Investigating the role of CD109 in skin immune homeostasis and disease

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Abstract

Immune surveillance of the skin is essential for tissue homeostasis and host defense. When the skin barrier gets disrupted during injury, it facilitates the invasion of pathogens and commensal microbiota to activate keratinocytes. Skin-resident immune cells, such as dendritic cells, are sensors of activated keratinocyte or microbe-derived products that, in turn, secrete cytokines such as interleukin (IL)-1 and IL-23. These cytokines subsequently lead to activation of IL-17-producing cells that reciprocally act to expel pathogens by recruiting phagocytes and restore barrier integrity by stimulating keratinocyte production of anti-microbial peptides. Unlike conventional αβT cells, IL-17-producing γδT (γδ17) cells are residents of the skin and can respond quickly upon inflammatory cytokine encounter in a TCR-independent manner. These unique activation requirements endow them with important protective activity, but also implicates them in chronic inflammatory skin diseases such as psoriasis. Although IL-17 is critical effector cytokine in protective and pathogenic skin inflammation, innate immune mechanisms that limit activation of cutaneous IL-17-producing cells remain poorly understood. CD109 is a GPI-anchored protein highly expressed by keratinocytes and previously shown to be downregulated in psoriatic lesions. Whether CD109 impacts cutaneous IL-17-producing cells has not been investigated. The focus of my thesis is to dissect the role of CD109 in regulating γδ17 in steady state conditions and during psoriasis-like inflammation. Our work identified three novel mechanisms by which CD109 regulates the cutaneous immune response: *1) CD109 restrains the activation of cutaneous γδ17 cells in an IL-23 dependent manner; 2) CD109 limits the commensal microbiota-induced γδ17 activation in the skin; 3) CD109 potentially binds to calcium-dependent proteases calpains to regulate the IL-1α but not IL-1β production by keratinocytes.* Collectively, our data not only provide a better understanding of how the activation of γδ17 cells are controlled by innate cell-derived CD109, but also provide insight into the mechanism of how structural cells, such as keratinocytes, regulate the immune response and maintain the skin homeostasis.

Résumé

La surveillance immunitaire de la peau est essentielle pour l'homéostasie tissulaire et la défense de l'hôte. Lorsque la barrière cutanée devient endommagée, comme lors d'une blessure, celà facilite l'invasion de pathogènes et le microbiote commensal active les kératinocytes. Les cellules immunitaires résidentes, telles que les cellules dendritiques, détectent les signaux émis par les kératinocytes activés ou les produits dérivés des microbes, pour entraîner une réponse immunitaire inflammatoire en sécrétant des cytokines telles que l'interleukine (IL) 1 et l'IL-23. Ces cytokines, initialement destinées à garder les micro-organismes à l'extérieur de l'hôte en renforçant l'intégrité de la barrière, produisant les peptides anti-microbiaux et en recrutant les neutrophils et macrophages, vont ensuite mener à l'activation des cellules produisant l'IL-17 dans la peau. Contrairement aux cellules αβT, les cellules γδT produisant l'IL-17 (γδ17) peuvent rapidement répondre aux cytokines inflammatoires au cours de la phase précoce inflammatoire de manière TCR-dépendante, afin de limiter l'invasion de pathogènes et ainsi réparer le tissue cutané. En plus du rôle des cellules γδ17 dans la défense de l'hôte, ces cellules sont également activées et contribuent aux maladies inflammatoires chroniques cutanées, telles que le psoriasis. Bien que l'IL-17 est une cytokine effectrice cruciale dans l'inflammation à la fois protective et pathogénique au niveau de la peau, les mécanismes immunitaires innés qui limitent l'activation des cellules cutanées produisant l'IL-17 restent mal compris. CD109 est une protéine à ancre GPI hautement exprimée dans les keratinocytes, mais dont l'expression est diminuée dans les lésions psoriasiques. Cependant, le rôle détrimental de CD109 sur les cellules cutanées produisant l'IL-17 n'a pas été étudié. L'objectif de cette thèse était de disséquer le rôle de CD109 dans la régulation des cellules produisant l'IL-17 en condition basale et au cours de l'inflammation de type psoriasique. Notre travail a permis d'identifier trois nouveaux mécanismes par lesquels CD109 régule la réponse immunitaire cutanée: *1) CD109 extrinsèque des cellules myéloïdes limite l'activation des cellules γδ17 cells de manière IL-23 dépendante; 2) CD109 limite l'activation des cellules γδ17 induites par le microbiote commensal au niveau de la peau; 3) CD109 se fixe potentiellement aux protéases calcium-dépendantes Calpaines pour réguler la production d'IL-1α mais pas d'IL-1β par les kératinocytes.* Collectivement, nos données permettent non seulement de mieux comprendre comment l'activation des cellules γδ17 est contrôlée par la protéine CD109 dérivée des cellules du système immunitaire inné, mais également fournissent un apercu du mécanisme des cellules

stromales, telles que les kératinocytes, pour réguler la réponse immunitaire response, en maintenant l'homéostasie de la peau.

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Contribution to Original Knowledge

In this thesis, chapter 2 and 3 significantly contribute to the fields of skin pathology and mucosal immunology. We have identified a keratinocyte-derived, GPI-anchored molecule CD109 that regulates cross-talk among commensals, stromal cells and immune cells, which plays a role in maintaining the skin homeostasis.

In the published manuscript Chapter 2 entitled "*CD109 restrains activation of cutaneous IL-17 producing γδ T cells by commensal microbiota"*, we demonstrated the following:

1. Genetic depletion of CD109 leads to spontaneous skin inflammation, with immune cell recruitment into the epidermis, such as neutrophils.
2. CD109 is a negative regulator of the cutaneous IL-23/IL-17 immune axis.
3. Deletion of CD109 amplifies IL-17 production by skin γδT cells, and this response is tissue-specific.
4. Loss of CD109 predisposed the skin to psoriasiform inflammation.
5. CD109 enforces skin barrier integrity and reactivity to commensal microbiota.

In the manuscript based Chapter 3 entitled *"The role of CD109 in regulating the cutaneous IL-1α/IL-17 immune axis"*, we highlighted the importance of CD109 in driving the keratinocyte-originated skin inflammation. Our preliminary data suggested that:

1. IL-1α, but not IL-1β, is a potential target of CD109 in keratinocytes.
2. Depletion of CD109 leads to spontaneous necrosis of keratinocytes.
3. Keratinocyte-derived IL-1α has the potential to activate the γδT cells *in vitro,* similar to IL-1β.
4. Short-term blockade of IL-1α *in vivo* partially reverses cutaneous inflammation induced by the loss of CD109; however, genetic depletion of IL-1R does not.
5. CD109 binds to the calcium-dependent, non-lysosomal cysteine protease calpain to regulate its activity.

Contribution of Authors

Chapter 1. Introduction

The introduction for this thesis was written by H.Z. and edited by I.L.K.

Chapter 2.

Zhang H, Carnevale G, Polese B, Simard M, Thurairajah B, Khan N, Gentile ME, Fontes G, Vinh DC, Pouliot R, King IL. **CD109 Restrains Activation of Cutaneous IL-17-Producing γδ T Cells by Commensal Microbiota.** *Cell Reports*. 2019 Oct 8; 29(2):391-405.e5. doi: 10.1016/j.celrep.2019.09.003.

H.Z and G.C. designed and performed experiments, analyzed the data and wrote the manuscript. B.P. performed the RNAscope experiments. N.K. performed tissue harvests and analyzed data. M.E.G. performed cell-sorting experiments and qPCR analysis of intestinal tissue. G.F. assisted with experiments, performed western blots and provided critical technical input. D.V. provided reagents and intellectual input. B.T. performed the qRT-PCR for inflammatory gene expression and skin microbiome analysis. M.S. performed percutaneous absorption assays. R.P. provided technical support and intellectual input for the percutaneous studies. I.L.K. conceptualized the study, designed and performed experiments and wrote the manuscript.

Chapter 3.

Zhang H, Fontes G, Thurairajah B, King IL. **The role of CD109 in regulating the cutaneous IL-1α/IL-17 Immune Axis.** *Manuscript in preparation.*

H.Z designed and performed the experiments, analyzed data and wrote the manuscript. G.F performed the western blots, Calpain activity tests and provided critical input. B.T. performed the primary keratinocyte culture from neonate skin. I.L.K. designed the project, analyzed data and wrote the manuscript.

Chapter 4. Conclusions

H.Z. wrote the conclusions and I.L.K edited.

List of Abbreviations

7, 12-dimethylbenz (α) anthracene (DMBA)
Activator 1 (Act1)
Activin receptor-like kinase (ALK)
Alpha-enolase (ENO1)
Anti-microbial peptides (AMPs)
B cell-activating factor belonging to the TNF family (BAFF)
Bone marrow (BM)
Brain tumor stem cell (BTSC)
Butyrophilin-like 1 (Btnl1)
CC chemokine ligand (CCL)
CC chemokine receptor (CCR)
Cisplatin (CDDP)
Cluster of differentiation (CD)
CXC chemokine ligand (CXCL)
Danger-associated molecular patterns (DAMPs)
Dendritic cells (DC)
Dendritic epidermal γδT cells (DETCs)
Double negative (DN)
Endoplasmic reticulum (ER)
Enzyme-linked immunosorbent assay (ELISA)
Forkhead box protein (Foxp)
Genome-wide association studies (GWAS)
Germ-free (GF)
Globin transcription factor (GATA)
Glycosyl phosphatidylinositol (GPI)
Granulocyte-macrophage colony-stimulating factor (GM-CSF)
Hair follicl (HFs)
Hematoxylin and eosin (H&E)
Herpes simplex virus (HSV)
Human leukocyte antigens (HLA)
Imiquimod (IMQ)
Immunoblot (IB)
Immunoglobulin (Ig)
Immunohistochemistry (IHC)
Immunoprecipitation (IP)
Innate lymphoid cells (ILCs)
Interferon (IFN)
Interleukin (IL)
Interleukin-17 producing γδ T (γδ17)
Janus kinase (JAK)
Keratinocyte (KC)
keratinocyte growth factor (KGF)
Knock-out (KO)
Lamellar bodies (LBs)
Langerhans cell (LC)
Leucine-leucine-37 (LL-37)
Lipoteichoic acid (LTA)
Major histocompatibility complex (MHC)
Mammalian target of Rapamycin complex (mTORC)
Natural killer (NK)
Nature killer T (NKT)
Nod-like receptors (NLRs)
Nuclear factor erythroid 2-related factor 2 (Nrf2)

Pathogen-associated molecular patterns (PAMPs)
Pattern recognition receptors (PRRs)
phycoerythrin (PE)
Polymerase chain reaction (PCR)
Promyelocytic leukemia zinc finger (PLZF)
Prostaglandin E2 (PGE2)
Receptor activator of NF-*k*B ligand (RANKEL)
Regulatory T (Treg)
Retinoid-related orphan receptor (ROR)
Rheumatoid arthritis (RA)
RIG-I-like receptors (RLR)
Severe combined immunodeficiency (SCID)
Signal transducer and activator of transcription (STAT)
Skin draining LN (sdLN)
Smad ubiquitination regulatory factor (Smurf)
Small mothers against decapentaplegic (Smad)
Squamous cell carcinoma (SCC)
T cell receptor (TCR)
T helper cell (Th)
T-box expressed in T Cells (T-bet)
TGF-β receptor (TβR)
Tissue resident memory T (TRM)
Toll-like receptors (TLRs)
Transforming growth factor (TGF)
Tumor necrosis factor (TNF)
Tyrosine kinase (Tyk)
Vascular cell adhesion molecule (VCAM)
Very late antigen-1 (VLA-1)
Wild-type (WT)
γ chain variable regions (Vγ)

Preface to Chapter 1

The central focus included in this thesis is to understand the mechanisms that are important for host defense and pathogenesis in the skin. IL-23/IL-17 immune axis has been regarded as the central network for cutaneous host defense. The cascade of IL-23/IL-17 immune axis starts from the irritation on keratinocytes by pathogen invasion or environmental stress, which activates the conventional dendritic cells to migrate to the skin draining lymph node, where they can activate and polarize the helper T cells into Th17 cells. Th17 cells, which are able to secrete IL-17A, IL-17F and IL-22, are then delivered to the skin, where those pro-inflammatory cytokines could act on the structural cells such as keratinocytes and endothelial cells, to induce the activation and proliferation of keratinocytes, the production of antimicrobial peptides, as well as the recruitment of leukocytes such as neutrophils. It is an efficient and fast way to clear the pathogens but aberrant activation of IL-23/IL-17 immune axis leads to psoriasis, and broad neutralization of either IL-23 or IL-17 achieved great success in alleviating symptoms of psoriasis patients. Despite irrefutable role of inflammation in psoriasis of IL-23/IL-17 immune axis, a complete understanding of what structural epithelial cells, immune cells and cytokines is crucial during the initiation and progression in psoriasis is barely explored. If we wish to develop effective therapies for psoriasis, it is important to acquire comprehensive knowledge of the inflammatory players that are involved in the initial phase of psoriasis. One open question that I felt very interesting to answer is, what subset of cells that hides under the skin, other than Th17 cells, orchestrates the initiation of psoriasis? Considering the dual roles of IL-23/IL-17 immune axis, is there an intrinsic regulator that only harnesses pathological but keeps host defense function of IL-23/IL-17 axis? Chapter 1 will provide an overview of skin including the structural cell, immune system and microbiota, with an emphasis on why the IL-23/IL-17 immune axis is so important in cutaneous homeostasis. Additionally, the GPI-anchored molecule CD109 will be briefly introduced.

Chapter 1. General Introduction

1.1. Overview of the skin.

As the largest barrier tissue, skin is the primary interface between the host and its environment. As a result, skin plays a critical role in thermoregulation, fluid balance and defense against environmental insults and infectious agents [1]. There are two main layers of the skin: the epidermis, made of closely-packed keratinocytes, and the dermis, composed of a dense network of connective tissues, blood and lymphatic vessels, neurons, hair follicles, sweat glands and a diverse repertoire of immune cells [2]. Although not of host origin, a thin layer of non-pathogenic microorganisms (i.e. the microbiota) form an additional barrier that contributes to tissue integrity, resistance to pathogen colonization and conditioning of the cutaneous immune system. Collectively, these compartments form the integumentary system [2]. Although skin is a highly plastic and regenerative tissue, dysfunction of this barrier organ can lead to a number of diseases from invasive infectious diseases, cancer and autoimmune diseases such as psoriasis [2]. Thus, skin homeostasis has important consequences on our health and survival.

Epidermis.

The outermost layer of the skin is the epidermis (Figure 1.1), composed predominantly of keratinocytes into four strata or layers [3]. The stratum basale is one row of undifferentiated keratinocytes which composes the most bottom layer of the epidermis. Keratinocytes divide frequently in the stratum basale and move up to the stratum spinosum (SS) [4]. These vertical cylindrical keratinocytes then change into multi-angled cells and start to synthesize keratins distinct from those made by the basal keratinocytes [4]. The upper layer is the stratum granulosum, containing the highest calcium levels among all four strata [4]. Keratinocytes in this layer are characterized by dark and compact cytoplasmic material; these cells actively produce keratin, proteins and lipids [4]. The stratum corneum is the most superficial layer of the epidermis, which provides the barrier against percutaneous chemical damage, pathogen infection and mechanical forces [4]. The keratinocytes finally differentiate into dead and organelle-free keratinocyte-derived cells, anucleated corneocytes, a process referred to as cornification [5]. In this layer, corneocytes are associated by corneo-desmosomes and surrounded with extracellular non-polar lipids to form

a hydrophobic matrix, to prevent dehydration [5]. In addition to keratinocytes, other cells residing in the epidermis include melanocytes, Merkel cells and immune cells, which will be discussed later [6].

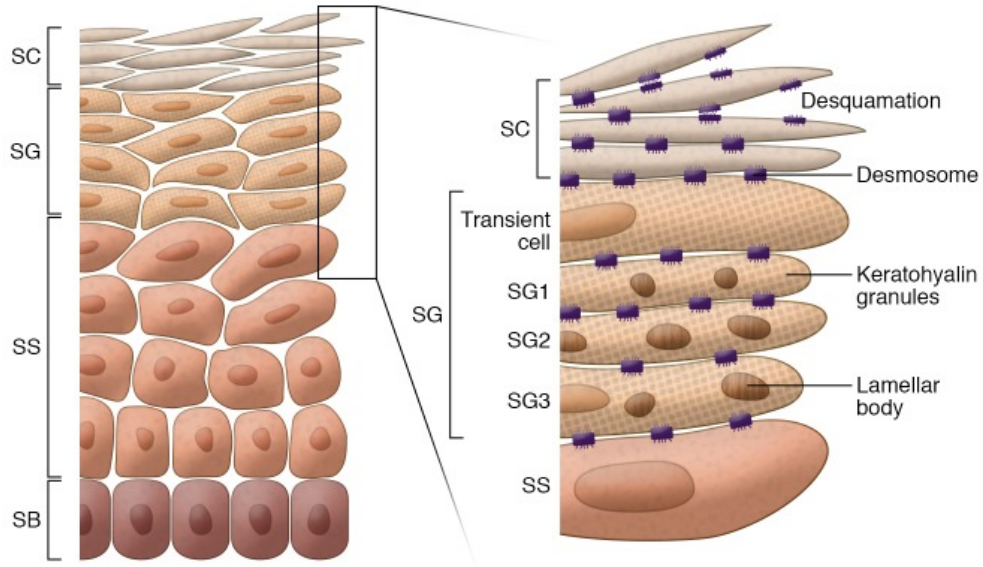

Fig.1.1 Layers of the human epidermis (Adapted from [3]) . See main text for details.
(A) Stratum basale (SB). The deepest portion of epidermis is stratum basale (SB), that cells found in this layer are cuboidal to cylindrical stem cells constantly producing keratinocytes.
(B) Stratum spinosom (SS) usually has 8~10 cell layers, containing irregular, polyhedral keratinocytes.
(C) Stratum granulosum (SG) contains 3~5 layers of diamond-shaped keratinocytes with karetohyalin granules and lamellar bodies.
(D) Stratum corneum (SC) is the uppermost layer filled with keratin and anucleate squamous cells. Those anucleate squamous cells, which are dead keratinocytes, could form horny scales.

Keratinocyte function as danger signal sensors and produce innate immune mediators. As the primary interface with the environment, keratinocytes constantly encounter injurious and inflammatory stimuli. They are the key sentinels of skin, that can recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), which can detect various pathogen and self-components [7-9]. Keratinocytes express several TLRs on their cell surface (TLR1,2,4,5 and 6) and in endosomes (TLR3 and 9), but they do not express TLR7 and 8 at steady state [7]. TLR7 expression of keratinocytes are triggered by TLR3 activation to facilitate the anti-viral response [8]. Other PRRs that could be expressed on keratinocytes include Nod-like receptors (NLRs), NLR pyrin domain-containing proteins, RIG-I-like receptors (RLR) and C-type lectins that respond to bacterial peptidoglycan, viral, fungal and self-constituents [9]. Together, these

PRRs expressed on keratinocytes make the epidermis a frontline against pathogen invasion and an early system for the underlying immune system.

Keratinocyte is also an important source of anti-microbial peptides (AMPs), which is an evolutionarily conserved defense mechanism of eukaryotes against pathogens [10]. AMPs are found to be highly expressed in the skin lesions of psoriasis patients, which could possibly explain their increased resistance to cutaneous infection [10].

In the healthy skin, keratinocytes constitutively express pro-IL-1α which is not processed and secreted into its most active form, cleaved IL-1α, at steady state [11]. In addition, the activation of IL-1α in keratinocytes is less clear. Caspase 8 seems to negatively regulate IL-1α, since it has been shown that loss of caspase 8 could increase IL-1α secretion from keratinocytes [12]. Additionally, IL-1α overexpression by basal keratinocytes in a transgenic model leads to an inflammatory skin phenotype, indicating that IL-1α could also participate in skin inflammation [13]. In addition, keratinocytes produce and release cytokines and chemokines in response to pathogen invasion. Chemokines including CXCL9, CXCL10 and CCL20 are important to recruit effector cells to the skin and cytokines including tumor necrosis factor (TNF), IL-1α, IL-1β and IL-33 could direct the immune response [1].

Lamellar bodies (LBs). The two most important barrier properties of the skin are permeability and antimicrobial production, which are localized primarily to the epidermal stratum corneum [14, 15]. The permeability barrier is the extracellular lipid-enriched lamellar membranes between corneocytes to block the movement of water and electrolytes [14, 15]. The antimicrobial barrier includes lipids and antimicrobial peptides within the extracellular spaces of the stratum corneum [14, 15]. Of note, the lipids and protein transportation of these two barriers are through the secretion of lamellar bodies [16].

