara treatment was combined with different doses of MC903 (1 nmol, 0.4 nmol, 0.1 nmol, 0.05 nmol and 0.01 nmol) (Fig. 2C). Results showed a dose-dependent decrease in the expression of IL-23p19, IL-23/IL-12p40, IL-17A, IL-22, S100A7A and S100A8 (Fig. 2B&C). These data indicate that MC903 dose-dependently inhibits

IL-23/IL-17/IL-22 axis in Aldara-induced psoriasis.

VDR but not TSLP mediates the inhibition of the IL-23/IL-17/IL-22 axis by MC903

Our previous studies have shown that MC903 induces TSLP expression and triggers an AD-like Th2 immune response through vitamin D receptor (VDR) [24, 25]. To examine whether MC903 inhibits the IL-23/IL-17/IL-22 axis via TSLP, wildtype Balb/c, TSLP$^{-/-}$ and VDR$^{-/-}$ mice were subjected to ETOH, MC903, Aldara+ETOH and Aldara+MC903 treatment as described in Fig. 1A. Quantitative RT-PCR analyses of ears showed that MC903-induced TSLP mRNA levels could be detected in the WT ears but not in TSLP$^{-/-}$ or VDR$^{-/-}$ mice ears (Fig. 3). In TSLP$^{-/-}$ skin, MC903 treatment led to a similar reduction in the expression of IL-23p19, IL-23/IL-12p40, IL-17A, IL-22, S100A7A and S100A8 as in WT skin (Fig. 3), indicating that the inhibition of IL-23/IL-17/IL-22 axis by MC903 is not mediated via TSLP. In contrast, in VDR$^{-/-}$ mice, MC903 treatment failed to inhibit the expression of IL-23p19, IL-23/IL-12p40, IL-17A, IL-22, S100A7A and S100A8 (Fig. 3). These data thus demonstrate that the inhibition of IL-23/IL-17/IL-22 axis by MC903 is mediated via VDR but not TSLP.

Keratinocytic VDR is required for the inhibition of IL-23/IL-17/IL-22 axis by MC903

As VDR can be expressed by various cell types in skin, we further examined whether VDR expressed by keratinocytes mediates the effect of MC903. Mice with the ablation of VDR selectively in keratinocytes (K14-Cre$^{tg/0}$/VDR$^{L2/L2}$ mice, also called VDR$^{ep-/-}$ mice [24]) were subjected to Aldara+ETOH or Aldara+MC903 treatment as described in Fig. 1A. Like in VDR$^{-/-}$ mice, MC903 also failed to inhibit the Aldara-triggered IL-23/IL-17/IL-22 axis in VDR$^{ep-/-}$ mice (Fig. 4A). In addition, IHC staining with IL-23p19 antibody showed that there was no reduction in the number of IL-23^{+} cells in VDR$^{ep-/-}$ (or in VDR$^{-/-}$) mouse skin treated with Aldara+MC903, compared with Aldara+ETOH treatment (Fig. 4B), indicating that the inhibition of IL-23/IL-17/IL-22 axis by MC903 is mediated by keratinocytic VDR.

In consistent with this, dermal infiltrate was comparable in VDR$^{ep-/-}$ (or VDR$^{-/-}$) skin upon Aldara+ETOH or Aldara+MC903 treatment (Fig. 4C), further confirming that the suppressing effect of MC903 on psoriatic inflammation is mediated by keratinocytic VDR.

Discussion

Using a psoriasis model induced by Aldara, we showed in this study that MC903 topical treatment resulted in the inhibition of IL-23/IL-17/IL-22 axis and neutrophilic skin inflammation. We further demonstrated that such effect of MC903 is independent of TSLP, although it is mediated by keratinocytic VDR. Our results suggest that epidermal keratinocyte-derived factor(s) is(are) implicated in that the inhibition of IL-23/IL-17/IL-22 axis by MC903.

It will be important to validate our findings in human psoriatic skin. In collaboration with Seneschal, J. and Boniface, K. (Bordeaux), we are examining the expression of genes related to IL-23/IL-17/IL-22 axis in human psoriasis skin prior to and after MC903 treatment. Our preliminary results indicated that MC903 treatment inhibited IL-23p19, IL-23/IL-12p40 and IL-17A mRNA expression in human psoriatic skin (data not shown), which is in consistent with the effect of MC903 in inhibiting IL-23/IL-17/IL-22 axis in mouse psoriatic skin.

What is(are) factor(s) activated by MC903 via VDR signaling in keratinocytes, and how it(they) exert(s) the inhibitory effect on IL-23/IL-17/IL-22 axis in psoriatic inflammation remain unknown. Interestingly, a recent study showed that the activation of aryl hydrocarbon receptor (AhR) signaling in keratinocytes by its endogenous ligand 6-Formylindolo (3,2-b)carbazole (FICZ) reduced the inflammatory response in the IMQ-induced mouse psoriasis [33]. We are thus wondering whether there exists a crosstalk between VDR and AhR signaling pathway in keratinocytes, as reported in macrophages [34]. We observed that the expression of Cyp1A1 and Cyp1B (genes downstream of AhR activation) was decreased upon Aldara treatment, but in the skin treated with Aldara+MC903, Cyp1A1 and Cyp1B mRNA level were restored (data not shown). My ongoing work is validating this observation. Such hypothesis will be further tested by using AHR$^{-/-}$ or AhR$^{ep-/-}$ mice.

MATERIALS AND METHODS

Mice

Balb/c mice were purchased from Charles River Laboratories. TSLP$^{-/-}$, VDR$^{-/-}$ and VDR$^{ep-/-}$ (K14-Cre$^{tg/0}$/VDR$^{L2/L2}$) mice were as previolyus described [24, 25], and were all on Balb/c genetic background. All mice used were 8-12-week-old females. Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the animal care and ethic committee of animal experimentation of the Institut de Ge´ne´tique et de Biologie Mole´culaire et Cellulaire (IGBMC) and Institut Clinique de la Souris (ICS).

MC903 and Aldara topical treatment

MC903 (calcipotriol; Leo Pharma, Denmark) was dissolved in ethanol (ETOH) and topically applied on mouse ears (25 ul per ear). Aldara cream (5% IMQ; 3M Pharmaceuticals) was topically applied on ears (30mg/ear).

Histopathology

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

Immunohistochemical (IHC) staining

For IHC staining of IL-23p19, paraffin sections were treated with 0.6% H_2O_2 (in PBS) to block the endogenous peroxidase activity, before antigen retrieving with citric buffer (10mM citric acid, pH 6). Slides were then blocked with 5% normal rabbit or goat serum (Vector Laboratories), followed by incubation of rabbit polyclonal IL-23p19 antibody (Abcam, ab45420, dilution: 1:100). Slides were then washed and incubated with biotinylated goat anti-rabbit IgG before treatment of AB complex (Vector Laboratories). Staining was finally visualized with AEC+ high sensitivity substrate chromogen solution (Dako).

For immunofluorescence staining of NIMP-R14, 10 μm cryosections were fixed in 4% paraformaldehyde, permeabilized with acetone, and blocked with 5% normal goat serum (Vector laboratories). Slides were then incubated with primary antibody [rat monoclonal anti-MINP-R14 (anti-neutrophil antibody, Abcam, ab2557, dilution: 1:3000)]. After washing, sections were incubated with CY3-conjugated goat-anti-rat IgG antibody, and mounted with Vectashield medium (Vector Laboratories) containing DAPI (Invitrogen).

RNA extraction

Whole ear tissue was homogenized with ultraturax in 1mL Trizol reagent, and incubated 5 min at room temperature. 200µL chloroforme was added into homogenised tissue solution, and then vortexed for 15 second, incubated 5 min at room temperature, and then centrifuged at 12000 rpm for 15 minutes at 4°C. The aqueous at the upper phase was taken to a new tube, added with 500µL isopropanol, incubated for 10 minutes at room temperature, and was centrifuged at 12000rpm for 10 minutes at 4°C. Supernatant was then removed, and precipitation was washed with 1mL 75% EtOH and was centrifuged at 7500 rpm for 5 minutes at 4°C. Pellet was dried and dissolved in RNase-free H_2O.

Quantitative RT-PCR

RNA was reverse-transcribed using random oligonucleotide hexamers and amplified by quantitative PCR with a Lightcycler 480 (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Relative RNA levels were calculated using hypoxanthine phosphoribosyltransferase (HPRT) as internal control. For the analyses of each set of gene expression, an arbitrary unit of 100 was given to the samples with the highest level, and the remaining samples were plotted relative to this value.

Gene name		Sequence (5' to 3')
HPRT (164 bp)	F	TGGATACAGGCCAGACTTTG
	R	GATTCAACTTGCGCTCATCTTA
IL-17A (239 bp)	F	CCAGGGAGAGCTTCATCTGT
	R	ACGTGGAACGGTTGAGGTAG
IL-22 (166 bp)	F	CCGAGGAGTCAGTGCT AAGG

	R	GCTGATGTGACAGGAGCTGA
IL-23p19 (213 bp)	F	AATAATGTGCCCCGTATCCA
	R	CTGGAGGAGTTGGCTGAGTC
IL-23/12p40 (176 bp)	F	CCTGAAGTGTGAAGCACCAA
	R	AGTCCCTTTGGTCCAGTGTG
S100A7A (167 bp)	F	AAGCCAAGCCTGCTGACGAT
	R	TGGCTATGTCTCCCAGCAAGG
S100A8 (178 bp)	F	AGCCCTGCATGTCTCTTGTCA
	R	GAACCAGACGTCTGCACCCT
TSLP (194 bp)	F	AGCTTGTCTCCTGAAAATCGAG
	R	AGGTTTGATTCAGGCAGATGTT