Proteomic analysis has identified over 900 components in the LBs [17]. LBs are specialized secretory organelles in the epidermis, derived from the *trans*-Golgi network [18]. It was first observed in the upper stratum spinosum, with increased numbers in the stratum granulosum [18]. LBs not only provide pro-barrier lipids conveying a fully lamellar internal structure, but also carry metabolizing and proteolytic enzymes, enzyme inhibitors and AMPs, which play a vital role in the formation of cutaneous permeability and antimicrobial barriers [19]. After acute barrier disruption, steady-existed LBs are quickly secreted by cells of the SG; simultaneously, lipids and enzymes

are produced to form new LBs [20]. When the permeability barrier is disrupted, AMP production is also stimulated and thus co-regulated with the permeability barrier function [14].

Calcium is also important for the epidermal barrier by regulating cell-cell adhesion and keratinocyte differentiation. The stratum granulosum, where the LBs are produced, has the highest calcium concentration, while the stratum corneum has very low concentrations due to its limited ability to dissolve ions in lipid contents [21]. A disrupted permeability barrier, that facilitates water influx to the stratum corneum, makes the epidermis unable to maintain the calcium gradient. One of the consequences of calcium loss is the exocytosis of LBs [21, 22]. Furthermore, it is reported that both calcium concentration and LBs are impaired in skin diseases such as psoriasis [23, 24].

Dermis.

The dermis lies beneath the epidermis, connecting with the SB. It is composed of three layers, which are papillary, reticular and hypodermis (Figure 1.2) [25]. The papillary dermis is the area directly below the epidermal basal membrane and supports epidermal appendages such as sweat glands and hair follicles [25]. The reticular dermis and hypodermis are the second and bottom layer, with the latter containing mainly white adipose tissue [25]. Additionally, both papillary and reticular dermis are enriched of fibroblasts, with different functions. Fibroblasts in the papillary layer provide a supportive niche for hair follicle development and maintenance, while reticular fibroblasts produce extracellular matrix for structural assistance [26, 27]. In addition to the fibroblast network and appendages, the dermis also contains structural cells for the neurovascular system and immune cells [28, 29]. These cells work together to ensure the proper function of skin barrier and immune protection. Hypodermis is the deepest layer of the skin which contains the adipose lobules and some skin appendages, sensory neurons and blood vessels [28].

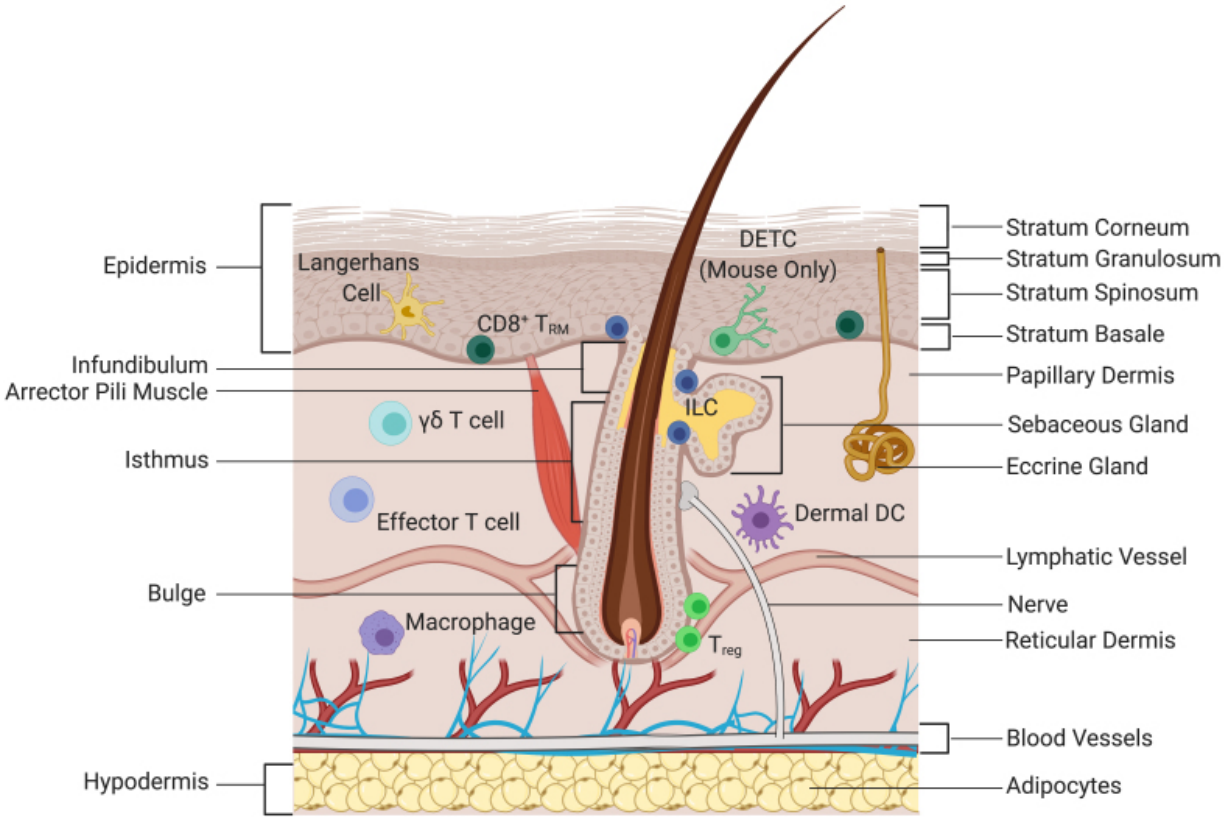

Figure 1.2. Structural and cellular stratification of the skin (Source: Adapted from [25]). See main text for details.

(A) Epidermis, which is composed of closely packed keratinocytes.

(B) Dermis is dense, irregular tissue that harbors hair follicle, sweat gland, blood vessel, nerve and other structures.

(C) Hypodermis is beneath the dermis that mainly composed of loose and fatty tissues.

Hair follicles.

The skin maintains appendages like "pilosebaceous units", that are composed of hair follicles (HF), sebaceous glands, arrector pili muscles and sweat glands (Figure 1.2) [25]. They are essential for thermoregulation, sensory input and physical protection. HFs consist of mainly keratinocytes, that are continuous with the epidermis and penetrate deep into the dermis [30]. HFs can be separated into distinct functional regions, based on the unique cellular and molecular features of their basal and superbasal cells. HF can be fractionated into infundibulum, isthmus and hair bulge, which are three regions from the skin surface toward the dermis. These three parts of the HF have distinct progenitors and interact with unique immune cell subsets to maintain local homeostasis [30-32].

The importance of hair follicles in skin immunity is not yet fully understood. Since HFs directly connect to sebaceous glands, they serve as a unique lipid-rich hydrophobic niche for distinct microbial constituents [33], that allow the immune cells positioned around the appendages to cross-talk with the microbiota [34]. Recent studies have shown that hair follicle-associated chemokines can influence the trafficking of immune cells, such as monocytes and Langerhans cells, between dermal and epidermal layers [35, 36].

1.2. The Cutaneous immune system

Langerhans cells and dendritic epidermal γδT cells (DETCs) are the major immune cells in the murine epidermis [25]. As the preliminary antigen-presenting cells in the skin, Langerhans cells continuously surveillance the epidermis, take up and process the antigens for the presentation to T cells [37]. Mouse epidermis uniquely contains DETCs, which will be discussed later.

The dermis is populated by various immune cells including dendritic cells, macrophages, T cells and innate lymphoid cells (ILCs) [38]. The papillary dermis is enriched for dendritic cells, and the CD4+ T cell population is closely associated with the dendritic cell populations [25]. Interestingly, either effector or regulatory T cell populations, which include αβT cells and γδT cells, are concentrated in proximity to the hair follicles [34]. Macrophages, whose populations is also associated with T cell populations, reside in the lower dermal layer [25]. Dermal γδT cells comprise nearly 50% of the total T cell population, and they mediate pathogen defense by augmenting neutrophil recruitment through IL-17 [34]. αβT cells could be further divided into CD4+, CD8+ and NKT cells, and the major part is tissue resident memory T (T_{RM}) cells. T_{RM} cells are most reported as CD8+ T cell lineage and express CD69 and CD103, which could quickly respond, to provide the local protection against infection independent from the circulation [39].

Dendritic cells (DCs)

Dendritic cells are present throughout the body and can be divided into at least five groups: plasmacytoid DCs, migratory and lymphoid tissue resident CD8+ DC-like DCs, migratory and lymphoid tissue resident CD11b+ DCs, Langerhans cells and monocyte-derived DCs [40]. However, in the skin, there are only three distinct subsets at steady state: Langerhans cells in the epidermis, CD11b+ and CD103+ DCs in the dermis. Langerhans cells are derived from the fetal liver and yolk sac during embryonic development [41]. Both CD11b+ and CD103+ DCs are derived from Pre-DCs, which are the downstream of common DC progenitors [42, 43]. Blood monocytes can also generate DCs, usually under inflammatory conditions, in various tissues including the skin, contributing to innate and adaptive immune responses [44, 45].

Langerhans cells (LCs) are antigen-presenting DCs that reside in epithelia of both human and mice, and they are the only DCs found in the epidermis at steady state. The precursors of LCs first

populate the epidermis at embryonic day 18, forming a self-renewing and radio-resistant dendritic cell population [46, 47]. They are placed in the epidermis above the basal keratinocytes with elongated dendrites upward toward the cornified layer of epidermis [46]. Dendrites of Langerhans cells can move cross the tight junctions, to sample a large area of epidermis and contact the keratinocyte, without compromising the epidermal barrier [37, 46, 48]. These dendrites, like other peripheral DCs, enable the LCs to efficiently acquire antigen from the periphery and migrate to regional lymph node, where they could present antigens to naïve and memory T cells [37]. LCs acquire a variety of antigens, including antigens from pathogens that invade the epidermis, self-antigen from keratinocytes and tumor antigen from neoplastic tissue [49]. For example, LCs have been reported to contribute to prime the skin pathogens such as *C. albicans* and *S. aureaus*, to favor the IL-17 producing T cell response, which is important for the host defense agianst these pathogens [50]. They could sample the bacterial toxins on the apical side of tight junctions, to avoid breaching the epithelial barrier and exposing these toxins to the deeper layers of the skin[48]. LCs could be immunosuppressive as well, by either the induction of T cell deletion or the activation of Treg cells [51, 52].

A unique feature of Langerhans cells is the expression of CD207, a C-type lectin receptor known as Langerin. Langerin is responsible for the generation of Birbeck's granules, which is an ultra-structural hallmark of LCs [37]. Moreover, LCs that are transiting through the dermis and migrated to the skin-draining lymph nodes could be identified by the expression of CD11b, EpCAM, Langerin and lack of CD103 expression [53, 54].

Dermal CD103+ DCs (cDC1), the mouse equivalence of human the CD141+ population, are members of the CD8+ DC-like DC group, with properties aligned to mouse splenic CD8+ DCs [43, 55]. They can capture dead cells, recognize virus and intracellular pathogens, drive Th1 response by the production of IL-12, and are very efficient at cross-presenting antigens to induce the CD8+ T cell response. For example, CD103+ DCs are found to efficiently present viral antigens to both CD4+ and CD8+ T cells, indicating their importance in viral immunity [56]. In addition, CD103+ DCs can cross-present cutaneous self-antigens to CD8+ T cells, suggesting a potential role in self-tolerance and immune homeostasis.

Dermal CD11b+ DCs (cDC2) are heterogeneous but phenotypically similar for most surface markers. It seems that CD11b+ DCs could be divided into two groups: one is similar with the splenic CD8- DCs derived from the pre-DCs, and the other from monocytes. As human

CD141+DCs match the CD103+ DCs, the CD1c+ DCs of pre-DC origin and CD14+ DCs of monocyte origin nicely match the two groups of murine CD11b+ DCs [55]. In the viral infection of HSV, dermal CD11b+ DCs has been shown to present antigen preferentially to CD4+ T cells, on one hand to activate the CD4+ T cells in both lymph node and skin, on the other hand to dampen the IFNγ-producing T cells by the antigen presentation to Treg cells [57, 58]. In addition, CD11b+ DCs but not Langerhans cells are the principal antigen-presenting cells in a model of leishmaniasis [59]. To note, CD301b+ DCs, which has been reported as a distinct population of CD11b+ DCs, could induce both Th2 and Th17 response, by the production of IL-4 and IL-23, in mouse models of *Nippostrongylus brasiliensis* and psoriasiform dermatitis, respectively [60-62].

Monocyte-derived DCs (mo-DCs) are phenotypically similar to dermal CD11b+ DCs that share the expression of CD11c, CD11b and CX3CR1 in mice. They have not been characterized in healthy human dermis but might be within the CD14+CD1c+ DCs. People could distinguish the mo-DCs from CD11b+ DCs by the requirement of CCR2 signaling for their development in skin. Further studies revealed that mo-DCs arise from Ly6C+ monocytes that enter the skin, and gradually lose Ly6C expression. To note, skin commensals could trigger their differentiation, as shown that germ-free mice have reduced mo-DCs in the skin. They could also activate CD4+ and CD8+ T cells and migrate to lymph node, even though they are inferior in this function compared to CD11b+ DCs [63].

Plasmacytoid DCs are usually absent from both human and mouse skin but could be recruited to the skin during inflammatory conditions. They are the major source of type I interferons, which play important roles in autoimmune diseases and anti-viral immunity [64]. In systemic lupus erythematosis (SLE), plasmacytoid DCs produce IL-6 and type I interferons in response to autoantibody-nucleic acid complexes, to drive the autoreactive B cell response [65]. In psoriasis, they can produce type I interferons to promote disease initiation [66].

Macrophages

Macrophages are only present in the dermis at steady state. In human, they are $MHCII^{br}$, CD14+, FXIIIa+ and highly auto-fluorescent, owing to their dense cytoplasmic content including melanin granules [67]. In mice, they are $MHCII^{int}$, CD115+ (CSF1R+), F4/80+, CD11b+, Ly6C+/-, CCR2-, CD64+ and MERTK+ [68]. Compared to DCs in the skin, dermal macrophages are

superior in phagocytic ability and inferior in T cell activation. To note, CSF1/CSF1R signaling is required for the development of dermal macrophages and they have two developmental origins [69]. The first and main source is circulating monocytes, that CCR2-/- mice exhibit a dramatic reduction of dermal macrophages; the second and minor source is prenatal embryo-derived macrophages [68, 69]. In human, CD14+ macrophages could be divided into two groups as well: non-auto-fluorescent ones which are blood CD14+ monocyte-derived, and auto-fluorescent macrophages containing melanin granules that are long-lived and turnover slowly [68]. The functions of dermal macrophages are not well described, but studies have shown their potential in scavenging and microorganism killing [68].

Innate lymphoid cells (ILCs)

Innate lymphoid cells (ILCs) are bone marrow-derived tissue-resident lymphocytes that, although arising from common lymphoid progenitors, do not express rearranged antigen-specific receptors. Recently, ILCs have been identified in the skin [70-73]. The nomenclature of ILCs is largely analogous to CD4+ T helper effector cell subsets, referred to as ILC1, ILC2 and ILC3 [74]. ILC1 populations are triggered by the cytokine IL-12 and IL-18 and participate in viral immunity. They express the transcriptional factor T-bet, make IFNγ and consist of conventional natural killer (NK) cells as well as non-lytic populations. ILC2 populations express GATA3 and produce the type 2 cytokines IL-5, IL-9 and IL-13 in response to the cytokines IL-25 and IL-33. ILC2 are involved in tissue repair, asthma and helminth infection. ILC3 populations express RORγt and produce cytokines such IL-17, IL-22 and some IFNγ. They respond to IL-1β and IL-23, participating in anti-bacterial response, tissue repair, and lymphoid tissue development.

So far, only a small number of NK cells of the ILC1 subset have been detected in healthy skin, while increased ILC1s have been observed in psoriatic skin [75, 76]. ILC2s in the other hand are much more common in the skin and are thought to maintain skin homeostasis. For example, ILC2s can promote wound healing by the production of IL-13 and amphiregulin [71, 72]. However overt ILC2 activation could lead to type 2-driven allergic disease as shown by ILC2 accumulation in the skin during atopic dermatitis, and depletion of ILC2s in T cell-deficient mice successfully reduced skin inflammation in a dermatitis model [71]. ILC3s have also been identified in mouse skin under

inflammatory conditions and might contribute to skin inflammation in an experimental psoriasis model [70].

Mast cells

Mast cells are multi-functional immune cells of myeloid lineage throughout the body including skin. They originate from the pluripotent progenitors of the bone marrow [77]. Mast cell progenitors migrate by engaging α4β7 integrin to adhesion molecule VCAM1, and differentiate into mast cells in the tissue, under the control of stem cell factors and cytokines. The hallmark of mast cells is large granules in the cytoplasm, to store the inflammatory mediators including histamine, heparin, cytokines, chondroitin sulfate and neutral protease. One of the first identified functions of mast cells was to produce anti-parasitic molecules when activated by IgE during helminth infections, which helps to expel parasites through increased vascular permeability and muscle contraction. It has also been shown that mast cells express PAMPs, and the response is dependent on which PAMPs is activated [78]. When TLR2 is activated by gram-positive bacteria and mycobacteria, mast cells secrete IL-4 with cell degranulation and histamine release. When TLR4 is activated by gram-negative bacteria, they release pro-inflammatory cytokines such as IL-1, IL-6 and TNFα without degranulation. Despite the role of mast cells in innate immunity, they could participate in adaptive immunity as antigen-presenting cells. Additionally, they produce IL-1 and TNFα, to promote DC migration from skin to the draining lymph node to activate cytotoxic T cells. Moreover, mast cells can be activated by IgE in the dermis, to release the histamine and vasoactive molecules, leading to urticaria and angioedema. Chronic mast cell activation could cause atopic dermatitis or eczema [79].

αβT cells

αβT cells are T cells that express α and β T cell receptor (TCR) chains. They originate from the fetal liver during embryonic life and bone marrow at post-natal periods. T cell progenitors from either fetal liver or bone marrow seed the thymus and undergo αβTCR-mediated positive and negative selection to become mature αβT cells. After this, αβT cells migrate to the peripheral tissue, where they can differentiate into effector αβT cells [80].

Human and mouse skin contains a large number of αβT cells, including both CD4+ and CD8+ T cells, with about 10% of Foxp3+ T cells indicating a regulatory T cell phenotype [81]. The majority of αβT cells reside in the dermis, most of which are CD4+ T cells, consisting of T helper (Th) cells and regulatory T cells (Tregs) [82]. Th cells, that are generated from naïve CD4+ T cells, could be further segregated into distinct subsets based on their cytokine profiles, including IFNγ-producing Th1, IL-4-producing Th2 and IL-17-producing Th17 cells [83]. In addition, naïve CD4+ T cells can also differentiate into regulatory T cells, to maintain immune tolerance and regulate the immune response by TGF-β production. To note, the lineage-specific transcriptional factors play the most critical roles during the differentiation process. T-bet is the master transcription factor for Th1 cell differentiation, by direct regulating a large number of Th1-specific genes, including remodeling of *Ifng* locus [84]. GATA3 is the master transcriptional factor to promote Th2 differentiation in different mechanisms, such as by directly regulating the *Il4*, *Il5*, *Il13* and *Il1rl1* (gene that encodes T1/ST2, IL-33 receptor) genes [85]. RORγt is the master transcription factor for Th17 cells by directly regulating IL-17a and IL-17f expression. And RORγt expression is induced by IL-6, IL-21 and/or IL-23 activated Stat3 [83, 86]. Finally, Foxp3 is the master transcription factor for Treg generation and function [87].

Moreover, the epidermis contains few αβT cell, which are CD8+ T cells. Nature killer T (NKT) cells, a subset of innate lymphocytes that express invariant TCRs, share nature killer cell functions, are present in the skin as well [82].

Cutaneous αβT cells could also be divided in two groups, by distinguishing between those cells that simply pass through the tissue and those that are permanently in the tissue as a memory population, those latter cells are referred to as tissue-resident memory (T_{RM}) cells [82]. Studies of HSV-1 infection in mice indicated a population of skin-associated T cells that are disconnected from the wider circulation [39, 88]. Most of the T_{RM} cells are of CD8+ T cell lineage, and express a unique set of markers including CD69, VLA-1 and CD103 [39]. Further study revealed that the CD4+ memory T cells and CD8+ T_{RM} cells have different tissue locations and recirculation patterns, that CD4+ memory T cells are seeded in the dermis and capable of recirculating to the blood while CD8+ T_{RM} cells reside in the epidermis and do not recirculate [88].