Statistic analysis

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

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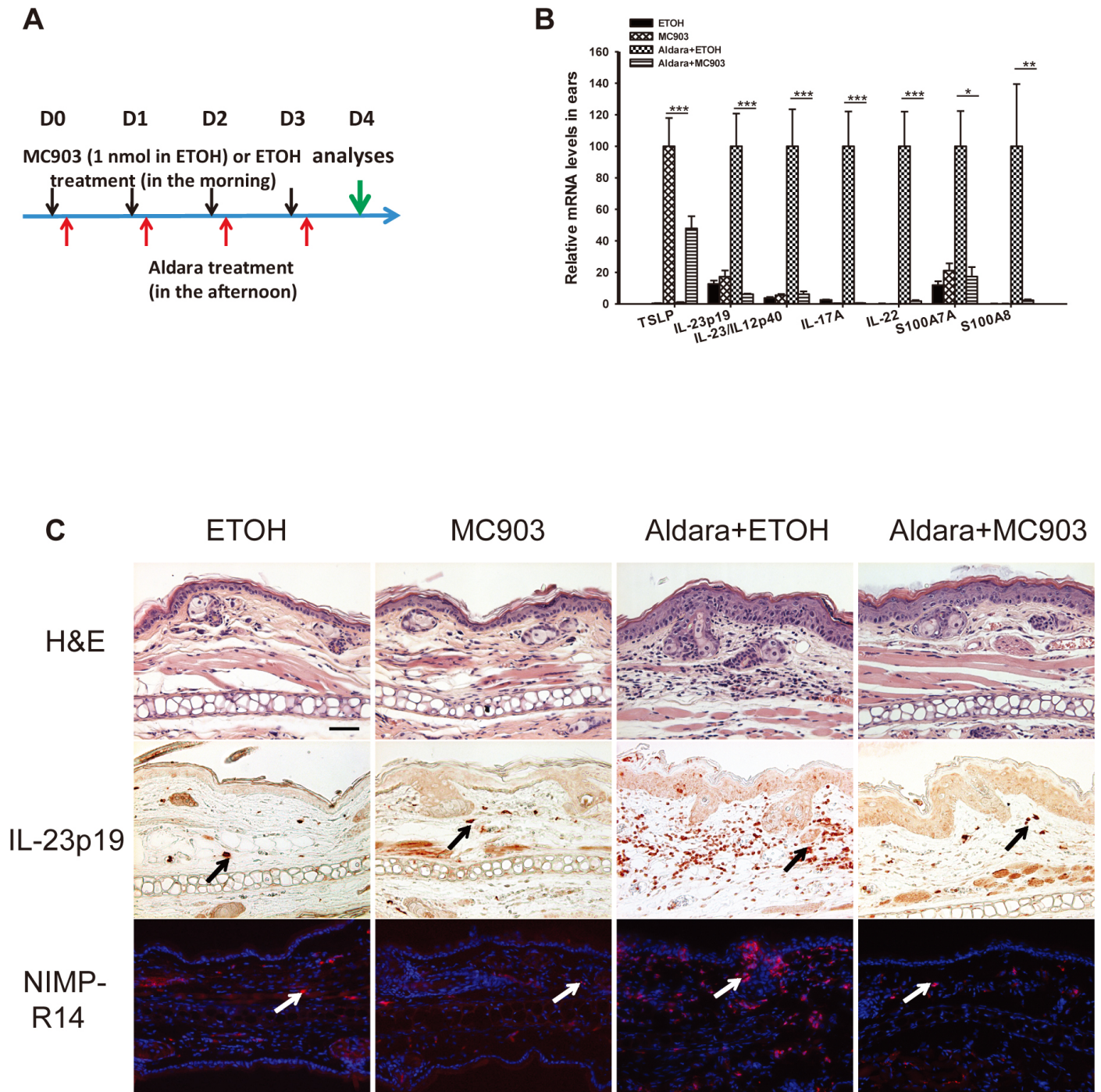
Figure 1. MC903 inhibits the IL-23/IL-17/IL-22 axis in mouse psoriatic skin. (A) Experimental protocol. Wildtype Balb/c mouse ears were topically treated with MC903 (1 nmol in ETOH), ethanol (ETOH; vehicle control), Aldara+ETOH or Aldara+MC903 every day from day (D) 0 to D3 and analysed at D4. (B) Quantitative RT-PCR analyses. *P<0.05; **P<0.01; ***P<0.001 (n=4) (student's t-test). (C) Hematoylin/eosin (H&E), immunohistochemical (IHC) staining with IL-23p19 antibody (in dark red) and immunoflorescent staining with NIMP-R14 antibody (for