γδT cells

The γδT cells are defined as T cells that express γ and δ TCR subunits. They develop in the embryonic thymus in sequential waves, then delineate into effector subsets based on the expression of either T-bet/IFNγ or RORγt/IL-17 (γδ1 or γδ17, respectively), and migrate to the peripheral tissues [89, 90]. There are three nomenclature systems for γδT cell subsets, but we will only use the one that proposed by Heilig and Tonegawa, which includes Vγ1-Vγ7 in mice [91].

- **γδT cell development**

Waves of γδT cell development (Table 1.1)**.** One distinct feature of γδT cells is that γδ thymocytes develop in a series of "waves" that can be defined by γ chain variable regions (Vγ) usage. This process begins early as the fetal thymus is seeded at embryonic day 13.5 (E13.5). Fetal liver progenitors generate the first wave of γδT cells, which are Vγ5+Vδ1+ DETCs [92]. The second wave of γδT cells are Vγ6+Vδ1+ subsets developing at E16, which preferentially seed the uterus, gingiva, lung, liver, placenta and dermis [93]. The next wave of γδT cells are Vγ4+ and Vγ1+ subsets that pair with several Vδ chains, which could be found in many tissues such as peripheral lymphoid organs, blood, lung, liver and dermis [94]. In addition, the development of Vγ4+ and Vγ1+ γδT cells are not restricted to the fetal window, and they can develop in neonatal and adult life. Vγ7+ γδT cells reside in the intraepithelial layer of the small intestine, and they are thought to be matured extrathymically.

Table 1.1 Waves of γδT cell development.

Subset	V(D)J diversity	Timing of development	Tissue residence	Major cytokine
Vγ1	High (Vγ1Vδ6.3+)	Perinatal and Adult	Liver, gingiva, lung, intestine, lymphoid tissues	IFNγ (IFNγ and IL-4)
Vγ4	Variable	E18~adult	Dermis, gingiva, liver, lung, intestine, lymphoid tissues	IL-17
Vγ5	Invariant (Vγ5Vδ1+)	E13~E16	Epidermis	IFNγ
Vγ6	Invariant (Vγ6Vδ1+)	E16~birth	Gingiva, uterus, lung, liver, dermis, placenta	IL-17
Vγ7	Intermediate	Neonatal	Intestine (intraepithelial)	IFNγ

TCR-dependent γδT cell development in thymus. Both αβT and γδT cells differentiate from common CD4-CD8- double negative (DN) progenitor where TCRγ, TCRδ and TCRβ rearrangement happens [95] (Figure 1.3). DN cells that receive strong TCR signals will adopt γδ fate, while the ones that receive weaker TCR signals will commit to the αβ lineage. Following the γδ lineage commitment, TCRγδ signaling also plays important role in thymic differentiation of γδT cell subsets. Studies on the progenitor cells of Vγ5+ and Vγ4+ γδT cells proposed that strong TCRγδ signals could drive the adoption of IFNγ secreting fate, and weaker TCRγδ signals could support the γδ17 cell differentiation [90]. These results are consistent with a bias toward γδ17 fate in the absence of ERK signaling. However, the fetal IL-17 producing γδT cells (Vγ6+, not Vγ4+) have been shown to be significantly decreased in mice heterozygous for CD3γ and CD3δ (CD3DH) mice, which lack TCRγδ signaling due to the reduced TCRγδ expression [96]. These contradictory results suggest that different γδT cell progenitors may require different TCRγδ signals, which needs to be further clarified. To note, even though both the γδ lineage commitment and γδT cell differentiation require TCRγδ signaling, these two process are likely to be temporally segregated, so that TCR signaling controls the distinct outcomes in sequential developmental windows.

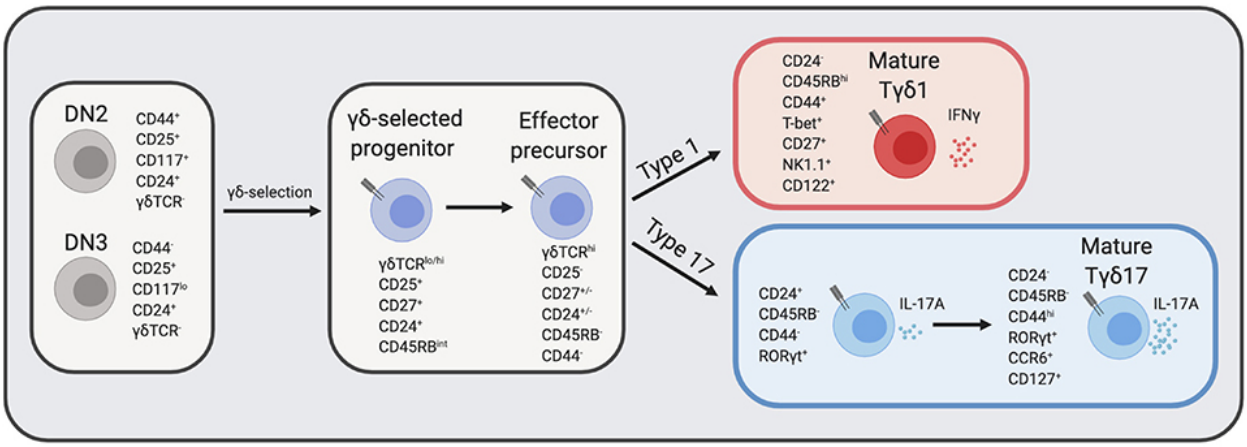

Fig.1.3. γδT cell development in the thymus. (Source: Adapted from [97]) See text for details.

DN thymocytes undergo γδ-selection and become immature γδ thymocytes, that diverge into either IFNγ or IL-17 produers. Type 1 and type 17 γδT cells have distinct surface markers and transcriptional factors that listed next to each cell type.

TCR-independent transcriptional networks in γδT cells. Cytokine production of T cells is tightly regulated at the transcriptional level [84, 98-101]. Studies have revealed that IFNγ vs IL-17 expression in γδT cell progenitors are controlled by lineage-specific transcriptional networks [102]. Like CD4+ T cells, the master transcriptional factors that regulate IFNγ vs IL-17 expression

in γδT cells are T-bet and RORγt, respectively (Figure 1.3). Mice deficient for T-bet or RORγt are entirely deficient in the corresponding cytokine-producing γδT cells. To note, even though these two transcription factors are core molecular determinants similar to CD4+T cells, the effector function differentiation of CD4+ T cells and γδT cells seems to be regulated differently. Indeed, the "auxiliary" transcriptional factors that contribute to Th1 and Th17 differentiation, such as Eomes, Stat3, RORα, BATF or IRF4, are dispensable for γδT cell differentiation [103].

The TCR-independent mechanisms of RORγt expression has been reported to be dependent on the transcriptional factors Sox4 and Sox13, which are expressed before TCR signaling events [104]. In support of this, mice with spontaneously mutated-Sox13 have a selective deficiency of Vγ4+ IL-17-producing γδT cells [105]. Together, these results indicate that Sox4/Sox13/RORγt/IL-17 program participate in γδ17 differentiation. When this program is counteracted, it will lead to γδ1 differentiation [96, 106, 107]. As mentioned before, strong TCRγδ signaling will lead to biased γδ1 differentiation. Egr2 and Egr3, which are transcriptional regulators of Id3, are involved in this process, by downregulating RORγt and Sox13 expression to not only prevent "default" into the IL-17 program but also allow IFNγ expression in γδT cells [106]. TCF1 and Lef1, have been shown to promote the development of IFNγ-producing γδT cells [104].

- **Features of γδT cells**

The "developmental preprogramming" of γδT cells in the thymus allows for rapid responsiveness to inflammatory cytokines in the periphery, without the requirement of TCR engagement. For example, IL-12 and IL-18 are sufficient induce IFNγ-producing γδT cells [103, 107], and IL-1β, IL-7 and IL-23 induce IL-17-producing γδT cells from mature, circulating γδ T cells [108, 109]. γδ1 and γδ17 cells can also be segregated by the expression of CD27 and CCR6, respectively [110, 111]. Notably, IFNγ and IL-17 production of γδT cells are tightly linked to the TCR somatic recombination and γ-chain variable region (Vγ) [89]. Vγ1+ subsets are mainly IFNγ-producing γδT cells. However, for the Vγ1Vδ6.3+ γδT subset, these cells could express the transcriptional factor PLZF and acquire the ability to produce Th1-cytokine IFNγ and Th2-cytokine IL-4 simultaneously. Vγ5+ γδT cells, which are known as the DETCs, produce neither IFNγ nor IL-17 [112]. Instead, they produce cytokines and chemokines that promote the wound healing process and tissue repair, such as keratinocyte growth factor, CCL10 and insulin-like growth factor-1 [113-115]. The main subset of γδ17 cells are Vγ4+ and Vγ6+ γδT cells, which are

shown to be clustered together but completely segregated from Vγ1+, Vγ5+ and Vγ7+ γδT cells by the high-resolution transcriptome analysis [116]. Even the proportions of Vγ4+ to Vγ6+ vary in each organ [117], both Vγ4+ and Vγ6+ γδT cells have the ability to produce IL-17 that facilitate neutrophil infiltration and bacterial clearance [111, 118, 119].

In spite of these "programmed γδT cells", there is a fairly large fraction of functionally immature γδT cells that leave the thymus with a naïve phenotype ($CD122^{lo}CD62+CD44^{lo}$) [120]. It has been reported that they only acquire the cytokine-producing capacity in the periphery. For example, the phycoerythrin (PE)-specific γδT cells differentiate into γδ17 cells when they encounter the antigen [121]. Furthermore, Vγ7+ intestinal intraepithelial lymphocytes (IELs) acquire the butyrophilin-like 1 (Btnl1) expression in intestinal epithelial cells to become IFNγ-producing cells [122].

Human γδT cells are distinguished by the δ-chain variable region, including Vδ1-Vδ3. Like murine γδT cells, human γδT cells are potent cytokine-producing cells. However, they are more plastic and dependent on TCR engagement for activation, as reported by studies showing that the Vγ9Vδ2+ subset can produce IFNγ, IL-4 or IL-17 [123].

- **Cutaneous γδT cells.**

DETCs and dermal γδT cells are the two main γδT cell populations in the murine skin. Over 98% of the DETCs express the Vγ5+ TCR, however the dermal γδT cells are more heterogeneous [124-126]. Even though the trafficking and migratory behavior of skin γδT cells varies, the depletion of CCR7, that mediates the emigration from the tissue to peripheral lymphoid organs of most cell types, has no effect on either DETCs or dermal γδT cells at steady state[125].

DETCs are the tissue-resident Vγ5+ γδT cells in the murine epidermis that do not return to the circulation. The precursors of DETCs appear in the first wave of fetal thymocyte development. They have extending dendrites oriented toward the apical epidermis, with cytoplasm - filled projections to detect the phosphorylated tyrosine signal [127]. Moreover, Vγ5+ TCRs are constitutively clustered and activated at steady state, forming the synapse between the projections of DETCs and tight junctions of squamous keratinocytes. CD103 is present in these synapses and interact with E-cadherin expressed on keratinocytes as an anchor of the apical dendrites. In response to tissue stress, the DETCs quickly retract their dendrites and re-localize toward the basal epidermis and Langerhans cells to maintain homeostasis [127]. Besides the stress sensor function

of DETCs, they can also produce growth factors and AMPs to promote epithelial proliferation and repair [115, 128].

Dermal γδT cells. Unlike DETCs, murine dermal γδT cells are more amoeboid in shape and express CCR2 and CCR6, enabling them to migrate to and from the periphery [117]. CCR6+ γδT cells, as expressed by polarized γδ17 cells, can be recruited to the dermis by keratinocyte-derived CCL20. Blockade of CCR6/CCL20 signaling in an IL-23-induced psoriasis model successfully prevents γδ17 cell infiltration to the dermis, which ameliorates epidermal hyperplasia and dermal edema [129]. However, another model of skin inflammation showed that CCR6 is downregulated and CCR2 is upregulated, to recruit more dermal γδT cells into the inflamed region [117, 130].

Both Vγ4+ and Vγ6+ γδT cells are present in the dermis and are able to elicit an IL-17 response similarly to Th17 cells during pathogen invasion such as *M.bovis* and *S. aureus* infection [124-126, 131]. However, the mechanisms for the activation of dermal Vγ4+ and Vγ6+ γδT cells are different. Recent study showed that Stat3 signaling is crucial for the effector functions of Vγ4+ γδ17 cells, while it is not required for Vγ6+ γδ17 cells [132]. Both TORC1 and mTORC2 are essential for dermal γδT cell proliferation, however, only loss of mTORC2 leads to decreased dermal γδ17 cells [132]. In addition, commensal microbes such as *C. mastitidis* and *S.epidermidis* activate γδT cells to provide heterologous protection against invasive pathogens [133, 134]. With the stimulation of IL-1β and IL-23, dermal γδT cells can be activated to produce IL-17, inducing neutrophil infiltration and bacterial clearance [124, 126].

Human γδT cells are present but rare in the skin, and Vδ1+ cells are the dominant subset [135]. It seems like Vδ1+ γδT cells recapitulate the role of DETCs in mice, as they are usually associated with IFNγ production, and are able to produce keratinocyte growth factors with the help of αβT cells[135, 136]. Additionally, human γδT cells can also produce IL-17 as shown in the psoriatic lesions [126].

1.3. The IL-23/IL-17 immune axis

IL-23/IL-17 axis in host defense.

- **IL-23 and IL-1.**

IL-23 is a dimer composed of a specific subunit p19 and a subunit p40 shared with IL-12. Sources of cutaneous IL-23 include monocyte-derived dendritic cells/macrophages in the dermis and Langerhans cells in the epidermis. IL-23 signals through a specific IL-23 receptor (IL-23R) paired with IL-12Rb1, expressed on RORγt+ cells, including αβT, γδT, natural killer T and innate lymphoid cells (ILCs). The binding of IL-23 to IL-23R subsequently induces JAK2/TKY2 and Stat3 phosphorylation [137], to drive IL-17 and IL-22 production. As the central factors to balance skin homeostasis, the IL-23/IL-17 immune axis is an essential regulator of bacterial and fungal infection, such as *S. aureus* and *C. albicans* [131, 138-140].

The combination of IL-23 plus IL-1 is synergistic in the induction of IL-17. IL-1 is a pleiotropic pro-inflammatory cytokine, containing two members IL-1α and IL-1β that induce systemic and local responses to infection. Nuclear factor κB (NF-κB) regulates the transcriptional activity of IL-1. Biological responses of IL-1 are mediated by the IL-1 receptor type 1 (IL-1R1), which is ubiquitously expressed [141]. Interestingly, IL-1R1 and TLRs share the same cytoplasmic signaling domain, the Toll/interleukin-1 receptor (TIR) domain, demonstrating the prominent role of IL-1 signaling for inflammation and innate immune sensing [142]. Compared with IL-1β, which is not constitutively expressed and is produced only by myeloid cells, the IL-1α precursor is present in mesenchymal cells of healthy humans and mice, including keratinocytes, the type 2 epithelial cells of the lung, the entire gastrointestinal tract and in brain astrocytes. There is no requirement of caspase-1 cleavage of the IL-1α precursor to process and release the active cytokine. However, the mature form of IL-1α has more potent biological activity [143].

There are various enzymes that have been reported to cleave pro-IL-1α, such as calcium-activated membrane Calpains, thrombin and Granzyme B [11, 144, 145]. IL-1α has dual functions, extracellularly and intracellularly. Extracellularly, like IL-1β, it binds to the IL-1R1 on the cell surface, which recruits the co-receptor IL-1R3 and initiates pro-inflammatory signals. Intracellularly, the precursor of IL-1α functions as a nuclear transcription factor [142]. Under natural apoptosis, IL-1α leaves the cytosol and binds tightly to chromatin. When that cell dies, no

inflammation ensues because IL-1α bound to chromatin does not bind to its cell surface receptor. In contrast, when the cell becomes necrotic, the IL-1α leaves the nucleus and is found in the cytosol. The IL-1α precursor is released with cell contents, where it binds to IL-1R1 on adjacent live cells [146]. Thus, IL-1α works as an "alarmin" during the inflammation.

- **Lymphoid sources of IL-17.**

IL-17 was discovered by a T cell gene expression screen as a homolog of HSV13 protein, sharing feature with other cytokines [147]. In that study, both IL-17 and HSV13 could induce cytokines such as IL-6, IL-8, G-CSF and prostaglandin E2 (PGE2) production from fibroblasts [148]. Further studies showed that IL-17 is a distinct class of cytokines, with six proteins showing their homology annotated as IL-17A to IL-17F. IL-17A (also known as IL-17) and IL-17F are produced by lymphoid cells as homodimers, while IL-17A/IL-17F heterodimeric complex could be generated as well [149, 150]. The source of IL-17 members is various, for example, the main producer of IL-17E (IL-25) is the epithelial cell, but we will only focus on the IL-17A-producing cells. There are five members of IL-17 receptors (IL-17Rs), from IL-17RA to IL-17RE [151]. The IL-17A/F response requires the ubiquitously expressed IL-17RA to pair with inducible IL-17RC. Both adaptive and innate immune cells are able to produce IL-17, including CD4+ T cell, CD8+ T cell, γδT cell, ILC and NKT cell (Table 1.2 and Figure 1.4). IL-17 production could be induced by a variety of pro-inflammatory cytokines, such as IL-1β and IL-23, and microbe-derived products. All these IL-17-producing immune cells participate in host defense and chronic inflammation [152].

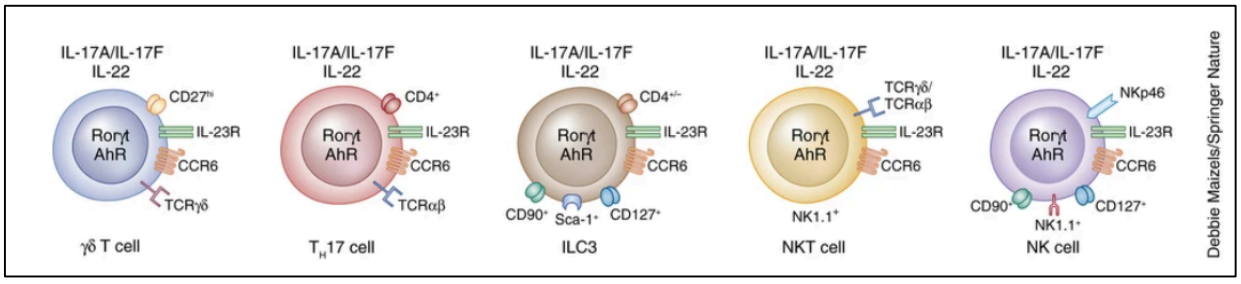

Fig.1.4 The major populations of lymphocytes that secrete IL-17 (Adapted from [153]). IL-17-producing cells share the surface receptors including CCR6 and IL-23R and the key transcriptional factor is RORγt.

Table 1.2. Type 17 immune cells (adapted from [154]).

Cell type	TCR usage	Developmental timing for RORγt and IL-23R	Requirements for IL-17 expression	Primary tissue location (at steady state)
Adaptive Th17 cells	Diverse TCR	Expression induced during thymic development from absence to adulthood	Antigen-specific TCR activation in the presence of IL-6, TGF-β, IL-1 and IL-23	Lymphoid organs
Natural Th17 cells	Diverse TCR Self-reactivity	Expression induced during thymic development from absence to adulthood	IL-1 and IL-23	Skin and mucosal tissues
γδ17 cells	Invariant TCR (Vγ4 & Vγ6)	Expression induced during fetal and neonatal thymic development	Dectins, TLRs, IL-1 and IL-23	Mucosal and peripheral tissues
	Variable TCR	Expression induced during thymic development from absence to adulthood	TCR, IL-1 and IL-23	Lymphoid organs
iNKT cells	Oligoclonal	Expression induced during thymic development from absence to adulthood	CD1 and glycolipds	Liver
ILC3s	None	Expressed throughout life	Dectins, TLRs, IL-1 and IL-23	Gut and skin

- **Functions of IL-17**

Ubiquitous and robust expression of IL-17RA and IL-17RC in epithelial cells enable the actions of IL-17 at barrier sites. Indeed, IL-17 seems to participate significantly in the barrier organs such as lung, intestine, oral cavity, vagina and skin [152, 155]. Of particular importance, IL-17 contributes to host defense by promoting the production of anti-microbial peptides (Figure 1.5), which act as natural antibiotics by directly inhibiting or even killing the invading microorganisms[156-159].

Another major feature of IL-17-producing cells is their involvement in immune cell recruitment (Figure 1.5). IL-17 has been reported *in vitro* to induce the expression of chemokines CXCL9 and CXCL10, which are ligands for CXCR3; CCL2, CCL7, CXCL1, CXCL2, CXCL5 and CXCL8, which are chemoattractants for neutrophil and monocytes ; and CCL20, which is the ligand for

CCR6 [160]. IL-17 deficient mice have reduced number of neutrophils at the site of inflammation and are highly susceptible to bacterial and fungal infections. For example, IL-17 has been reported to be essential for host defense against cutaneous *S. aureus* infection in mice [131]. Consistent with the studies in mice, IL-17 has been shown to be critical in human defense against fungal and bacterial infections. Patients with hyper-IgE syndrome, which is driven by a dominant negative mutation of Stat3 have compromised generation of IL-17 producing cells. These patients suffer from repeated mucocutaneous candidiasis and pulmonary *S. aureus* infections [161, 162]. A further study showed that this is due to the critical role of Stat3 downstream of IL-6 in the development of Th17 cells [163]. The fungal cell wall component, β-Glucan, could be recognized by the PRRs dectin1 and dectin2 in DCs and macrophages, and CARD9 acts as a downstream adaptor molecule of dectin1 and dectin2 to drive IL-17-mediated immunity. Similarly with Stat3 mutation, autosomal - recessive mutation of CARD9 also leads to susceptibility to Candida infection [164]. In addition, *Klebsiella pneumoniae* administration to IL-17RA knock-out mice exhibited a significant reduction of lung neutrophil numbers with 100% mortality [165]. Conversely, ectopic expression of IL-17 could induce a strong neutrophil response [166]. Subsequent studies revealed that IL-17 could stimulate stromal cells, mostly epithelial cells, to produce CXC chemokines, including the CXCL1 and CXCL5 in mice and IL-8 in human, which are neutrophil attracting molecules [148, 167].

Th17 cells have a known role in IgA production in Peyer's patches as well [168]. IL-17R signaling requires the adaptor protein Act1, which also binds to BAFF to negative regulate its signaling. IL-17 induces the sequestration of IL-17R and Act1, thereby reduces the interaction of Act1 and BAFF. As a result, IL-17 enhances the retention of B cells in the germinal center (Figure 1.5, IgA production). B cell retention by IL-17, can markedly boost the generation of high-affinity antibodies, thus controlling the expansion of potentially harmful microorganisms [169]. By this synergistic action, IL-17-producing immune cells enhance the long-term protection against infectious agents and after vaccination [170, 171].

There are no data to support the idea that IL-17 and IFNγ inhibit each other's expression programs. Conversely, both IFNγ and IL-17 are found at inflammation sites, that could be produced by Th17 cells or individually by IFNγ-producing and IL-17-producing cells. Their roles could be synergistic (Figure 1.5, IL-17/IFNγ balance), as shown that IL-17-induced nitric oxide could be increased in the presence of IFNγ [172]. In addition, collaborative IFNγ and IL-17 could

protect the oral mucosa from *S. aureus*, by the induction of T cell, macrophage and neutrophil recruitment [173]. However, neutralization of either IFNγ or IL-17 could lead to increased expression of the other cytokine, and result in immunopathology, even when they do not contribute to clearance during the helminth infection [174-176].

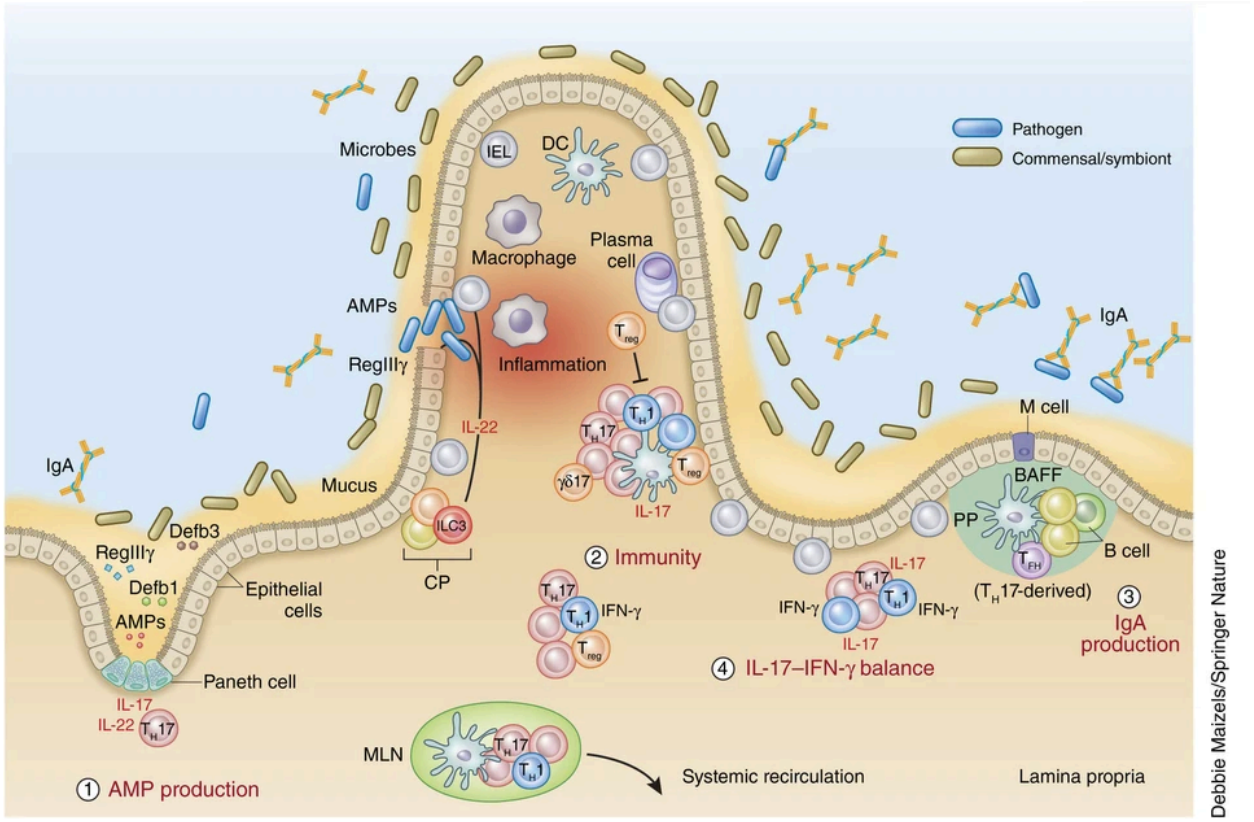

Fig.1.5 The role of IL-17 at epithelial barrier sites (small intestine). (Adapted from [153]).

(1) AMP production. IL-17 acts partially in synergy with IL-22, to induce the expression of AMPs such as LCN2, β-defensins (Defbs) and RegIIIγ.
(2) Immunity. IL-17 could recruit the phagocytic cells to prevent the infection when the barrier is breached.
(3) IgA production. IL-17 is important for the generation of IgA to offer a life-long protection against invading microorganisms.
(4) IL-17/IFNγ balance. IL-17 and IFNγ reponses are specifically and synergistically, to induce a successful immunity with minimal immunopathology at the same site of inflammation.

- **IL-17 in response to intracellular and extracellular organisms.**

Epithelial layers are the primary entry site for microorganisms. IL-17 response is primarily intended to keep the microorganisms out of the host, via strengthening the barrier integrity and producing the AMPs. If the barrier is breached it could induce the attack of these microorganisms by neutrophils and macrophages. For examples, loss of barrier integrity will result in the cytokine

expression such as IL-1β, IL-6 and IL-23, that drives the IL-17 production from innate-like lymphocytes such as γδT cells and ILCs [119, 177, 178]. IL-17, by acting with IL-22 and/or TNF, in turn, could attract more immune cells, such as neutrophils and macrophages, to the site of injury.

IL-17 plays a role in anti-viral immunity and virus-specific IL-17-producing immune cells are detectable during virus infection [179, 180]. They could increase the RSV-induced neutrophil-attracting chemokine production, increasing neutrophil recruitment and immunopathology [181]. However, during influenza infection, IL-17 is not required for viral clearance, even though it is critical for the regulation of immunopathology after infection [182]. To note, some viruses such as HSV, have integrated genes to encode an IL-17 homolog suggesting that IL-17 may somehow benefit the virus [147].

Additionally, IL-17 has an important role against bacteria, especially extracellular and gram-negative species such as *K. pneumoniae*, *Bacteroides fragilis*, *Shigella flexneri*, *C. rodentium*, *Francisella tularensis* and *Pseudomonas aeruginosa* [183-185]. Moreover, IL-17 is involved in the defense against some gram-positive bacteria, such as *P. acnes*, *M. tuberculosis* and *B. burgdorferi* [186]. In line with its capacity to enhance the response of neutrophils, IL-17 defends against extracellular bacteria by the large phagocytic capacity of neutrophils, acting as the first cells to respond to infectious agents [187]. Even though IL-17 is not thought to act primarily against intracellular bacteria, which classically require an IFNγ dominant response, it has been shown to be important but not critical in neutrophil recruitment [188].

IL-17 is essential to fungal immunity, again, owing to its ability to recruit neutrophils [140, 189, 190]. It is of particular importance in defense against fungi such as *C. albicans* and *A. fumigatus*, as well as fungal-like *Pneumocystis spp* [140, 189, 191, 192]. Interestingly, nociception (pain sensation) seems to be upstream of IL-23/IL-17 immune axis [61]. This is supported by evidence showing that IL-23 production from CD301b+ CD11b+ DCs require the intact nociception in *C. albicans* skin infection [61]. Consistently, ablation of nociceptive response in mice can lead to decreased IL-23 and IL-17 levels in IMQ-induced skin inflammation [61].

- **Dermal γδ17 cell as first line of defense.**

Dermal γδT cell is an important early source of IL-17, contributing to cutaneous host defense. It has been shown that germ-free mice have a decrease of total IL-17-producing γδT cells [193]. *S. epidermidis*, which represents the most commonly isolated bacterial species from human healthy

skin, is highly capable to modulate host immune response in a host beneficial manner. It induces IL-1 signaling to promote the pro-inflammatory effector T cell response. Furthermore, germ-free mice showed increased bacterial burden of *L. major* due to the decreased IL-17 and IFNγ production of γδT and αβT cells [193].

It has also been demonstrated that dermal γδT cells have anti-bacterial and anti-fungal activity through the recruitment of neutrophils to the skin. γδT cells could produce protective IL-17, in response to infection with BCG, *S. aureus* and *C. albicans* [194]. For example, IL-23 from dermal CD301b+ CD11b+ DCs is required for the expansion and production of IL-17 during *C. albicans* skin infection [61]. Additonally, in an atopic dermatitis model induced by a filaggrin mutation, LCs were reported to be required for *S. aureus* dysbiosis and IL-17 production from CD4+ T and γδT cells, thus mediating immune pathology [195]. In addition, sensory nociceptive neuron activation, such as the activation of cutaneous TRPV-1+ neurons, was efficient to elicit the local γδ17 immune response and augment host defense to *S. aureus* and *C. albicans* [61, 196], indicating that sensory neurons are able to regulate the γδ17 response in the skin.

Activation of γδ17 cells (Figure 1.6)**.** Strong TCR recognition is prone to trigger activation-induced cell death, as shown by *in vitro* stimulation of γδT cells with TCR or CD3 agonist stimulation [107]. However, low avidity TCR-ligand interactions can successfully induce γδ17 cells, as shown in an *in vivo* model where γδT cells with well-defined TCRs could recognize phycoerythrin and differentiate into γδ17 cells [121]. Furthermore, γδ17 cells that are activated by phycoerythrin not only acquire the $CD44^{hi}CD62L^{lo}$ phenotype, but also upregulate the expression of cytokine receptors for IL-1 and IL-23 [121]. IL-1β and IL-23 in the absence of TCR stimulation is sufficient to induce IL-17 production of CD27-CCR6+ γδT cells *in vitro*, while TGF-β or IL-6 is not [111, 118, 119]. To note, the activation of liver γδ17 cells is independent of IL-1β and IL-23, but dependent on lipid antigen that presented by CD1d on hepatocytes [197].

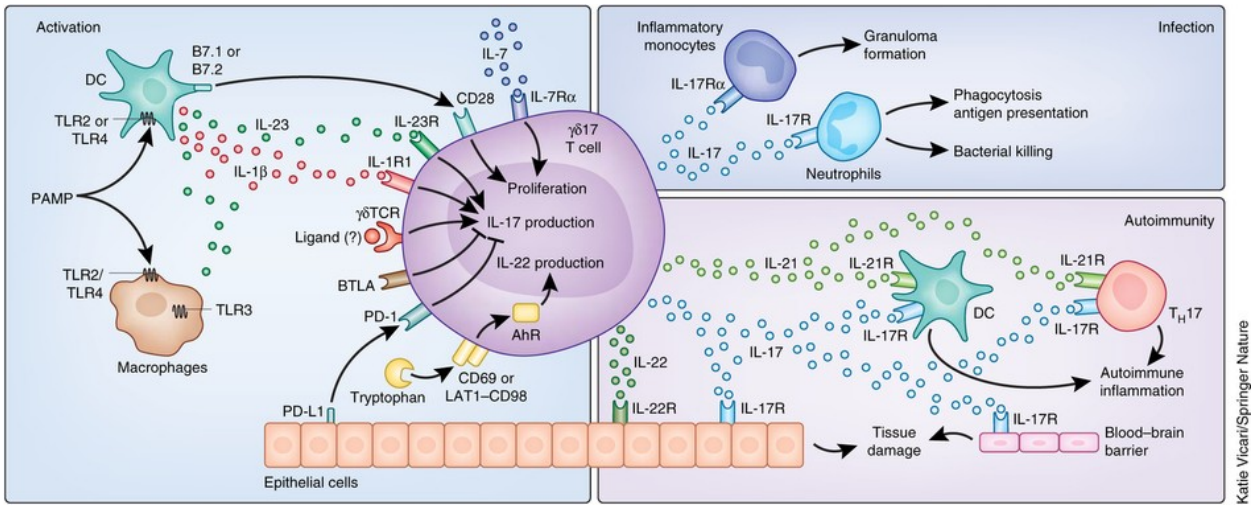

Fig.1.6 γδ17 cell activation. (Adapted from [198]) See text for details. Cellular and molecular mechanisms for γδ17 cell activation and reproduction, focusing on the cytokines and costimulatory receptors, as well as the effector functions of γδ17 cells in infection and autoimmunity.

IL-23/IL-17 immune axis in psoriasis

- **IL-23/IL-17 immune axis strongly links to the genetic background of psoriasis.**

Psoriasis is a chronic inflammatory skin disease characterized by skin patches with (1) abnormal proliferation/differentiation of keratinocytes leading to epidermal hyperplasia, (2) epidermal and dermal excessive immune cell infiltration and (3) increased dermal capillary density, with enhanced permeability in wide caliper vessels [199]. It is the most common and researched immune-mediated skin disease, that affects 2~5% of the adult population in developed countries, which has a great impact on the quality of life [200, 201].

It is now well-known that the incidence and severity of this disease are connected with environmental factors and a complex genetic background [200]. Genome wide association studies (GWAS) showed that the risk of psoriasis is associated with genes that encode factors of antigen-presentation, innate and adaptive immune cells, such as human leukocyte antigens (HLA) [202, 203]. Patients with specific HLA genotypes could be designated to certain clinical features of psoriasis. Autoantigens like keratin 17 that share similar sequences with streptococcal M-proteins, LL37 and the melanocyte autoantigen ADAMTSL5 may also predispose individuals to psoriasis [204-207].

To note, genes of S100 family members are reported as psoriasis-susceptibility loci as well [208]. They are a family of low molecular (9~13kDa) proteins characterized by two calcium-

binding EF-hand motifs, which are engaged in a wide variety of functions in different cells and tissues [209]. S100a7, 8, 9, 12 and 15 have been reported to exhibit anti-microbial effects and be increased in psoriatic lesions [210]. Particularly, S100a7, also called psoriasin, was first isolated in active psoriatic lesions, whose level has been shown to be elevated in both lesioned skin and serum of psoriasis patients [211]. High levels of S100a7 possibly induced by calcium, microbial products and IL-17/IL-22, could recruit neutrophils and T cells into the lesioned skin of psoriasis [211].

Interestingly, genes encoding transcriptional factors such as Tyi2 and Stat3 are also shown to be psoriasis-associated polymorphisms [212]. Tyk2 is associated with signal transduction of interferons and cytokines such as IL-12 and IL-23. Stat3 is of most interest in this scenario because it is not only essential for the differentiation of Th17 cells as well as IL-23R expression, but also has a proliferation promoting effect on keratinocytes [213, 214]. In addition, GWAS analysis also revealed the psoriasis-associated genes encoding cytokines including the IL-12B, IL-23A and IL-23R loci [212]. IL-12B (p40) and IL-23A (p19) are the heterodimers to form IL-23.

Despite the differences between human and rodent skin, animal models of psoriasiform inflammation have been established to identify the immunological mechanisms during the disease development [215]. Either topically applying imiquimod (IMQ), a TLR7/8 agonist, or intradermal injection of IL-23 could successfully induce a psoriasis-like skin phenotype, including epidermal hyperplasia, parakeratosis and rete edges of the skin [156, 216]. In addition, experiments using IL-17-, IL-23a- and IL-23R-deficient mice, as well as blocking antibodies, have demonstrated the requirement of these cytokines in the development of psoriasis, which highlighted the IL-23/IL-17 immune axis in psoriasis [216, 217].

- **Activation of the IL-23/IL-17 immune axis in psoriasis** (Figure 1.7)**.**

IL-23 signaling in psoriasis.

The pathogenic involvement of IL-23 in psoriasis is supported by the increased transcripts of IL-23 in psoriatic lesions. In addition, intradermal IL-23 administration could successfully induce psoriasiform characteristics in the mouse model, including erythema, induration and prominent dermal papillary blood vessels [156, 218]. Compare with IL-12-induced skin lesions, IL-23-induced pathology is more severe and dysregulated exposure of T cells to IL-23, but not IL-12, triggers downstream signaling that results in the production of IL-17 [156]. To note, TNFα and

IL-20R2 has also been shown to participate in the IL-23 induced psoriasis-like inflammation [218], indicating the interplay between IL-23/IL-17 immune axis and TNFα axis.

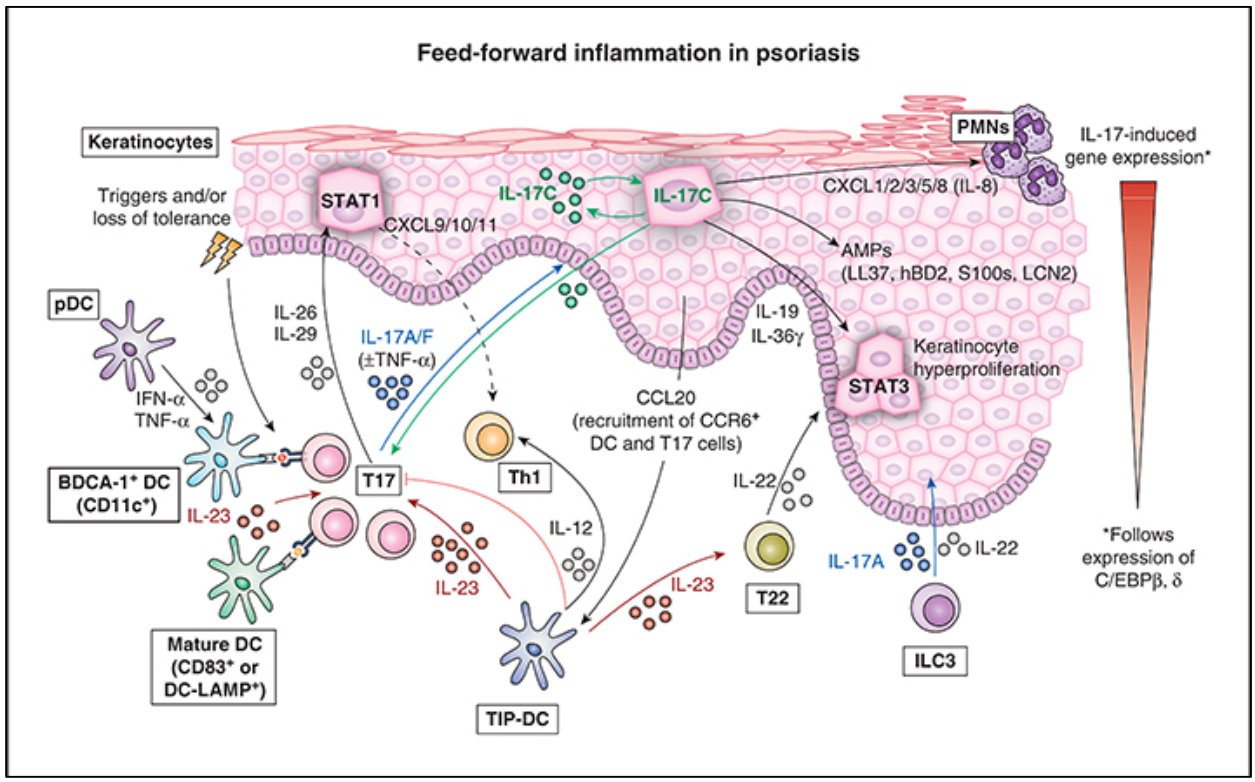

Fig.1.7. Current pathogenic model of psoriasis. (Adapted from [219]).