neutrophils; red corresponds to positive signal whereas blue corresponds to DAPI staining of nuclei) of ear sections. Arrows point to one of positive signals. Bar = 50 μm.
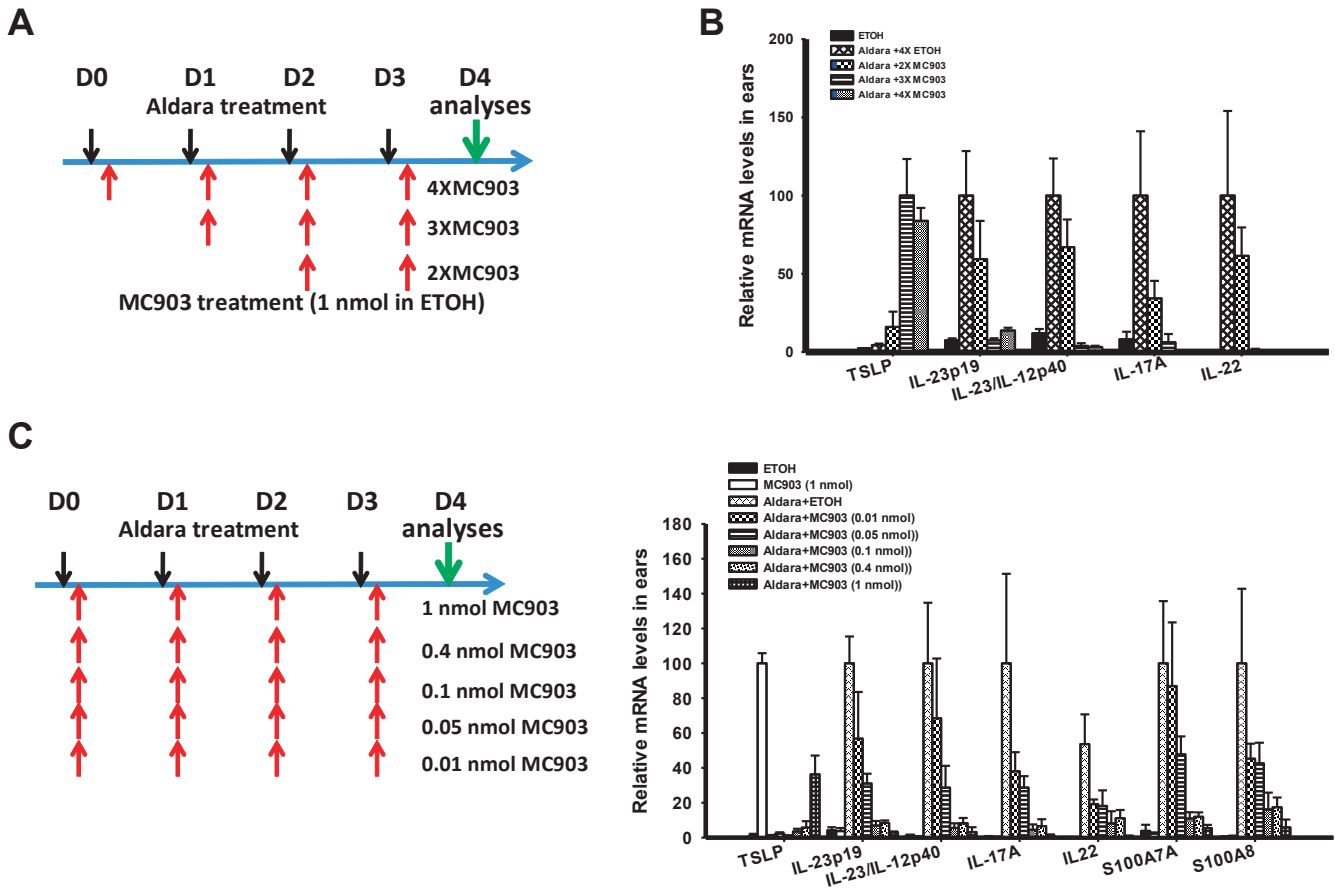
Figure 2. The inhibition of IL-23/IL-17/IL-22 axis by MC903 is dose dependent. (A) Experimental protocol. Wildtype Balb/c mouse ears were treated with 4X Aldara (at D0, D1, D2, D3), combined with 4X MC903 (at D0, D1, D2, D3) or 3X MC903 (at D1, D2, D3) or 2X MC903 (at D2, D3). Mouse ears were analyzed at D4. (B) Quantitative RT-PCR analyses. (C) Experimental protocol. Balb/c mouse ears were treated with 4X Aldara plus 4X MC903 with different doses (1, 0.4, 0.1, 0.05, 0.001 nmol). Mouse ears were analyzed at D4. (D) Quantitative RT-PCR analyses. (n=4)
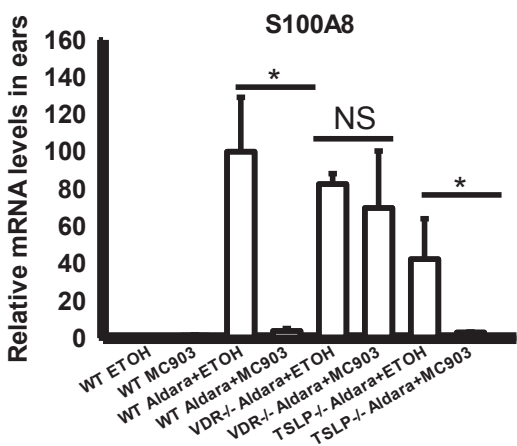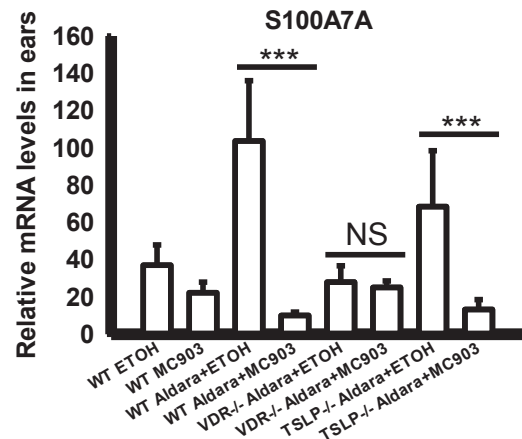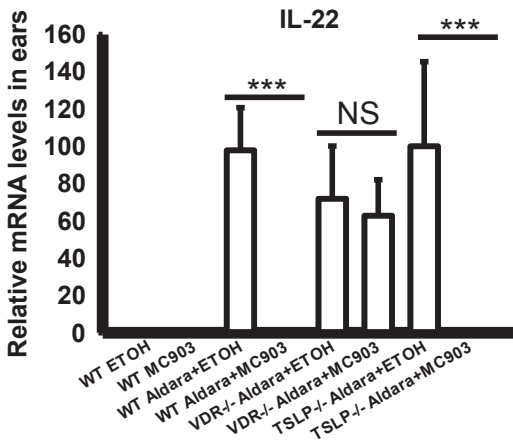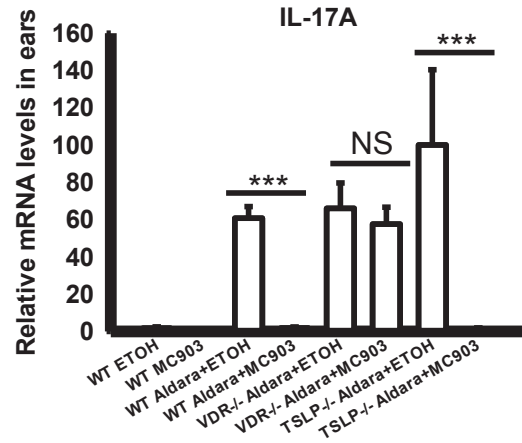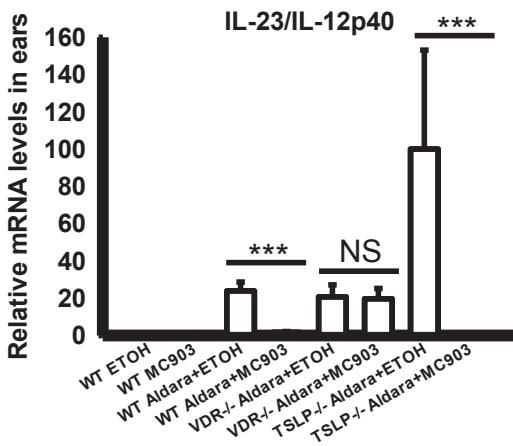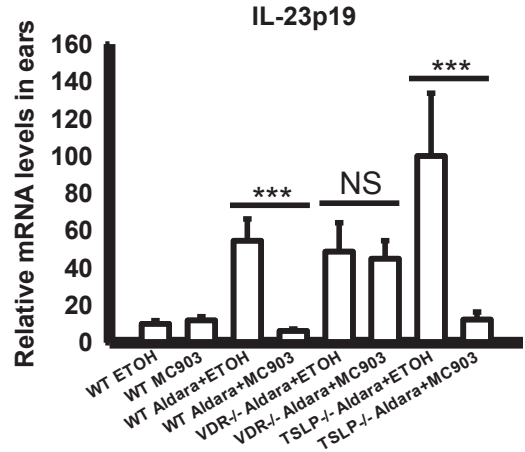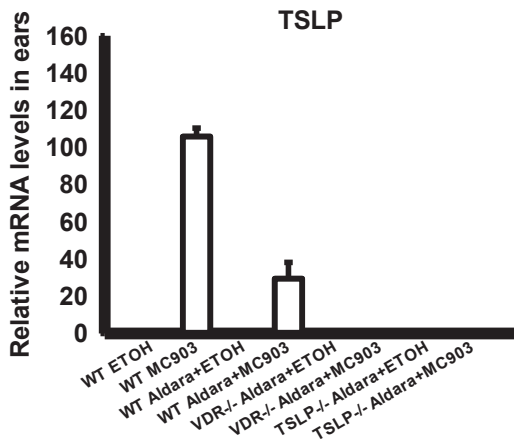
Figure 3. VDR but not TSLP mediates the inhibition of IL-23/IL-17/IL-22 axis by MC903. Wildtype Balb/c, VDR$^{-/-}$ or TSLP$^{-/-}$ mouse ears were treated with ETOH, MC903, Aldara+ETOH or Aldara+MC903, as described in Fig. 1A. Ears were analyzed by quantitative RT-PCR at D4. *P<0.05; ***P<0.001 (student's t-test). n=4 .NS = no significant difference.
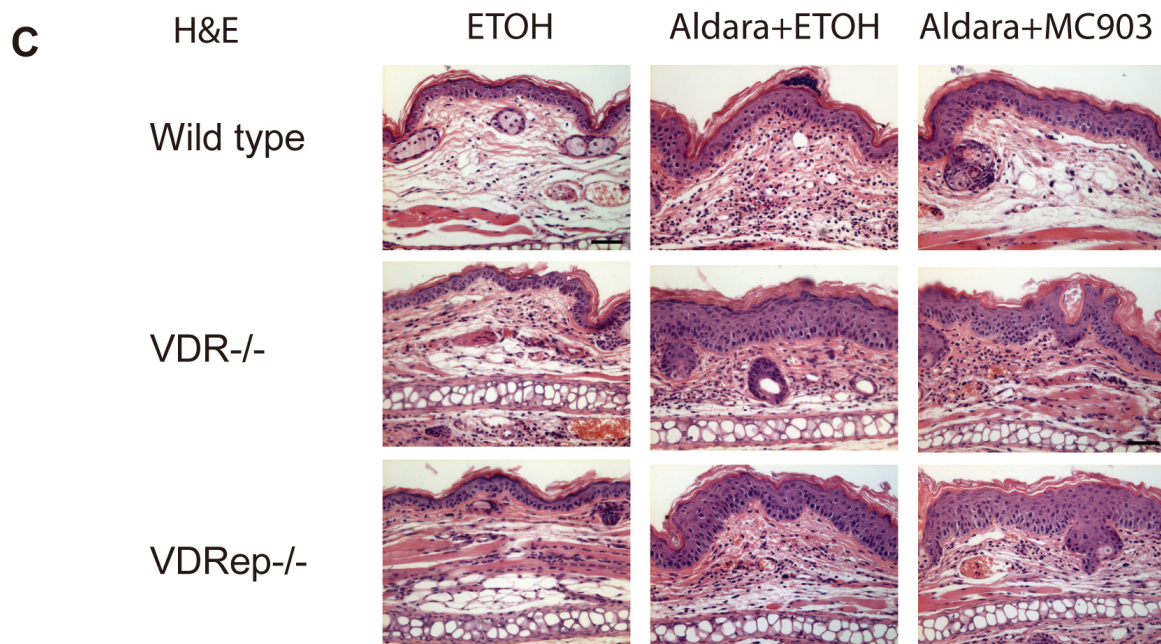
A