The initiation of psoriasis begins with environmental triggers and/or loss-of tolerance, that lead to the activation of IL-23-producing dermal DCs and plasmacytoid DCs. These pro-inflammatory DC subsets could either present the autoantigens or produce cytokines to trigger the polarization and clonal expansion of IL-17 producing immune cells. Activated IL-17-producing immune cells release cytokines, including IL-17, IL-22 and TNFα, that act on the epidermal keratinocytes to promote a feed-forward cutaneous inflammation. IL-17, alone or synergistically with TNFα, induces the expression of psoriasis-related genes of keratinocytes, resulting in the epidermal hyperplasia and AMP productions (e.g. S100s and LL37). CCL20 produced by keratinocytes, could recruit the CCR6+ cell (e.g. IL-23-producing DCs and IL-17-producing immune cells), to further promote the skin inflammation. In addition, keratinocytes could also produce CXCL1/2/3/5/8 to recruit the neutrophils and macrophages into the inflamed skin. IL-22 induced by IL-23, could act together with IL-19/IL-36γ to alter the terminal differentiation and proliferation of keratinocytes. Activated DC-derived IL-12, together with keratinocyte-derived CXCL9/10, could promote the influx of Th1 cells into the lesioned skin. ILC3s in the skin, which are capable to produce IL-17 and IL-22, could contribute to the development of psoriatic skin inflammation.

IL-1 signaling in psoriasis.

IL-1 has also been shown to be a potential effector molecule in psoriatic disease. It has been reported that IL-1 and IL-23 could induce innate IL-17 production from γδT cells, amplifying Th17 responses and autoimmunity[118]. By blocking IL-1R3, which is the co-receptor of the IL-1 family

members, it significantly attenuated the disease severity of psoriasis [220]. At steady state, IL-1 is present at large amounts in the epidermis, to support the keratinocyte proliferation by stimulating the fibroblasts in dermis that, in turn, produce the keratinocyte growth factor (KGF) [221]. KGF induces the proliferation but not differentiation of keratinocytes [222]. In addition, IL-1β expression is significantly increased in the psoriatic lesions, as the ratio of IL-1α to IL-1β decreased to 1:1 from 40:1 in the normal skin [223].

IL-17 signaling in psoriasis.

IL-17 plasma levels have been reported to be correlated with the disease severity of psoriasis [29]. Moreover, IL-17-producing CD4+ T cells, CD8+ T cells, γδT cells and ILCs have been reported in psoriatic skin [73]. Even though IL-23 could induce both IL-17 and IL-22 production, which are shown to be involved in the pathogenesis of psoriasis in mouse models [156, 216, 224], the failure of IL-22 blocking antibodies and success of IL-17 blocking antibodies in clinical trials turned the focus on the effector functions of IL-17 rather than IL-22 [224]. Patients with psoriasis who received the IL-17 blocking antibodies showed great outcomes including decreased keratinocyte hyper-proliferation, epidermal thickening, inflammatory markers, and resolution of histological features of psoriasis [225]. Consistent with the clinical trials, IL-17R deficient mice showed reduced and delayed phenotypes in IMQ-induced skin inflammation. Keratinocytes were involved in this process, since keratinocyte-specific depletion of IL-17R could also significantly reduce the skin inflammation induced by IMQ [226].

The pathological cycle of psoriasis is completed by the IL-17-mediated activation of skin resident cells, such as epidermal keratinocytes, dermal fibroblasts and endothelial cells, which subsequently respond to IL-17 to promote the cell proliferation and neo-angiogenesis. All these steps intensively contribute to disease progression. In addition, appropriate production of AMPs and chemokines, such as LL-37, S100a7, CXCL1, CXCL2, CCL20, which are results of IL-17 stimulation on keratinocytes [158], could lead to the recruitment and activation of neutrophils, mast cells and inflammatory macrophages [227], to induce tissue thickening, enhance cell death, and cause scaling of the inflamed skin [217].

γδT cells in psoriasis.

Murine models of both IMQ- and IL-23 induced psoriasiform inflammation indicate that γδT cells participate in the progression of psoriasis. Mice genetically depleted of γδT cells (TCRδ-/-) have decreased psoriasis-like inflammation in both animal models, compared to αβT cell-deficient mice [70, 126]. Additionally, reconstitution of either Vγ4+ or Vγ6+ γδT cells into the TCRδ-/- mice could successfully restore disease susceptibility [228]. However, selective depletion of Vγ4+ or Vγ6+ γδT cells showed that each subset of γδT cells are sufficient for IMQ-induced inflammation [105, 229], indicating that they could compensate when the other subset is absent. Compared with the Vγ6+ subset, Vγ4+ γδT cells could acquire memory-like characteristics, as shown in two studies that Vγ4+ γδT cells respond faster in the same area or even distant sites, upon the secondary IMQ treatment [130, 230]. In addition, inflamed skins in both psoriasis models have increased expression of CCL20, which induces the recruitment of dermal CCR6+ γδ17 cells into the epidermis. Thus, blockade of CCL20 significantly decreased the recruitment of γδ17 cells during IL-23-induced skin inflammation [119, 129].

IL-1 signaling has been shown to be important for γδT cell function in psoriasis as well. IL-1β promotes γδT cell proliferation in vitro, and the production of IL-17 from Vγ4+ subset is more dependent on the IL-1 signaling than Vγ6+ γδT cells. Additionally, mice genetically deficient of IL-R1 have reduced skin inflammation in an IMQ-treated model [228]. Interestingly, Stat3 deficiency in dermal γδT cells, which blocks IL-23 signaling, could not rescue the IMQ-induced skin inflammation. However, the absence of mTORC2, which leads to reduced dermal γδT cells, could successfully ameliorate psoriasis-like inflammation [132]. IL-38, another IL-1 family member produced by keratinocytes, has the ability to limit γδT cell activation at steady state, by inhibiting the IL1RAPL1 on the γδT cell surface [231]. Consistent with this result, the expression of IL-38 has been reported to be downregulated in human psoriatic lesions, as well as IMQ-treated mouse skin [231, 232].

In humans, IL-17-producing γδT cells are reported to accumulate in inflamed skin as well. A recent study showed that Vγ9Vδ2+ subset, which is CCR6+ and able to produce IFNγ, IL-17 and TNFα, is observed in the psoriatic lesions [233]. Additionally, the elevated frequency of Vγ9Vδ2+ γδT cells in the psoriatic skin is positively correlated with the disease severity, and negatively correlated with the Vγ9Vδ2+ γδT cell proportion in the circulation [233]. Thus, even though the majority of IL-17-producing cells in human psoriatic skin are αβT cells [234], γδT cells could also contribute to disease progression.

1.4. The cutaneous microbiota

The skin is the most exposed interface between the environment and the body which acts as the first line of physical and immunological defense. It also harbors a complex and dynamic ecosystem, inhabiting by many microorganisms, including viruses, bacteria and fungi. Thus, the skin has a difficult task to protect the host from microbial invasion, as well as to maintain a peaceful co-existence with its resident microbiota.

Studies have shown that the rich diversity of commensal microbes have great importance to cutaneous health [235]. Thus, a proper balance of the microbial composition is critical for host homeostasis. Dysbiosis of the skin microbiome has been associated with several skin disorders, such as atopic dermatitis, psoriasis and rosacea [236-240].

Highly diverse microbial communities reside on the skin surface, as well as in associated appendages such as hair follicles and sebaceous glands (Figure 1.8) [241].

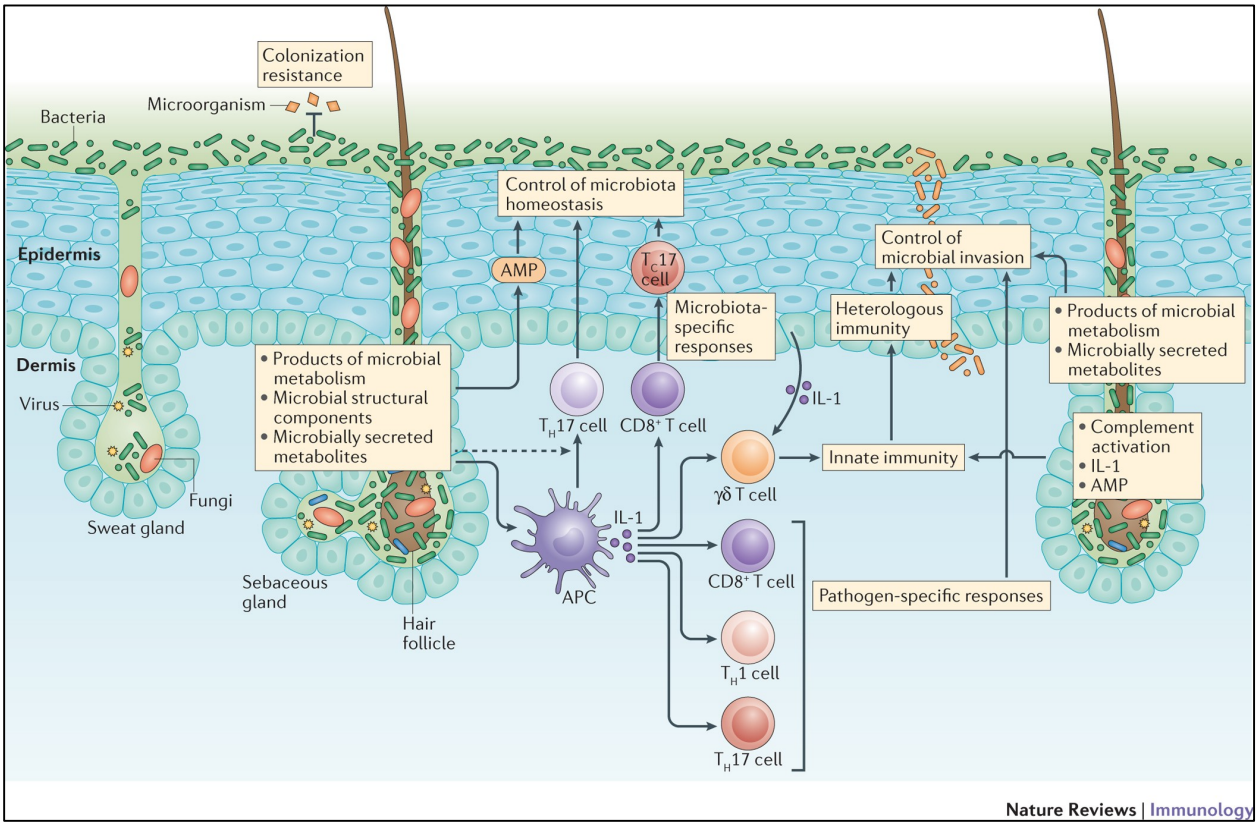

Fig.1.8 Structure and cellular components of the skin and control of immunity by skin-resident microorganisms. (Adapted from [235]).

Microorganisms such as viruses, fungi and bacteria cover the skin surface and reside in skin appendages, including hair follicle, sebaceous glands and sweat glands.
(1) Colonization resistance. Microorganisms can themselves limit the expansion of pathogenic microorganisms.
(2) Control of microbiota homeostasis & invasion. Skin-resident microorganisms can promote the production of antimicrobial peptides (AMPs) by keratinocytes and the production of central mediators of immunity, such as complement and IL-1 by antigen-presenting cells (APCs) and/or other innate immune cells. These molecules can directly or indirectly enhance skin immunity by promoting cytokine production, increasing antimicrobial functions, promoting the recruitment of effector cells and favoring the priming of pathogen-specific T cell responses. These factors can also promote adaptive immunity to the microbiota that in turn can promote heterologous protection against invasive microorganisms. Enhanced production of IL-17 that is induced by the microbiota can promote keratinocyte effector function against invading microorganisms.

The skin microbiota provides many benefits to host physiology including wound repair and protection against pathogens, induction of host cell antimicrobials, conversion of host products into antimicrobials or by altering the nature of the adaptive immune response. For example, after mechanical or pathological skin injury, TLR3 stimulation could activate keratinocytes to induce inflammatory responses to necrotic cells [242]. However, overt inflammation could delay wound repair and damage the tissue. Lipoteichoic acid (LTA) produced by *S. epidermidis*, a commensal bacterial species, could balance the TLR3-induced inflammatory response by signal through TLR2. Specifically, TLR2-induced TRAF1 could impair the TLR3-dependent production of IL-6 and TNFα to limit the inflammation and immune cell recruitment (Figure 1.9) [243]. In addition, activation of TLR2 by *S. epidermidis* could also increase AMP production to defend against pathogen infection [244]. In mouse models of skin colonized with *S. epidermidis* or *C. accolens*, it has been shown that commensal bacteria could lead to the accumulation of dermal αβT and γδT cells, in an IL-23 dependent way [134, 245].

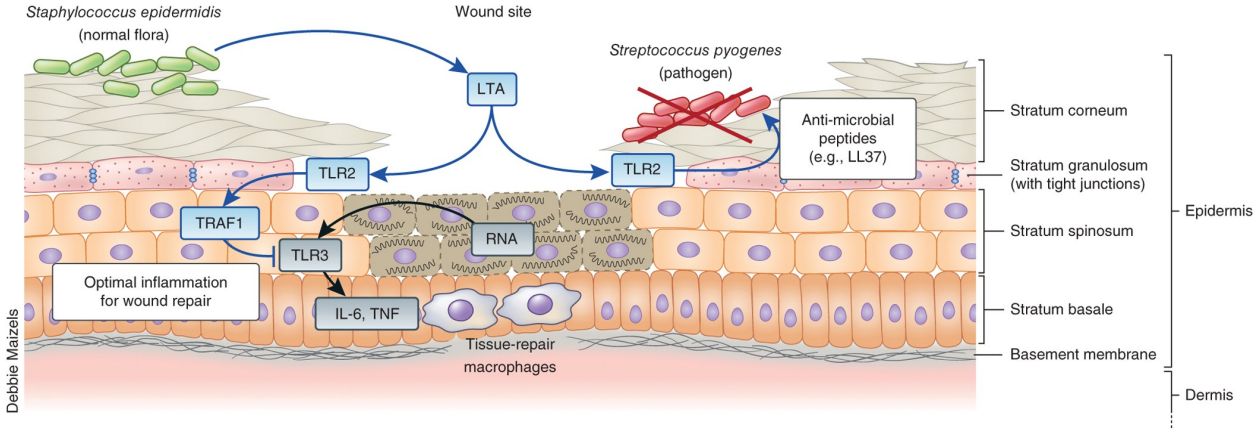

Fig.1.9. Resident microbiota in the skin contribute to immunity and wound repair. (Source: Adapted from [9]). The mechanisms of skin microflora benefit host physiology, including wound healing and protection against pathogens. See text for details.

Commensal microbiomes are considered to maintain the skin homeostasis, and the composition of the commensal communities can influence the immune status of the skin. Human and animal research has provided hints linking commensal microbiota and the pathogenesis of psoriasis [246, 247]. Even though the link is not yet proven, there is evidence showing that the microbiome inhabiting the psoriatic lesion is much more diverse than on the healthy skin either from psoriasis patients or unaffected individuals [248]. Consistent with these clinical results, mice treated with oral antibiotics exhibited decreased IMQ-induced inflammation and a reduction of IL-17 production, indicating that the microbiome regulates both cutaneous health and disease [246]. However, the factors regulating microbiota-dependent activation of the skin IL-23/IL-17 immune axis are only beginning to be revealed.

1.5. Biological function of CD109

The structure of CD109.

CD109 is a glycosyl phosphatidylinositol (GPI)-anchored protein of approximately 170kDa and a member of the α2-macroglobulin/ C3, C4, C5 family of thioester-containing proteins, whose gene is located in chromosome 6q13 in human and chromosome 9qE1 in mouse [249, 250]. It contains a N-terminal leader peptide, a putative bait region, a thioester binding site, a thioester reactivity defining hexapeptide and a C-terminal consensus GPI-anchor signal sequence with the cleavage site [249]. The N-terminal leader peptide anchors the protein to the inner membrane within the periplasm. As an α2-macroglobulin thioester-containing protein, CD109 has the potential to be activated after proteolytic cleavage of a largely disordered bait region, leading to a conformational change that exposes the thioester bond, that covalently traps the protease in a cage-like structure [251]. This "snap-trap" inhibition potentially prevents the damage of large cell envelop components, but not host digestion [251, 252]. In addition, the C-terminal consensus GPI-anchor signal sequence is also known as a receptor-binding domain [251].

Maturation and secretion of CD109.

The glycosylation and secretion of CD109 varies in different cell types and tissues. Generally, in the endoplasmic reticulum (ER), the 155kDa CD109 core protein is linked to the GPI and glycosylated as a 205kDa glycoprotein [253]. It is then transported to the Golgi apparatus. In the Golgi apparatus, glycosylated CD109 is further glycosylated and cleaved by furinase into the 180kDa and 25kDa fragments [253]. The 180/25kDa complex of CD109 is then transferred to the cell surface and enriched on lipid rafts, while the 180kDa isoform is released out of the cell [253].

Recently, CD109 has also been reported to be detected in exosomes, a type of extracellular vesicle that contains cell constituents [254]. Both immunoblot and immune-electron microscopy of isolated exosomes confirmed the presence of CD109 180kDa and 25kDa fragments, suggesting that the 180/25kDa complex could also be secreted into culture medium [254]. In addition, CD109 has been detected to be enriched in lamellar bodies [17], which are lysosome-related and trans-Golgi network-derived organelles of keratinocytes [255]. However, the function of CD109 in lamellar bodies needs to be further analyzed.

CD109 is a membrane protein that works in multifunctional ways.

CD109 was first identified as a cell surface antigen by a monoclonal antibody against the human lymphoid/myeloid cell line KG1a [256]. Structure analysis of CD109 revealed that it carries $Gov^{a/b}$ alloantigens, possibly contributing to the platelet transfusion refractoriness and neonatal alloimmune thrombocytopenia [257, 258]. It has a broad expression profile, such as platelets, activated T cells, endothelial cells, $CD34^{+}$ megakaryocyte progenitors, mesenchymal stem cell subsets and malignant tumor cells [190, 256, 259-261]. At steady state, CD109 expression could be detected in mammary, salivary and lachrymal glands myoepithelial cells, prostate basal cells, skin epidermis and bone osteoblasts and osteoclasts [262-264]. Previous studies have also shown that CD109 can be expressed in various malignant tumor cell lines and tissues, and its expression levels are correlated with patient prognosis [261, 265-268].

- **The TGF-β signaling pathway and CD109.**

The TGF-β signaling pathway controls a diverse set of cellular responses including cell proliferation, differentiation and apoptosis. It figures prominently in animal development from embryogenesis to mature tissues [269]. TGF-β1, 2 and 3, which are three important TGF-β ligands, can bind to transmembrane type I and type II serine/threonine kinase receptors (TβR1 and TβR2) [269, 270]. Thus, TβR1 is activated to multiply the signals by phosphorylating its intracellular R-Smad proteins, namely Smad2 and Smad3 [271]. Activated R-Smads, in turn, form a heteromeric complex with Smad4 that accumulates in the nucleus to control the gene expression in a cell-type-specific and ligand-dose-dependent way [269, 270].