Relative mRNA levels in ears

Legend:
- WT ETOH
- WT Aldara+ETOH
- WT Aldara+MC903
- VDRep-/- ETOH
- VDRep-/- Aldara+ETOH
- VDRep-/- Aldara+MC903

X-axis categories: IL-23p19, IL-23/IL-12p40, IL-17A, IL-22, S100A7A, S100A8

B

IHC-IL-23p19 | ETOH | Aldara+ETOH | Aldara+MC903

Wild type

VDR-/-

VDRep-/-

C

H&E | ETOH | Aldara+ETOH | Aldara+MC903

Wild type

VDR-/-

VDRep-/-

Figure 4. Keratinocytic VDR is required for the inhibition of IL-23/IL-17/IL-22 axis by MC903. (A) VDR$^{ep-/-}$ mice (K14-Cre$^{Tg/0}$ /VDR$^{L2/L2}$) and wildtype (WT) control (K14-Cre$^{0/0}$ /VDR$^{L2/L2}$) mice were treated with ETOH, MC903, Aldara+ETOH or Aldara+MC903 (as describe in Fig. 1A). Ea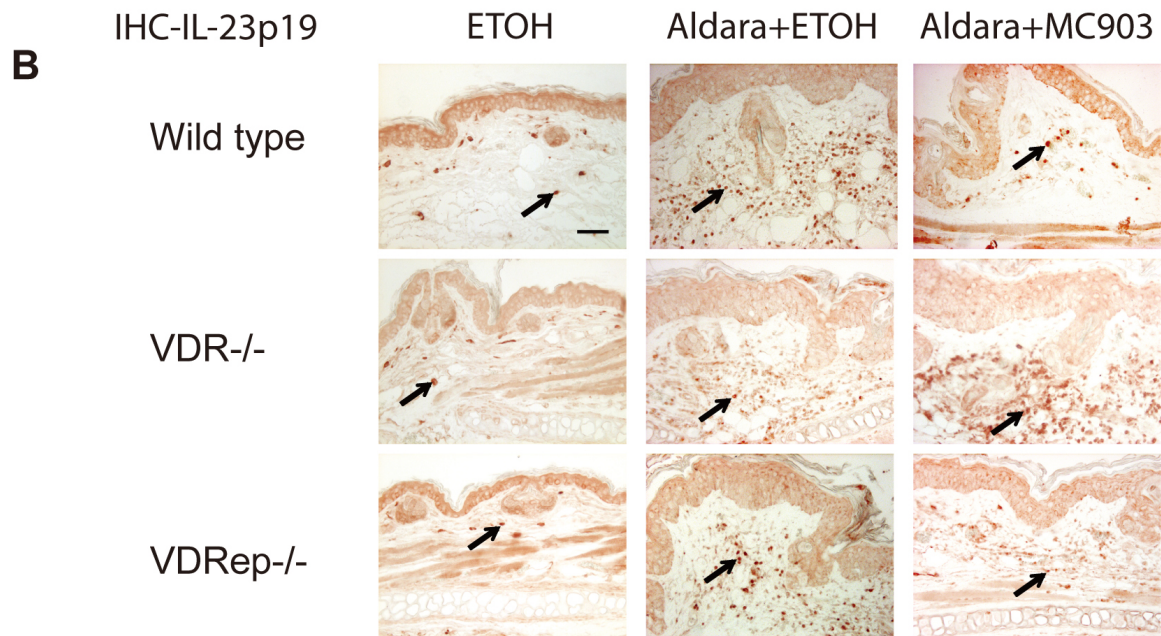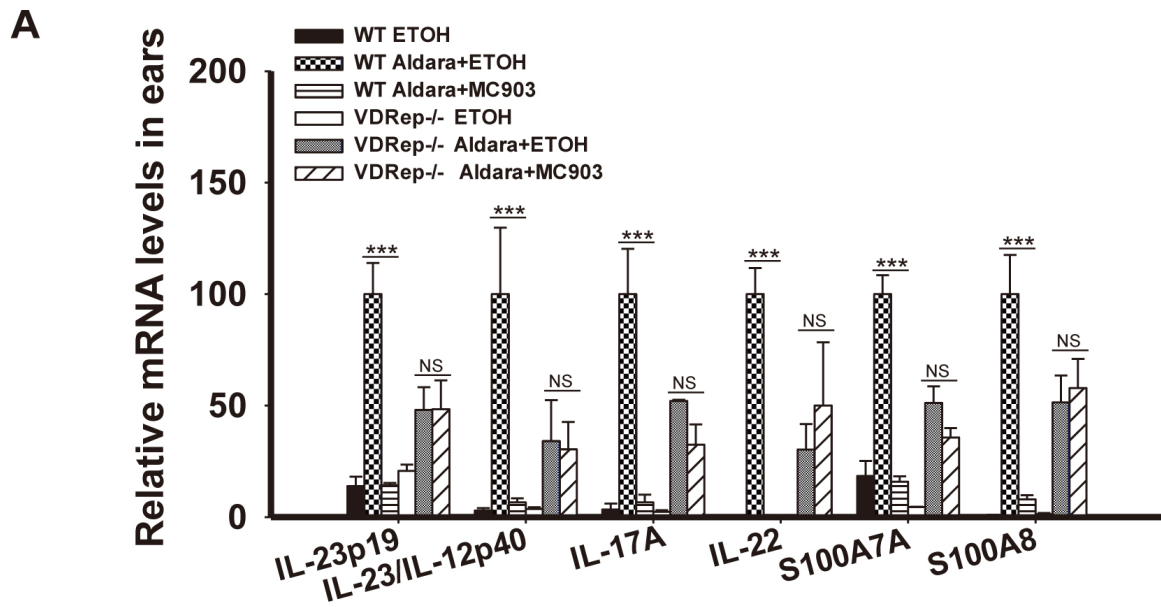rs were analyzed by quantitative RT-PCR at D4. *P<0.05; ***P<0.001 (student's t-test), n=4. (B) Comparison of immunohistochemical (IHC) staining with IL-23p19 antibody among WT, VDR$^{-/-}$ and VDR$^{ep-/-}$ ear sections. Positive cells are in dark red. Bar = 50 µm. (C) Hematoxylin/eosin (H&E) staining. Bar = 50 µm.