The endocytosis of TβRs is an important mechanism to regulate TGFβ signal transduction. The internalization of TβRs by clathrin- and caveolae-dependent ways lead to different outcomes. The activation of R-Smads are dependent on the internalization of TβRs through clathrin-coated pits, which links with receptor recycling. Conversely, when TβRs locate to the caveolae, they will be degraded following ubiquitination by the E3-ubiquitin ligase Smurf1 and 2 [272, 273]. Moreover, the inhibitory Smad (I-Smad), Smad7, can move from the nucleus to the plasma membrane upon the TGF-β stimulation, forming a stable complex with Smurf1 and Smurf2, where I-Smad directly binds to activated TβR1 and inhibits the phosphorylation of R-Smads [274]. Activated TβR1 at the same time undergoes ubiquitination and degradation, resulting in the termination of TGF-β signaling [270].

CD109 has been first reported as a TGF-β co-receptor that modulates the TβR activity *in vitro* [275]. In addition, the 180/25kDa cell surface and 180kDa soluble CD109 have been shown to inhibit TGF-β signaling [253, 275-277]. On the cell surface, GPI-anchored CD109 can interact with TβR1 and TβR2 to form a TβR/CD109 complex and promote TβR degradation by Smad7/Smurf2 mediated ubiquitination [275]. In addition, the TβR/CD109 complex associates with caveolin-1 and facilitates the endocytosis of TβR, indicating that the internalization of TβR /CD109 is dependent on caveolae [277]. Furthermore, CD109 can be released from the cell surface and directly bind TGF-β1 to antagonize cytokine receptor binding and decrease the phosphorylation of Smad2/3 [276]. When the furinase cleavage site of CD109 was mutated, 205kDa CD109 could be expressed on the cell surface but TGF-β signaling was not affected, indicating that the furinase cleavage is necessary for the biological activity of CD109 [253]. Another study showed that in tumor cells, CD109 can serve as a partner of 78kDa glucose-regulated protein (GRP78), to promote the TβR/CD109/GRP78 complex routing to caveolae for degradation [278].

Another study reported that CD109 differently regulated the ALK5(TβR1)-Smad2/3 and ALK1(TβR1)-Smad1/5 signaling pathways [279]. Murine keratinocytes that overexpress CD109 have decreased ALK5-Smad2/3 but enhanced ALK1-Smad1/5 activation [279]. In summary, CD109 regulates TGF-β signaling by internalization and degradation of TβR.

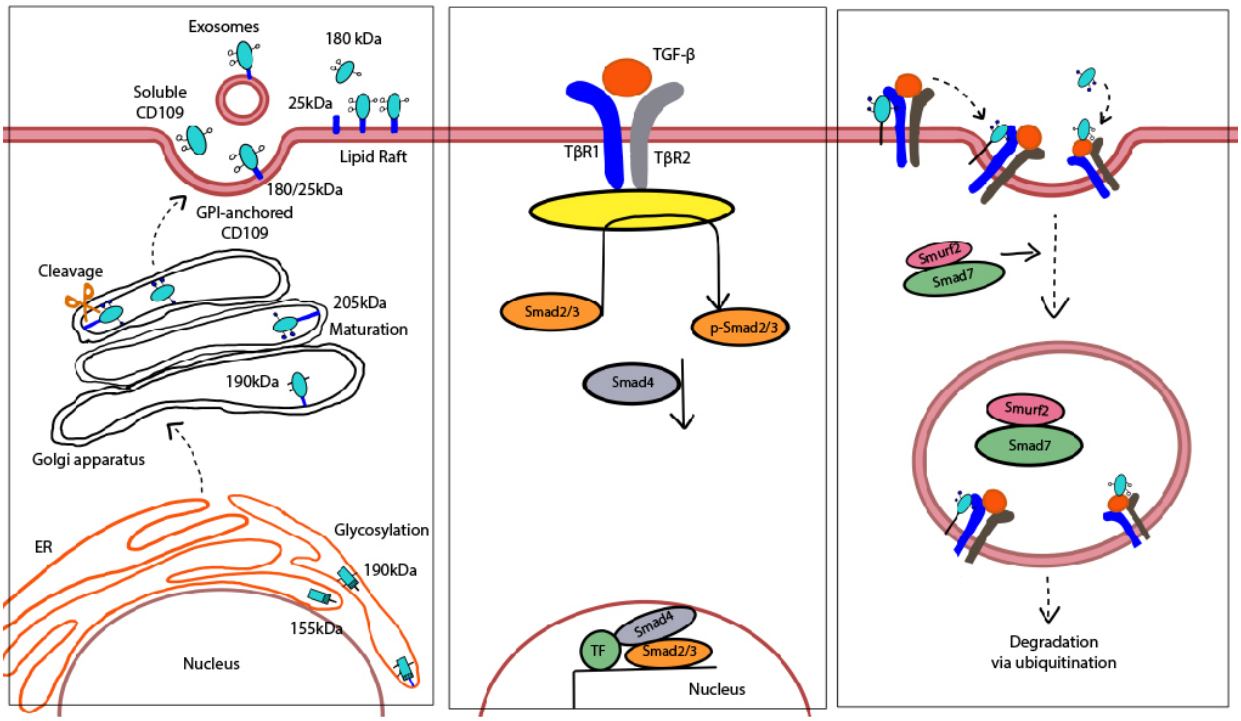

Fig 1.10. Cell biology of CD109. (Source: Adapted from [280, 281]).

(A) Illustration of CD109 maturation and secretion. The 150kDa CD109 core protein is first linked to the GPI and undergoes glycosylation in ER to become as a GPI-anchored, glycosylated 190kDa CD109. Then CD109 is transported to Golgi apparatus, being further glycosylated and cleaved into 180kDa and 25kDa fragments by furinase. The 180/25kDa complex can either stay on the cell surface or be released into the cell culture medium as exosomes, and the 180kDa fragments are secreted out of the cells.

(B) TβR activation. TGF-β can bind to TβR2 to recruit and phosphorylate TβR1. Activated TβR1 directly phosphorylates R-Smads, then the activated R-Smads form a heteromeric complex with Smad4 to relocate into the nucleus. The Smad2/3/4 complex combines with the transcriptional factors to regulate the gene transcription.

(C) CD109 helps the degradation of TβRs. CD109 binds to TGF-β or TβR1, that promotes TβRs localization to caveolae, ultimately promotes TβRs endocytosis and facilitates TβRs degradation. This process requires Smad7 expression and Smurf2 ubiquitin ligase activity.

- **Stat3 signaling and CD109**

Stat3 signaling is another pathway that plays vital roles in cell fate such proliferation, differentiation and survival [282]. CD109 could take part in regulating the Stat3 signaling pathways. But results differ between studies. For example, it was first discovered that the loss of CD109 leads to enhanced phosphorylation of Stat3 in the epidermis while TGF-β signaling was not affected [263]. However, a recent study on lung adenocarcinoma showed that CD109 could interact with gp130 to increase the Stat3 phosphorylation in a Janus kinase (JAK) dependent way [261], suggesting that CD109 regulates Stat3 signaling in a more complex manner. To date, the relation between CD109 and Stat3 signaling is not clear.

The role of CD109 at steady state and during disease progression.

- **Lung, brain and bone.**

Previous studies have demonstrated that CD109 is preferentially expressed in the basal cells of bronchial, bronchiolar epithelia and myoepithelial cells of bronchial secretary glands from human biopsy samples [283]. Moreover, compared with other types of lung carcinoma, squamous cell carcinoma has higher CD109 expression [283]. However, in a study of lung adenocarcinoma, CD109 has been reported to be required for the metastasis of tumor cells without affecting the proliferation ability [261]. Even though TGF-β signaling is regulated by CD109 in the tumor cell lines, Jak-Stat3 signaling is critical for the pro-metastatic function of CD109 [261]. It has also been reported that brain tumor stem cells (BTSCs) isolated from mouse glioma have high levels of CD109 expression. Further analysis showed that $CD109^+$ BTSCs have higher proliferation and are resistant to chemotherapy than $CD109^-$ BTSCs [266]. Recently, CD109 has been reported to be associated with the tumor-initiating population located in the tumor core of gliomas, serving as a downstream mediator of HDAC1-derived intercellular signals from core to edge glioblastoma cells [268, 284].

CD109 expression is also upregulated during osteoclastogenesis and knockdown of CD109 induces less fusion capacity of RAW264.7 cells in an *in vitro* RANKL-induced osteoclastogenesis model [285]. In addition, CD109 expression has been verified in both osteoblasts and osteoclasts *in vivo*, which are important for bone remolding [264]. CD109-/- mice have a high-turnover osteoporosis phenotype, which was described as reduced bone/tissue volume, decreased trabecular number and increased trabecular separation [264]. Related to bone disease, CD109 is abundantly expressed in synovial tissues from Rheumatoid arthritis (RA) patients and in collagen-induced arthritis (CIA), a mouse model of RA [286]. Further analysis showed that its expression could be upregulated by inflammatory stimuli such as IL-1β and TNF-α in fibroblast-like synoviocytes (FLS). Treatment using anti-CD109 blocking antibodies and CD109 depletion significantly ameliorated the arthritis phenotype during CIA [286]. In this study, CD109 interacted with and stabilized alpha-enolase (ENO1) to regulate the inflammatory response of RA in a TGF-β independent manner [286].

- **Skin.**

The function of CD109 in the skin has been studied both *in vitro* and *in vivo* by using human keratinocytes and different mouse models. CD109 protein level is significantly decreased in the lesioned epidermis of psoriasis patients as compared with the adjacent uninvolved skin, while the mRNA level remains unchanged, indicating that its expression may be regulated at protein level during psoriasis development [287]. Further analysis revealed that silencing of CD109 expression in cultured human keratinocytes could significantly inhibit STAT3 phosphorylation [287]. In the murine skin, CD109-deficient (CD109-/-) mice have delayed hair growth compared with the wild-type (WT) mice [263]. Hematoxylin and eosin (H&E) staining showed that CD109-/- skin has persistent epidermis and sebaceous gland hyperplasia. Basal and super-basal layers of epidermis, where the CD109 is abundantly expressed in the WT mice, are thickened in CD109-/- mice [263]. Accumulation of sebum in the ectatic hair follicles is also observed in the CD109-/- mice shown by Oil Red O staining [263]. Even though CD109 has been reported as a TGF-β receptor and negatively regulate the TGF-β/Smad *in vitro*, further analysis showed that activation of Smad2 is undetectable. Moreover, Stat3 phosphorylation level is enhanced in the CD109-/- epidermis [263], which is opposite to the *in vitro* results from human keratinocytes.

CD109 can also regulate wound healing, functioning as a TGF-β antagonist through the Smad2/3 pathway [288, 289]. In murine models of incisional and hypoxic wounds, overexpression of CD109, using K14.CD109-Tg mice, was associated with decreased dermal thickness and extracellular matrix deposition compared to control mice [288, 289]. During the healing of excisional wounds, CD109 overexpression in Keratin14^{+} cells, which are basal keratinocytes, could markedly reduce the granulation tissue area, accompanied by decreased macrophage and neutrophil recruitment to the epidermis, whereas wound closure remains unaffected as compared with WT littermates [288]. In K14.CD109-Tg mice, the mRNA levels of IL-1α, monocyte chemoattractant protein-1 (MCP-1) and extracellular matrix components were all significantly decreased [288].

In a bleomycin-induced skin fibrosis mouse model, K14.CD109-Tg mice showed resistance to fibrosis as compared with their WT littermates [290]. In this model, overexpression of CD109 in the epidermis showed decreased phosphorylation of Smad2/3, as well as reduced dermal thickness and collagen deposition than that of WT skin [290].

The role of CD109 has been also studied in skin squamous cell carcinoma (SCC) [291, 292]. It was reported that CD109 expression level is inversely correlated with the tumor grade and the

activation of TGF-β pathway. *In vitro* knock-out of CD109 by CRISPR/Cas9 system in cultured SCC cells inhibited epithelial traits and promoted the mesenchymal phenotype [292]. In another study, compared with that of WT skin, primary keratinocytes isolated from the CD109-/- skin had increased resistance to 7, 12-dimethylbenz (α) anthracene (DMBA)- or cisplatin (CDDP)- induced cytotoxicity, which is TGF-β/p21/Nrf2 pathway dependent [291]. Moreover, CD109-deficiency *in vivo* induces the increase of TNF-α, TGF-β1 and GM-CSF transcripts, as well as macrophage infiltration into the skin at steady state [291]. Loss of CD109 suppresses the sensitivity of skin to DMBA, as $p53^{+}$ and cleaved caspase-3^{+} cell numbers decreased in the epidermis, and DMBA/TPA-induced skin tumorigenesis is suppressed in the CD109-/- mice [291]. Thus, *in vivo* results showed that CD109 negatively regulating cutaneous Stat3 signaling and TGF-β signaling.

1.6. Rationale and specific aims

As described in Chapter 1, dermal IL-17-producing γδT cells play an important role in the maintenance of skin homeostasis, by participating in both host defense and disease pathogenesis. These γδ17 cells, including Vγ4+ and Vγ6+ subsets, can be activated by cytokines such as IL-1 and IL-23. Activated keratinocytes triggered by stress such as injury and microbial products from either commensals or pathogens, could subsequently release IL-1α or activate the dermal antigen-presenting cells, to release the cytokine IL-1β and IL-23, to induce the activation of IL-17-producing cells including γδT cells. While chronic exposure to IL-1 and IL-23 will lead to skin pathology such as psoriasis. CD109 has been reported to be abundantly expressed in the normal skin, and keratinocytes are enriched of CD109. Even though it has been reported as a co-receptor of TGF-β to negatively regulate the TGF-β signaling, functions of CD109 in keratinocytes remains further discovered. Thus, CD109 may have other roles in the skin. Regardless, CD109 has been reported to be down-regulated in human psoriatic lesions and mice deficient in CD109 expression exhibited epidermal hyperplasia and cutaneous leukocyte infiltration, both hallmarks of psoriatic disease. *Chapter 2 of this thesis aimed to understand the role of CD109 in the maintenance of IL-23/IL-17 immune axis, especially the γδ17 cells*. *In order to better understand the contributions of keratinocyte-derived CD109 in regulating the skin homeostasis, Chapter 3 aimed to identify the specific functions of CD109 in keratinocyte biology.*

Preface to Chapter 2

"Blue agony of the morning-glory,
The fuchsia's psoriasis;
These flowers unscab the heart. Sick skin, sick skin;
Pus flares in the daisy chain."

Charles Wright, *Victory Garden.*

Psoriasis is a common chronic, recurrent autoimmune skin-tropic disease that affects as much as 2~3% world population [199]. Although psoriasis is not life-threatening, it can have negative impact on the physical, emotional and psychosocial wellbeing of affected patients [293]. Over the past twenty years, clinical data and pre-clinical models have shown that IL-23/IL-17 immune axis plays a central role in psoriasis. Despite the success of neutralizing antibodies targeting IL-23 and IL-17, it is still debatable what is the game changer for the initiation of psoriasis, the keratinocyte, the immune cells, or just one molecule? CD109 is a GPI-anchored protein that is enriched in keratinocytes, which is down-regulated in the psoriatic lesions compared with non-lesioned skin. Our goal in this chapter was to identify the events that occur in the skin when deficient in CD109, thus to further our understanding on the role of CD109 in regulating tissue homeostasis of the skin.

Chapter 2. CD109 restrains activation of cutaneous IL-17 producing γδ T cells by commensal microbiota

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2.1 Summary

Interleukin-17 producing γδ T (γδ17) cells play a central role in protective and pathogenic immune responses. However, tissue-specific mechanisms that control activation of these innate lymphocytes are not known. Here we demonstrate that CD109, a GPI-anchored protein highly expressed by keratinocytes, is an important regulator of skin homeostasis and γδ17 cell activation. Genetic deletion of CD109 results in spontaneous epidermal hyperplasia, aberrant accumulation of dermal-derived γδ17 cells and enhanced susceptibility to psoriasiform inflammation. In this context, γδ17 activation requires IL-23 signals and can be reversed by transient depletion of the skin microbiota. Mechanistically, CD109 restrains γδ17 cell activation in a cell-extrinsic manner by fortifying skin barrier integrity. Collectively, our data provide new insight into the regulation of the skin IL-23/IL-17 immune axis and how homeostasis is maintained at this important barrier site.

Keywords: skin, CD109, IL-17, gamma delta T cells, barrier tissue, immunity, microbiota, psoriasis

2.2 Introduction

Mammalian skin is a multi-layered barrier organ indispensable for host protection from environmental insults and invasion by pathogenic microorganisms. The outermost layer of the skin, the epidermis, is predominantly composed of a stratified epithelial cell network maintained by a single layer of self-renewing basal progenitor cells [294]. The underlying dermal tissue harbors a diverse leukocyte compartment that, upon epidermal barrier disruption or infection, are positioned to rapidly respond to injury or infection [295]. Recently, a subset of innate-like γδ T lymphocytes have been described that seed the dermis within the first days of life and exhibit self-renewal capacity [124-126]. Dermal γδ T cells express a limited, but polyclonal TCR repertoire enriched for Vγ4 usage and are imprinted in the thymus to express the lineage-specific transcription factor Rorγt endowing them with the ability to produce interleukin (IL)-17 (referred hereafter as γδ17 cells) [110]. Once resident in the skin, γδ17 cells can be activated upon exposure to inflammatory cytokines, such as IL-23, in a TCR-independent manner [118]. Their unique localization and effector functions make γδ17 cells important for early resistance against cutaneous pathogens such as *Staphylococcus aureus* and *Candida albicans* [61, 131]. In these settings, IL-17 stimulates keratinocytes to produce anti-microbial factors and chemokines that recruit microbicidal neutrophils that together limit microbial dissemination [295]. Although controversial, γδ17 cells have also been implicated in the chronic inflammatory disease psoriasis. Indeed, imiquimod-driven psoriasiform inflammation - characterized by epidermal hyperplasia and parakeratosis - in mice requires Vγ4+ γδ17 cells [105] and two independent studies of human psoriatic disease identified increased numbers of γδ17 cells in lesioned skin compared to non-lesioned patient skin [126, 233]. Thus, γδ17 cells must be tightly controlled to protect against pathogen invasion, but also prevent dysregulated responses that lead to chronic inflammation and disease.

The anatomical niche of cutaneous γδ17 cells positions them to be influenced by commensal microbial communities (i.e. the microbiota) on or within the skin barrier. For example, germ-free mice contain decreased numbers of dermal γδ17 cells compared to specific pathogen-free (SPF) mice [193]. Conversely, colonization of mice with commensal bacteria including *Corynebacterium* and *Staphylococcus* species results in the non-inflammatory accumulation of γδ17 cells [245]. In this context as well as in overt inflammatory conditions, IL-23 signaling is necessary for γδ17 cell cytokine production [61]. The IL-23/IL-17 immune axis is known to be

triggered when injury or infection-induced epithelial damage and barrier disruption exposes dermal mononuclear phagocytes to microbial-derived TLR ligands that initiate activation of innate tissue-resident cell types [154]. However, keratinocytes actively maintain a physical and chemical barrier that limits exposure of leukocytes to commensal or pathogenic microbes. Currently, the mechanisms that control activation of the cutaneous IL-23/IL-17 immune axis in general, and dermal γδ17 cells, specifically, to the commensal microbiota and other inflammatory stimuli are not clearly defined. Moreover, the diverse roles of this axis in the skin versus other barrier tissues such as the intestine – where γδ17 cells promote barrier integrity [296] – provide the impetus for identifying tissue-specific mechanisms of immune regulation that may be harnessed to design therapies targeted to enhance barrier integrity, but also alleviate the pathology associated with psoriasis and other immune-mediated skin disorders.

CD109 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein highly expressed in healthy skin and malignant tumor cells such as lung adenocarcinoma [261, 263]. Mice lacking CD109 exhibit a delay in hair development, persistent epidermal hyperplasia and high bone turnover [263, 264]. However, the specific impact of CD109 of the cutaneous immune system has not been examined. Here we report CD109 as a selective regulator of the skin IL-23/IL-17 immune axis and γδ17 cells. Deficiency of CD109 in mice resulted in spontaneous inflammation of the skin including epidermal hyper-proliferation and thickening, neutrophil accumulation, aberrant IL-23-dependent activation of dermal γδ17 cells. Consistent with a dysregulated IL-23/IL-17 pathway, the absence of CD109 led to enhanced psoriasiform inflammation following imiquimod application. We also demonstrate that CD109 acts in a skin-specific and cell-extrinsic manner to limit IL-23 dependent γδ17 activation by the commensal microbiota. Collectively, our data reveal CD109 as a negative regulator of host-microbiota crosstalk that restricts activation of the cutaneous IL-23/IL-17 immune axis.