Perspectives

In the first part of my thesis study, I identified the critical role of Langerin+ DCs in promoting Tfh cell differentiation, and uncovered the roles of TSLP and OX40L signaling in the maintenance of Tfh cells and IL-4 commitment. There are still several aspects needed to investigate in future.

First we have identified that in MC903-induced AD, Langerin+ DCs play a crucial role in promoting Tfh cell differentiation, but it is not clear which Langerin+ DCs (epidermal LCs or Langerin+ dermal DCs) is responsible for Tfh cell linage commitment. It has been reported that after DT-mediated Langerin+ DC depletion, LCs and Langerin+ dermal DCs reconstitute in the epidermis and dermis at different time [1]. [2]We may then perform experiments at 13 days after DT injection to investigate whether LCs are responsible for Tfh cell linage commitment. Alternatively, we could use huLangerin-DTR in which LCs, but not Langerin+ dDC are specifically ablated to address this issue.

Second, we observed that TLSP and OX40L signaling are crucial for IL-4 induction in Tfh cells at the early stage of Tfh cell generation. These findings raise the possibility that there may be a TSLP(KCs) -OX40L(Langerin+ DCs) axis that plays a crucial role in IL-4 commitment in Tfh cells. To test this possibility, we could treat TSLP+/+ mice and TSLP-/- mice with MC903 and then examine the expression of OX40L in LCs. We should further explore the signaling pathway involved in IL-4 expression in Tfh cells. It has been reported that Batf is important for IL-4 expression in Tfh cells rather than in canonical Th2 cells. Batf in cooperation with interferon regulatory factor (IRF) 4 trigger IL-4 production in Tfh cells by directly binding to and activation of the CNS2 region in the IL-4 locus[3]. It could be interesting to study whether the regulation of IL-4 expression in Tfh cells by TSLP or OX40L involves Batf. To address this question, we could compare Batf and IRF4 mRNA expression in sorted Tfh cells from TSLP-/- and TSLP+/+ mice, as well as from anti-OX40L antibody-injected and Isotype antibody-injected mice. In addition, we can also perform chromatin immunoprecipitation (ChIP) analysis to examine the binding of Batf to the IL-4 locus in these sorted Tfh cells.

Third, in addition to DCs, OX40L can be also expressed by B cells. Is OX40L expressed by B cells or GC B cells implicated in the expansion/maintenance of Tfh cells? We could

generate conditional gene knockout mice in which OX40L is selectively ablated in DCs or B cells to investigate which cells take part in OX40L-mediated expansion/maintenance of Tfh cells.

Finally, we need to further explore the pathogenic role of Tfh cells in the context of AD. We may use CD4Cretg/0Bcl6fl/fl or CD4CreERT2Bcl6fl/fl mice that are lack of Tfh cells to investigate the influence on MC903-induced AD and the induction of humoral response in the absence of Tfh cells.

In the second part of my thesis study, I examined the role of MC903 in regulating the psoriatic inflammation using Aldara-induced psoriasis mouse model. We found that MC903 topical treatment inhibit the IL-23/IL-17/IL-22 axis and neutrophilic skin inflammation, which is mediated by keratinocytic VDR but not TSLP.