2.3 Results

2.3.1 Deletion of CD109 results in spontaneous skin inflammation

Genetic deletion of CD109 in mice results in delayed hair growth that reaches normal density by 6-8 weeks of age [263]. In addition, we observed that at 10-12 weeks of age, CD109-/- mice began to exhibit patchy hair loss that increased in area with age (Figure 2.1A). Consistently, we found CD109 to be highly expressed in the skin, but undetectable in lymph node cells and circulating leukocytes of wild-type (WT) animals (Figure 2.1B). Tissues from CD109-/- mice were used as negative controls (Figure 2.1B). In addition, markers of inflammation (e.g. *S100a8* and *S100a9*) [297] increased with age in the skin of CD109-/- mice coupled with significant ear thickening and epidermal hyperplasia by 10 weeks of age (Figure 2.1C-F). These results prompted us to examine the skin tissue of CD109-/- mice in greater detail. Epidermal hyperplasia in 8-12 week old CD109-/- mice was associated with an increase in the presence of epidermal and dermal cell proliferation as determined by Ki67 expression using immunofluorescence microscopy and *ex vivo* flow cytometry analysis in hematopoietic (CD45+) and non-hematopoietic (CD45-) compartments (Figure 2.1G and H). Increased cell proliferation was associated with inflammation, as ears from CD109-/- mice contained Ly6G+ neutrophils in both epidermal and dermal layers that were essentially absent in WT controls (Figure 2.1G, I and J). These results indicate that loss of CD109 results in spontaneous and progressive skin inflammation.

2.3.2 Dysregulation of the cutaneous IL-23/IL-17 immune axis in the absence of CD109

To determine the immune pathways associated with the observed spontaneous skin inflammation in CD109-/- mice, we extracted total RNA from epidermal and dermal layers of ears from WT and CD109-/- mice and performed targeted gene expression analysis using Nanostring® technology. Transcript analysis indicated a significant increase in cytokine gene expression in skin from CD109-/- compared to WT controls associated with the IL-23/IL-17 immune axis including *Il17a*, *Il17f*, *Il19*, *Il20*, *Il22* and *Il23a* (Figure 2.2A). RT-qPCR analysis of selected transcripts confirmed a significant increase in *Il17a* expression from CD109-/- skin, but no significant differences in cytokine mRNA characteristic of type 1 or type 2 immunity such as *Ifng* or *Il4*, *Il5, respectively,* with the exception of *Il13* that was decreased in the epidermis of CD109-/- mice compared to controls (Figure 2.2B and C). Consistent with these data, spontaneous secretion of IL-17A but not IFN or IL-13 was detected in cultures of total skin cells from CD109-/-, but not

WT, mice (Figure 2.2D). Furthermore, deletion of IL-17 producing cells in CD109-/- mice by crossing these animals to Rorγt$^{GFP/GFP}$ mice in which GFP is knocked into the *Rorc* locus, a transcription factor required for the differentiation of IL-17 producing cells [298] prevented epidermal hyperplasia in comparison to WT or CD109-/- Rorγt$^{GFP/+}$ controls (Figure 2.2E and F).

Previous studies indicate that CD109 negatively regulates canonical TGFβ signaling [287]. However, we were unable to detect a difference in SMAD2/3 activation between WT or CD109-/- total skin cells *ex vivo* or following *in vitro* stimulation with recombinant TGFβ1 (Figure 2.2G). By contrast and consistent with a previous report [263], we observed spontaneous activation of STAT3 in adult and neonatal CD109-/- but not WT skin cells, a transcription factor downstream of numerous cytokines associated with the IL-23/IL-17 immune axis and keratinocyte dysregulation (Figure 2.2G and H) [214]. Thus, the absence of CD109 results in spontaneous and selective activation of the cutaneous IL-23/IL-17 immune axis.

As IL-17 can be produced by diverse immune cell types, we determined the source of this cytokine by flow cytometric analysis. As our gene expression analysis indicated increased expression of *Il17a* and *Il17f* in both epidermal and dermal skin layers of CD109-/- mice (Figure 2.2A), we analyzed each compartment separately. C57BL/6 mice contain a monoclonal population of epidermal-resident TCRδhiVγ5+δ1+ T cells (referred to as dendritic epidermal T cells or DETCs) and a population of polyclonal dermal αβ and γδ T cells [125]. As expected, CD45+ epidermal cells from WT mice were almost exclusively CD3hiTCRδhi T cells (mean, 94.3%; SD, 1.3 from 4 representative mice) whereas the dermis contained TCRδlo and TCRδ- cell subsets (Figures 2.3A and S2.1). Dermal cells from WT also contained a small population of TCRδhi cells that were likely contaminating DETCs (mean, 6.4%; SD, 2.7 from 4 representative mice) (Figure 2.3B). Consistent with previous reports, WT mice contained a population of dermal TCRδlo cells that produced IL-17A upon *in vitro* stimulation that was rare in epidermal preparations (Figure S2.1A-C) [193]. By comparison, CD109-/- mice harbored a substantial population of γδ17 cells in the epidermis (Figure S2.1A-C). To determine whether γδT cells were not only capable of producing IL-17 following *in vitro* stimulation, but were producing this cytokine *in vivo*, we assessed spontaneous production of IL-17A by *ex vivo* antibody labeling. While spontaneous IL-17 production was not detectable in WT skin cells, spontaneous IL-17A production was observed in both epidermal and dermal cells from CD109-/- skin and was limited to the TCRδ^{lo} compartment indicating that these were dermal-derived γδT cells (Figure 2.3A, B; see Figure S.1D and E for

further phenotyping). Next, we determined if the γδ17 cells in the skin of CD109-/- mice were selectively derived from the $V\delta4^+$ lineage, a γδT cell subset imprinted with IL-17 producing potential [110]. Indeed, a large percentage of epidermal and dermal γδ17 cells from CD109-/- mice also expressed the Vγ4 T cell receptor subunit (Figure 2.3C, D). Although the dermal γδT cell population also has been reported to include Vγ1+ and Vγ6+ subsets, we were unable to detect Vγ1+ cells in the skin of WT or CD109-/- mice from our animal colony despite their clear presence in the skin-draining lymph nodes (Figure S2.2A-C). As antibodies detecting the Vγ6 subset are not readily available, we considered Vγ4-Vγ1- γδ T cells as Vγ6+ cells. As shown in Figure S2.2B, a significant proportion of the IL-17+ γδ T cell population was Vγ4-Vγ1- suggesting that both Vγ4+ and Vγ6+ subsets of dermal γδ17 cells accumulate in the absence of CD109. Although all of our experiments comparing WT and CD109-/- were performed with mice initially reared from the same founder dams, environmental differences can occur following cage separation that may have impacted our results. To rule out this concern, we enumerated cutaneous γδ17 cells isolated from WT, CD109+/- and CD109-/- mice littermates raised in the same cage until sacrifice. Consistent with the results shown in Figure 2.3, CD109-/- mice still harbored significantly more epidermal and dermal γδ17 cells compared to WT controls (Figure S2.3A and B). Interestingly, we did observe a gene dosage effect in that CD109+/- more closely resembled CD109-/- mice in terms of cutaneous γδ17 cell number (Figure S2.3A and B).

To confirm the epidermal localization of dermal-derived γδTCR+ cells, we performed confocal immunofluorescent microscopy of WT and CD109-/- skin (Figure 2.3E-G). Whole mount microscopy of epidermal sheets revealed the presence of $TCR\delta^{hi}$ DETCs extending processes across the more superficial layers of the epidermis in both WT and CD109-/- mice as previously described (Figure 2.3E)[135]. However, consistent with our flow cytometry analyses, clusters of rounded Vγ4+ and Vγ4-$TCR\delta^{lo}$ cells localized to the deeper layers of the epidermis of CD109-/-, but not WT, skin (Figure 2.3E). Labeling of cross-sectioned skin samples confirmed the accumulation of Vγ4+ and Vγ4-$TCR\delta^{lo}$ cells in the epidermis of CD109-/- mice, in close proximity to the hair follicle (Figure 2.3F). Finally, whole-mount microscopy of inverted epidermal sheets further demonstrated an increase of Vγ4+$TCR\delta^{lo}$ clusters surrounding the hair follicle in CD109-/- but not WT skin. (Figure 2.3G). Consistently, the neutrophils were also present within the CD109-/- but not WT hair follicles (Figure S2.3C). These results coincided with an increase in mRNA expression of CCL20, a chemokine that promotes leukocyte migration to the hair follicle

[299], in CD109-/- mice compared to controls (Figure 2.3H). Taken together, the absence of CD109 results in polyclonal accumulation of dermal γδ17 cells and their mislocalization to the epidermal layer of the skin.

Accumulation of γδ17 cells in both epidermis and dermis of CD109-/- mice as well as increased expression of *Il23a* and *Il12b* (that encode for IL-23p19 and IL-12/23p40, respectively), but not *Il12a* (IL-12p35), mRNA in these tissues (Figure 2.3I and nanostring analysis) prompted us to determine the relevance of IL-23 in this setting, given that production of IL-17 by γδ T cells can occur in both IL-23-dependent and independent manners [245, 296]. To this end, we administered anti-IL-12/23p40 neutralizing antibodies or isotype control to 10-12 week old CD109-/- mice. Following two weeks of antibody treatment, the number of γδ17 cells were detected by flow cytometric analysis. Temporary anti-IL-12/23p40 blockade significantly decreased the number of IL-17 producing γδT cells in both epidermis and dermis of the skin to numbers approximating age-matched WT controls (Figure 2.3J). Thus, spontaneous accumulation of cutaneous γδ17 cells requires IL-23 activity.

2.3.3 CD109 deficiency enhances the severity of psoriasiform inflammation

Dysregulation of the IL-23/IL-17 immune axis and STAT3 activation are associated with psoriasiform inflammation in both humans and mice [126, 214]. Given that CD109-/- mice exhibited similar dysregulation of these pathways and that decreased amounts of CD109 protein have been found in lesioned compared to non-lesioned skin of individuals with psoriasis [287], we hypothesized that loss of CD109 may predispose mice to a more severe form of psoriasiform inflammation. To this end, we treated the ear skin of CD109+/+ and CD109-/- mice with low-dose imiquimod (IMQ, 3.75% w/v), a TLR7/8 agonist that triggers many of the clinical characteristics of psoriasis in an IL-23 and IL-17 dependent manner [216]. Compared to CD109+/+ controls, CD109-/- mice exhibited a greater absolute and relative increase in ear thickness over 7 days of treatment (Figure 2.4A, B). Histological cross-sections of ear skin before and after IMQ treatment revealed enhanced epidermal hyperplasia, greater disorganization of the stratum corneum and increased dermal leukocyte infiltration in CD109-/- mice relative to controls (Figure 2.4C). Consistent with these results, we detected an increased number of both epidermal and dermal γδ17 cells in treated CD109-/- mice even greater than untreated mice (Figure 2.4D, E and compare to Figure 2.3A, B). Notably, the enhanced inflammation persisted in CD109-/- mice even after

cessation of IMQ treatment compared to control mice (Figure S2.4). Thus, loss of CD109 not only disrupts skin homeostasis but also enhances activation of the IL-23/IL-17 immune axis following overt inflammatory challenge.

2.3.4 CD109 regulates γδ17 cells in a skin-specific and cell-extrinsic manner

The activation requirements and function of the IL-23/IL-17 immune axis at barrier sites are tissue-specific [193, 296] and have important implications for biologics targeting this cellular pathway. To determine whether the absence of CD109 affects the spontaneous activation of γδ17 cells at sites other than the skin, we crossed CD109-/- mice to Rorγt$^{GFP/+}$ mice that faithfully report all leukocytes with IL-17 producing potential as indicated above [298]. Consistent with the increased frequency and number of skin γδ17 cells described in Figure 2.3, the number of Vγ4+GFP+ γδ T cells in the epidermis and dermis of CD109-/-Rorγt$^{GFP/+}$ mice were significantly greater than control mice (Figure 2.5A, B). We also detected a greater frequency and number of Vγ4+GFP+ γδ T cells in the skin-draining lymph nodes (sdLN) and spleen of CD109-/- mice compared to WT controls (Figure 2.5C, D). In contrast, the number of GFP+ γδ T cells in other tissues such as lung, small intestine, mesenteric LN and thymus were similar between both groups (Figure 2.5C, D). Similar results were obtained by assessing the number of γδ17 cells upon *in vitro* stimulation of cells from the same tissues (Figure S2.5). These results are consistent with robust expression of *Cd109* mRNA expression in keratinocytes but not epithelial cells from other barrier sites such as the intestine (Figure S2.6). Taken together, we conclude that CD109 acts in a tissue-specific manner to regulate γδ17 cell homeostasis. Furthermore, these data suggest that CD109 may act in a cell-extrinsic manner to regulate cutaneous γδ17 cells.

To test the cell-extrinsic effects of CD109 on γδ17 cell activation, we first performed *in vitro* cultures in which total skin cells from WT or CD109-/- mice were cultured with purified CD3+ T cells from sdLN (Figure 2.6A). Stimulation of T cells with IL-1β and IL-23 was used as a positive control to induce IL-17 secretion. T cells cultured with skin from CD109-/- mice produced significantly more IL-17A compared to cultures using WT skin. In line with our *in vivo* studies using anti-IL-23p40 antibody treatment (Figure 2.3J), IL-17A production was abrogated in the presence of anti-IL-23p19 antibodies (Figure 2.6A). To confirm the cell-extrinsic regulation of γδ17 cells *in vivo*, we generated bone marrow (BM) chimeras in which BM from CD45.1+ WT or CD45.2+CD109-/- mice was transplanted into irradiated CD45.2+ WT, CD45.1+ WT or CD109-

/- recipient mice (Figure 2.6B-G). Although dermal γδ T cells have been shown to predominantly accumulate in the dermis within a narrow time period (postnatal day 0-4) and are largely radio-resistant [125, 228], we speculated that the inflammatory milieu present in the skin of CD109-/- mice may be sufficient to elicit *de novo* generation of γδ17 cells from adult BM. Although we observed a selective increase in dermal γδ T cells from the skin of WT → CD109-/- chimeras, dermal γδ T cells in all groups were almost exclusively derived from recipient mice precluding our ability to assess a donor population (Figure 2.6B, C). Although we observed a similar increase in the sdLN of WT → CD109-/- chimeras, the majority of γδ T cells from sdLN in all groups were donor-derived (Figure 2.6D). Interestingly, however, WT → CD109-/- chimeras contained a substantial number of host-derived γδ T cells that were almost exclusively Vγ4+Rorγt+ cells (Figure 2.6E, F). In addition, only WT → CD109-/- chimeras harbored CD45.1+ donor Vγ4+ cells expressing Rorγt (Figure 2.6F, G). While these results demonstrated a cell-extrinsic effect on γδ17 cell development, we sought to confirm this observation in skin-resident γδ T cells. To this end, we adopted an approach developed by Gray et al. in which supplementation of standard bone marrow chimeras with neonatal thymocytes reconstitutes a detectable donor population of dermal γδ T cells [124]. To this end, we co-transplanted adult bone marrow and neonatal thymocytes from CD45.1+ WT or CD45.2+CD109-/- mice into irradiated CD45.2+ WT or CD109-/- recipient mice. This approach efficiently reconstituted the skin with a detectable population of donor γδ T cells as determined by congenic CD45 discrimination (Figure 2.6H). Consistently, only CD109-/- recipients, regardless of donor genotype, exhibited an increase in the frequency and number of epidermal and dermal γδ17 cells (Figure 2.6I, J). Collectively, these results indicate that CD109 expression by a radioresistant cell type acts in cell-extrinsic manner to limit the localization and activation of IL-17 producing γδ T cells.

2.3.5 CD109 is required to limit γδ17 cell activation by commensal microbiota

The cell-extrinsic nature of CD109 on γδ17 cell activation prompted us to explore other factors that regulate the cutaneous immune system. The commensal microbial community of the skin, referred to as the microbiota, plays an important role in host defense by promoting anti-microbial peptide secretion by keratinocytes and endowing lymphocytes, including γδT cells, with the potential to produce IL-17 and protect the host from subsequent infectious challenge [193]. Consistently, there was a significant increase in anti-microbial gene expression in epidermal and

dermal sheets from CD109-/- mice including *S100a7*, *S100a8*, *S100a9* and *Defb14* mRNA as determined by Nanostring and RT-qPCR analysis compared to control mice (Figure 2.7A, B and Figure S2.7A, B). In addition, pathway analysis of our Nanostring data indicated that "cell-cell adhesion" and "defense response to bacterium" was among the top gene sets significantly different between WT and CD109-/- epidermal and dermal tissue (Figure 2.7C and Figure S2.7C). To test whether activation of these pathways was a consequence of alterations to commensal skin microbiota, we performed 16S rRNA sequencing of skin swabs from WT and CD109-/- littermates. However, no significant differences at 6 or 12 weeks of age in terms of overall composition, alpha diversity (as determined by unweighted Unifrac analysis) or beta diversity (e.g. Shannon and Faith indices) were observed between genotypes (Figure 2.7D-F). An alternative explanation is that CD109 regulates skin permeability resulting in aberrant exposure or activation to commensal microorganisms. To test skin barrier integrity, we used two approaches. First, an *ex vivo* caffeine diffusion assay was performed in which penetration of caffeine, an amphiphilic small molecule, through the depilated skin is measured in the supernatant by high performance liquid chromatography (Figure 2.7G)[300]. This assay revealed a significant increase in caffeine absorption over time in skin from CD109-/- mice compared to WT controls. As an additional measure of barrier integrity, lucifer yellow, a hydrophilic dye that does not breach the stratum corneum under normal conditions, was applied to depilated mouse back skin sections and transdermal penetration was quantified by fluorescence spectrophotometry. Similarly, this assay showed that skin from CD109-/- mice was significantly more permeable to lucifer yellow compared to WT control skin (Figure 2.7H)[301]. This difference was age-dependent as no differences in skin barrier permeability were observed using skin from WT and CD109-/- neonates (Figure 2.7I).

Consistent with the aberrant anti-bacterial response and compromised barrier integrity of skin from CD109-/- mice, *in situ* mRNA labelling indicated that CD109 transcripts were highly expressed within the basal layer of the epidermis, with particular abundance in the hair follicles, a site with a dense microbial constituency (Figure 2.7G)[193, 302]. To directly test whether the commensal microbiota is required for the activation of the IL-23/IL-17 axis in CD109-/- mice, we topically applied broad-spectrum antibiotics or a vehicle control to the ear skin of WT and CD109-/- skin and cutaneous γδ17 cell numbers and neutrophil accumulation were used as readouts. Remarkably, short-term treatment of CD109-/- with antibiotics abrogated the epidermal

accumulation of γδ17 cells, restored the number of dermal γδ17 cells to WT numbers and eliminated epidermal neutrophil accumulation (Figure 2.7H, I and S2.7D). Consistently, *Il17a* and *S100a8* mRNA in CD109-/- skin were also significantly reduced after antibiotic treatment while pSTAT3 expression remained elevated (Figure S2.7E-G).

2.4 Discussion

Immune cells residing in barrier tissues such as the skin, lung, intestine and female reproductive tract face the complex task of protecting the host from pathogens while living in a mutualistic relationship with the commensal microbiota. However, the primary function of the immune system is to recognize and destroy microorganisms. Therefore, mechanisms that counter or temper this function at barrier sites must be put in place to tolerate the microbial shield that maximizes host fitness in a dynamic environment. While several cell-intrinsic pathways have been described that limit cutaneous immune cell proliferation and activation, the molecular mechanisms by which structural cells negatively regulate the immune system are less well understood. Here we identify CD109 as a negative regulator of the IL-23/IL-17 immune axis and γδ17 cell reactivity to the cutaneous microbiota. Consistent with the critical role of this immune axis in psoriasiform inflammation [154], deletion of CD109 led to enhanced disease severity following IMQ-induced sensitization. Although CD109 has been shown to be expressed by activated human T cells [256], our results indicate a cell-extrinsic role for regulating the localization and production of IL-17 by γδ T cells. By contrast, no dysregulation of γδ17 cells was apparent at the other barrier sites we examined including the gut and lung. While it will be important to investigate the role of CD109 during overt inflammation at these alternative barrier sites, our data point towards a tissue-specific function for CD109 in regulating skin homeostasis.