It will be interesting to investigate whether MC903 could reduce skin inflammation when Aldara-induced psoriatic skin inflammation has been established.

It is not clear how IL-23/IL-17/ IL-22 axis is inhibited. There are several candidates in keratinocytes to be considered. First, a recent study showed that the activation of aryl hydrocarbon receptor (AhR) signaling in keratinocytes by its endogenous ligand 6-Formylindolo (3,2-b) carbazole (FICZ) reduced the inflammatory response in the IMQ-induced mouse psoriasis [4]. We are thus wondering whether there exists a crosstalk between VDR and AhR signaling pathway in keratinocytes. This possibility exists as we observed that the expression of Cyp1A1 and Cyp1B (genes downstream of AhR activation) was decreased upon Aldara treatment, but in the skin treated with Aldara+MC903, Cyp1A1 and Cyp1B mRNA level were restored. Such hypothesis will be further tested using AHR-/- or AhRep-/- mice. It has been found that IL-36 is master regulator of the IL-23/IL-17/ IL-22 axis and development of cutaneous pathology in response to IMQ. It would be interesting to investigate whether MC903 inhibits IL-36 expression so as to inhibit IL-23/IL-17/ IL-22 axis. In addition, we could perform RNAseq analysis in sorted Keratinocytes from Aldara-treated mice, MC903-treated mice and Aldara+MC903-treated mice to identify these factors.

Although Calcipotriene cream and ointment (comprising MC903) have been used to treat mild to moderate plaque psoriasis, it may have side effects (e.g. skin irritation, calcium related side effect). Recently, Calcipotriol is used in combination with a steroid medicine called betamethasone, which improves efficacy and decreases skin irritation [5]. Although

we demonstrated its effect in inhibiting IL-23/IL-17/ IL-22 axis, we also showed that its effect is dose-dependent. The current dose (750 µg of Calcipotriol each day indicated for the topical treatment of stable plaque psoriasis vulgaris in adults 18 years of age and above) may not reach the optimal efficacy. Novel vitamin D analogs could be tested in order to achieve better therapeutic effects with fewer side effects. The discovery of the mechanism may also provide new targeting strategies.

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Ruicheng WEI

Etudes des mécanismes cellulaires et moléculaires de la réponse immunitaire de type 2 dans la dermatite atopique

UNIVERSITÉ DE STRASBOURG

École Doctorale
des Sciences de la Vie
et de la Santé
S T R A S B O U R G

Résumé

Mon travail, lors de ma thèse, avait pour but d'étudier la différentiation des lymphocytes Tfh, ainsi que leur fonction et leur régulation dans la pathogenèse de la DA. Pour cela, j'ai utilisé un modèle murin précédemment établi au sein de notre laboratoire consistant en l'application topique de MC903 (un analogue de la vitamine D3) induisant la production de TSLP par les kératinocytes et, par conséquence, la réponse immunitaire Th2 et la pathogenèse de la DA. mon travail doctoral s'est porté sur la différentiation des lymphocytes Tfh, leur production cytokinique ainsi que la formation des centres germinatifs dans le contexte d'un modèle murin de DA induite par le MC903. Mes études ont démontré un rôle critique joué par TSLP dans la réponse Tfh et ont exploré le rôle potentiellement joué par les cellules dendritiques langerine^{+} et la signalisation OX40L dans le développement des réponses Tfh et de type 2. Ceci nous a permis d'approfondir nos connaissances concernant les mécanismes sous-tendant la réponse immunitaire de type 2 dans la pathogenèse de la DA.

Dans la deuxième partie de ma thèse, nous avons examiné le rôle de MC903 dans la régulation de l'inflammation due au psoriasis, en utilisant un modèle de psoriasis induit par l'Aldara. Nous avons montré que MC903 inhibe l'axe 23/IL-17/IL-22 chez les souris souffrant de psoriasis. De plus, cette inhibition semblait être dose-dépendante. Nous avons en outre exploré le rôle de TSLP et VDR dans la médiation de cet effet dû au MC903.

Mots clés : Dermatite atopique, cellules Tfh, formation des centres germinatifs, cellules dendritiques

Résumé en anglais

My thesis aimed at studying the Tfh cell differentiation, function and regulation in AD pathogenesis. To this aim, I employed our previously established AD mouse model in which MC903 (a vitamin D analog) topical treatment on the skin induces TSLP production by keratinocytes, promotes Th2 cell response and drives the pathogenesis of AD. my thesis work investigated Tfh cell differentiation, its cytokine expression and germinal center formation using MC903-induced AD mouse model. By exploring the role of TSLP, Langerin+ DCs and OX40L signaling in Tfh cell differentiation and regulation, my study provides novel insights into the mechanisms underlying the type 2 immune response in AD pathogenesis.

In the second part of my study, we examined the role of MC903 in regulating the psoriatic inflammation using Aldara-induced psoriasis model. We showed that MC903 inhibited IL-23/IL-17/IL-22 axis in mouse psoriasis. Moreover, this inhibition exhibited a dose-dependent manner. We further explored the role of TSLP and VDR in mediating such effect of MC903.

Key words : Atopic dermatitis, Tfh cells, germinal center formation, Dendritic cells