Prior to our study, little was known regarding the role of CD109 in cutaneous immune regulation. Although Mii et al found accumulation of leukocytes in the skin of CD109-/- mice, the immune pathways activated *in vivo* and the mechanisms driving inflammation were not examined [263]. Our studies show that deletion of CD109 in mice results in dysregulated keratinocyte activation and spontaneous activation of the cutaneous IL-23/IL-17 immune axis including neutrophil accumulation, increased expression of alarmins with anti-microbial properties such as S100A7, S100A8 and S100A9 and multiple cytokines previously associated with psoriatic skin inflammation including members of the IL-20R-dependent cytokine family such as IL-19 and IL-20 that signal via STAT3 dependent pathways. Consistently, we found constitutive activation of STAT3 in CD109-/- skin lysates even in the absence of *ex vivo* stimulation. In line with these data and previous studies, dysregulated STAT3 activation in keratinocytes is strongly associated with the IL-23/IL-17 immune axis and psoriasiform inflammation [214]. While we cannot equate our animal studies with human disease, a previous study showed decreased protein production of

CD109 within psoriasis lesions compared to non-lesional skin from the same patients [287]. However, genomic analyses of patients with psoriasis have yet to identify risk alleles in the CD109 gene. This may not be surprising, however, as no change was observed in *cd109* mRNA expression between skin from patients with psoriasis and healthy controls [287]. Although these clinical results are based on small patient cohorts, they suggest that post-translation modification of CD109 may be a critical determinant of disease initiation and/or progression and provide the impetus for understanding how CD109 is regulated.

Deletion of CD109 was prominently associated with accumulation of γδ17 cells, a population previously implicated in early protective immunity to extracellular bacterial and fungal infection, but also potential initiators of pathological skin inflammatory conditions as in the case of psoriasis [61, 126, 131, 233]. Prinz and colleagues recently described the aberrant localization of dermal-derived γδ17 cells to the epidermis in the context of IMQ-induced inflammation, a result we also observed [303]. However, in accordance with the inflammatory nature of CD109-/- skin, we observed spontaneous accumulation of γδ17 cells in the epidermal layer even in the absence of intentional immune challenge. In our analysis of Vγ4+ γδ T cell localization, a subset enriched for IL-17 production, we observed the preferential localization of these cells around the hair follicle. Recent data indicates that this region of the epidermis is particularly immunological active as these sites are also enriched for CD4+ T regulatory cells (Treg) that promote the hair cycle and serve as a conduit for capture of epicutaneous antigens by dermal dendritic cells [299, 304]. In addition, hair follicles are migration portals for monocytes to reconstitute the epidermal Langerhans cell population during tissue stress [35]. Notably, Treg and Langerhans cell expression of CCR6 is required for accumulation at the follicle [35, 299]. Similarly, CCR6 is highly expressed on dermal IL-17 producing γδ T cells and is required for IMQ-induced psoriasiform inflammation [105]. Consistent with these data, we observed prominent expression of CD109 within the hair follicle and following its deletion, progressive hair loss and a concomitant increase of CCL20 expression, the ligand for CCR6, in epidermal sheets. Thus, CD109 may act as a rheostat to balance CCL20-CCR6 dependent regulatory and inflammatory immune elements to mediate barrier homeostasis and tissue regeneration at the hair follicle.

CD109 has been reported as a TGF-β co-receptor and negative regulator of canonical TGF-β signaling [287]. Consistent with the phenotype of CD109-/- mice, overexpression of TGF-β signaling in basal keratinocytes results in aberrant epidermal thickening and IL-17 production

[305]. However, examining SMAD2/3 activation of total skin cells, we were unable to detect differences in responsiveness to TGF-β signaling as determined by phosphorylated SMAD2/3. Although these results do not rule out a potential role for CD109 in regulating TGF-β signaling in certain conditions, our data suggest that CD109 may have additional and/or alternative roles in cutaneous immune regulation. We found CD109 to be highly expressed in the basal layer of the epidermis and the hair follicle. We chose to examine the location of CD109 mRNA expression by *in situ* mRNA detection because, once translated, its GPI anchor is sensitive to phospholipase cleavage resulting in a soluble protein making its cellular source difficult to determine [287]. Although our histological and imaging results indicated epidermal hyperplasia and aberrant skin thickening in the absence of CD109, whether this protein directly regulates keratinocyte proliferation and the specific stage of cell differentiation that CD109 becomes functionally active remain to be determined. Interestingly, CD109 has been demonstrated to localize to lamellar bodies, lipid-rich vesicles secreted by granular keratinocytes that join corneocytes at the most superficial region of the stratum corneum and are critical for barrier formation [17]. We are currently investigating whether CD109 activity promotes the formation and/or function of lamellar bodies to fortify the skin barrier [15].

Despite the low microbial biomass compared to other barrier tissues such as the intestine, it is well-established that microbes residing on mammalian skin condition the cutaneous immune system to respond towards a host of environmental challenges [306]. Indeed, microbial colonization of the skin regulates susceptibility to diverse pathogens as well as infection-induced tissue damage [193, 307]. Thus, limiting reactivity to microbiota-dependent inflammation is central to orchestrating a balanced immune response. Our results indicate that CD109 limits expression of anti-microbial factors including members of the S100 and β-defensin families. Importantly, a brief course of topical antibiotics to CD109-/- mice was sufficient to reduce the number of γδ17 cells and neutrophils to numbers comparable to WT mice. These results indicate that continuous exposure to the microbiota is required to drive activation of IL-23/IL-17 immune axis. Although the mechanisms by which microbial colonization of the epidermis transmits signals to the dermal immune compartment are not well understood, the signals stimulating IL-17 production in this context are likely cytokine-driven and not antigen-restricted because 1) both Vγ4+ and Vγ4- γδ T cell subsets produced increased amounts of IL-17 in the absence of CD109, 2) production of IL-17 by γδ T cells does not require TCR engagement and 3) we did not detect

any spontaneous cytokine production by cutaneous αβ T cells, a lineage that requires antigen encounter for activation. We are currently investigating the composition of the skin microbiota in CD109-/- mice to determine whether specific members of the microbial community are dysregulated. If so, this will be an exciting opportunity to identify microbes that exploit a compromised skin barrier and activate the underlying immune system.

Based on our data we propose a scenario whereby the absence of CD109 production by keratinocytes results in a breach of the skin barrier sufficient to expose the dermal immune compartment to chronic stimulation by epicutaneous commensal microbes. Dermal-infiltrating microbes or microbial-derived products are subsequently sensed by pattern recognition receptor-expressing innate effector cells, such as dermal dendritic cells, macrophages or possibly keratinocytes themselves [134, 308], that release inflammatory cytokines promoting neutrophil recruitment and IL-23-dependent proliferation and activation of IL-17-producing, dermal-derived γδ T cells. A compromised barrier stimulates an epidermal stress response inducing CCL20 that promotes γδ17 cell migration to the hair follicle, a position that predisposes the host to enhanced reactivity towards overt inflammatory stimuli. Identifying CD109 as an endogenous regulator of cutaneous inflammation and host tolerance to the microbiota motivates further investigations into tissue-specific mechanisms that maintain tissue homeostasis and skin health.

2.5 STAR Methods

Key resource table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies (Clone)		
Mouse monoclonal anti-mouse CD45.1-eFluor 450 (A20)	eBioscience	Cat# 48-0453-82; RRID: AB_1272189
Mouse monoclonal anti-mouse CD45.2-PE-eFluor 610 (104)	eBioscience	Cat# 61-0454-82; RRID: AB_2574562
Rat monoclonal anti-mouse B220-Alexa Fluor 700 (RA3-6B2)	eBioscience	Cat# 56-0452-80; RRID: AB_891460
Hamster monoclonal anti-Mouse CD3e-BV650 (145-2C11)	BD	Cat# 564378; RRID: AB_2738779
Hamster monoclonal anti-Mouse TCRβ-APC-eFluor 780 (H57-597)	eBioscience	Cat# 47-5961-8; RRID: AB_1272173
Hamster monoclonal anti-Mouse TCRγ/δ-FITC (GL3)	Biolegend	Cat# 118106; RRID: AB_313830
Hamster monoclonal anti-Mouse TCRγ/δ-PE (GL3)	Biolegend	Cat# 118108; RRID: AB_313832
Hamster monoclonal anti-Mouse TCRγ/δ-PerCP/Cy5.5 (GL3)	Biolegend	Cat# 118118; RRID: AB_10612756
Hamster monoclonal anti-Mouse TCR Vγ2(4)-PE-Cyanine7 (UC3-10A6)	eBioscience	Cat# 25-5828-82; RRID: AB_2573474
Hamster monoclonal anti-Mouse TCR Vγ2(4)-PE (UC3-10A6)	Biolegend	Cat# 137706; RRID: AB_10643577
Rat monoclonal anti-mouse IL-17a-PE (eBio17B7)	eBioscience	Cat# 12-7177-81; RRID: AB_763582
Rat monoclonal anti-mouse IFN-γ-Alexa Fluor 488 (XMG1.2)	eBioscience	Cat# 53-7311-82; RRID: AB_469932
Rat monoclonal anti-mouse Ki-67-FITC (SolA15)	eBioscience	Cat# 11-5698-82; RRID: AB_11151330
Rat monoclonal anti-mouse Ly6G-Alexa Fluor 488 (1A8-Ly6g)	eBioscience	Cat # 11-9668-82; RRID: AB_2572532
Rat monoclonal anti-mouse CD11-APC (M1/70)	eBioscience	Cat# 17-0112-82; RRID: AB_469343
Mouse CD109 Affinity Purified Polyclonal Ab	R&D	Cat# AF7717
Mouse monoclonal anti-mouse Lamin B1 (A11)	Santa Cruz	Cat# sc-377000
Goat Anti-Rabbit IgG (H + L)-HRP Conjugate	BioRad	Cat# 170-6515
Goat Anti-Mouse IgG (H + L)-HRP Conjugate	BioRad	Cat# 170-6516
Rabbit Anti-Sheep IgG (H+L)-HRP Conjugate	BioRad	Cat# 1721017
Phospho-stat3 (Tyr705) rabbit mAb (D3A7)	Cell signaling	Cat# 9145S
Stat3 mouse mAb (124H6)	Cell signaling	Cat# 9139S
Phospho-smad2 (Ser465/467)/Smad3 (Ser423/425) Rabbit mAb (D27F4)	Cell signaling	Cat# 8828S
Smad2/3 Rabbit mAb (D7G7)	Cell signaling	Cat# 8685S;
Rat *InVivo*MAb anti-mouse IL-12 p40 (C17.8)	BioXell	Cat# BE0051; RRID: AB_1107698
*InVivo*MAb rat IgG2a isotype control, anti-trinitrophenol (2A3)	BioXell	Cat# BE0089; RRID: AB_1107769
Chemicals, peptides and recombinant peptides		
Recombinant mouse IL-23	Biolegend	Cat# 589004
Recombinant Human TGF-β1	R&D	Cat# 240-B-002
Fixable Viability Dye-eFluor 506	eBioscience	Cat# 65-0866-18
Fixable Viability Dye-eFluor 780	eBioscience	Cat# 65-0865-18
Fixation/Permeabilization Concentrate	eBioscience	Cat# 00-5123-43
Fixation/Permeabilization Diluent	eBioscience	Cat# 00-5223-56

Permeabilization Buffer (10X)	eBioscience	Cat# 00-8333-56
Collagenase from *Clostridium histolyticum* (Collagenase, Type IV)	Sigma-Aldrich	Cat# C5138
DNase I	Roche	Cat# 11284932001
Collagenase/Dispase	Sigma-Aldrich	Cat# 11097113001
Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate	PerkinElmer	Cat# NEL104001EA
PMA	Sigma-Aldrich	Cat# P1585
Ionomycin calcium salt from *Streptomyces conglobatus*	Sigma-Aldrich	Cat# I0634
EDTA (Ethylene diamine tetra acetic acid) disodium salt	BDH	Cat# ACS 345
RPMI 1640 Medium	Gibco	Cat# 11875119
HBSS, calcium, magnesium	Gibco	Cat# 24020117
DPBS, no calcium, no magnesium	Gibco	Cat# 14190144
FBS (fetal bovine serum)	Wisent	Cat# 081-105
EvaGreen 2X qPCR mastermix without ROX	ABM	Cat# ABMMmix-S-XL
DNase/RNase-Free Distilled Water	Invitrogen	Cat# 10977015
Aquaphor Healing Ointment	Eucerin	DIN 02009609
3.75% Imiquimod (Zyclara) cream	Valeant	DIN 02340445
NEOSPORIN® Original Ointment	HeliDerm	DIN 00666122
Lucifer Yellow CH dilithium salt	Sigma-Aldrich	Cat# L0259
DAPI	Sigma-Aldrich	Cat# D9542
Acetone	Thermo Fisher	Cat# A16P-4
Paraformaldehyde solution 4% in PBS	Affymetrix	Cat# AAJ19943K2
10% Formalin	Thermo Fisher	Cat# SF100-4
Sulfuric acid solution, 1M	Honeywell Fluka	Cat# 15644920
Critical Commercial Assays		
IL-17A Mouse Uncoated ELISA Kit	Invitrogen	Cat# 88-7371-88
IL-13 Mouse Uncoated ELISA Kit	Invitrogen	Cat# 88-7137-88
IFN-γ Mouse Uncoated ELISA Kit	Invitrogen	Cat# 88-7314-88
DNeasy Blood & Tissue Kit	Qiagen	Cat# 69506
RNeasy Mini Kit	Qiagen	Cat# 74104
RNAscope 2.5 HD Detection Reagent - RED kit	Advanced Cell Diagnostic	Cat# 322360
Oligonucleotides		
Primer: mouse CD109 Forward	This paper	
GAGCTTATGCGCTAGATCC		
Primer: mouse CD109 Reverse	This paper	
ATGCCTGTCCTCTCTGAACC		
Primer: mouse S100a7 Forward	This paper	
CAGGCAGTCTCTCATCACCA		
Primer: mouse S100a7 Reverse	This paper	
CCCAGAACCATGACCTGAGT		
Primer: mouse S100a8 Forward	This paper	
GGAAATCACCATGCCCTCTA		

Primer: mouse S100a8 Reverse	This paper	
TCCTTGTGGCTGTCTTTGTG		
Primer: mouse S100a9 Forward	This paper	
CAGCATAACCACCATCATCG		
Primer: mouse S100a9 Reverse	This paper	
GTCCTGGTTTGTGTCCAGGT		
Primer: mouse RegIIIg Forward	This paper	
AACAGAGGTGGATGGGAGTG		
Primer: mouse RegIIIg Reverse	This paper	
ATTTGGGATCTTGCTTGTGG		
Primer: mouse CRAMP Forward	This paper	
CTCCGCAAAGGTGGGGAGAAG		
Primer: mouse CRAMP Reverse	This paper	
GGGAATCCAGAAACAGGCCAAGG		
Primer: mouse IL-4 Forward	This paper	
TCACAGCAACGAAGAACACC		
Primer: mouse IL-4 Reverse	This paper	
ATTCATGGTGCAGCTTATCG		
Primer: mouse IL-5 Forward	This paper	
TCATGAAGTGCTGGAGATGG		
Primer: mouse IL-5 Reverse	This paper	
CATCTGGACCAAGAGTTCAGG		
Primer: mouse IL-13 Forward	This paper	
ATTGCATGGCCTCTGTAACC		
Primer: mouse IL-13 Reverse	This paper	
TGAGTCCACAGCTGAGATGC		
Primer: mouse IL-17A Forward	This paper	
ACTCTCCACCGCAATGAAGA		
Primer: mouse IL-17A Reverse	This paper	
CTCTCAGGCTCCCTCTTCAG		
Primer: mouse IFN-γ Forward	This paper	
TTCTTCAGCAACAGCAAGGC		
Primer: mouse IFN-γ Reverse	This paper	
ACTCCTTTTCCGCTTCCTGA		
Primer: mouse IL-23p19 Forward	This paper	
GACTCAGCCAACTCCTCCAG		
Primer: mouse IL-23p19 Reverse	This paper	
GGCACTAAGGGCTCAGTCAG		
Primer: mouse CCL20 Forward	This paper	
AATCTGTGTGCGCTGATCC		
Primer: mouse CCL20 Reverse	This paper	
AGGTCTGTGCAGTGATGTGC		
Primer: mouse Hprt Forward	This paper	
AGGACCTCTCGAAGTGTTGG		
Primer: mouse Hprt Reverse	This paper	

AACTTGCGCTCATCTTAGGC		
Primer: bacteria 16S-V2 Forward	This paper	
AGYGGCGIACGGGTGAGTAA		
Primer: bacteria 16S-V2 Reverse	This paper	
CYIACTGCTGCCTCCCGTAG		
Primer: bacteria 16S-V6 Forward	This paper	
AGGATTAGATACCCTGGTA		
Primer: bacteria 16S-V6 Reverse	This paper	
CRRCACGAGCTGACGAC		
Primer: mpIgRgenomic Forward	This paper	
TTTGCTCCTGGGCCTCCAAGTT		
Primer: mpIgRgenomic Reverse	This paper	
AGCCCGTGACTGCCACAAATCA		
Experimental Models: Mouse strains		
Mouse: C57BL/6 (B6)	Jackson Lab	Stock No: 000664 | Black 6
Mouse: CD109-/-	Masahide Takahashi (Japan)	PMID: 22846721
Mouse: Pep Boy, B6 Cd45.1	Jackson Lab	Stock No.002014 | B6 Cd45.1
Mouse: B6.129P2(Cg)-Rorc^{tm2Litt}/J	Jackson lab	Stock No: 007572 | Rorc(γt)-EGFP
Software		
FlowJo	BD	https://www.flowjo.com
GraphPad Prism 7	GraphPad Software	https://www.graphpad.com
nSolver4.0	NanoString Technologies	https://www.nanostring.com
Others		
VWR clear frozen section compound	VWR	Cat# 95057-838
ProLong diamond antifade mountant	Invitrogen	Cat# P36961

Animals

The CD109+/+, CD109+/-, CD109-/-, CD45.1+, Rorγt$^{GFP/+}$ and Rorγt$^{GFP/GFP}$ (8-24 weeks old) on a C57BL/6 background were bred and maintained under specific pathogen-free conditions at the Comparative Medicine & Animal Resources Centre at McGill University. CD109-/- mice on a C57BL/6J/129S6 background were generated by Dr. Masahide Takahashi (Nagoya University) (REF). Embryos were generously provided by Dr. Takahashi and injected into 0.5 pseudopregnant CD1 females. Progeny were crossed to C57BL/6 mice at least five times before use. All experiments involving CD109-/- mice used littermates as controls or mice originally derived from CD109+/- breeders. Rorγt$^{GFP/GFP}$mice were original purchased from Jackson (stock #007572) and

bred with our C57BL/6 colony. All animal studies were approved by the McGill University Animal Care Committee.

Immunoblots

Total protein concentration of ultrasonicated samples was assessed by protein quantification assay under the manufacturer's instructions. The lysates were then diluted in a 6× loading buffer, and incubated at 100 °C for 5 min. Next, samples were separated by SDS-PAGE and transferred on polyvinylidene difluoride membranes by wet blotting. The membrane was blocked by 5% milk powder in 1×TBS/0.1 % Tween-20 (TBST) at room temperature for 1 hour and incubated in primary antibody overnight at 4 °C. Membrane was washed 3 times and incubated with secondary antibody for 1 hour at room temperature. The blots were detected by the chemiluminescence light-detecting kit.

Histology and RNA scope

For histology, skin tissue was fixed in 10% formalin, paraffin embedded, sectioned and stained with hematoxylin and eosin by the MUHC-RI Histology Core. For RNAscope, formalin-fixed paraffin-embedded skin samples were stained with RNAscope® 2.5 HD red detection reagent kit including sequence-specific probes for CD109. Stained slides were scanned at either 20× or 40× magnification. Slides were visualized using Image J software.

Isolation of skin cells

To prepare single cell suspensions from skin tissue, ears were cut across the hairline and manually partitioned into rostral and caudal sides using fine scalpels. To partition the epidermis from the dermis, each side was incubated dermal side down in separation buffer (RPMI 1640 containing 1mg/ml Collagenase/Dispase) for 1.5 hours in a 35 mm petri dish at 37°C. The epidermis and dermis were then rinsed in cold PBS, cut into ~2mm^2 pieces and incubated with digestion buffer (RPMI 1640 containing 154 U/ml Collagenase IV, 200 U/ml Dnase I and 2% FBS) for 2 hours in 24 well plates at 37°C. For spontaneous cytokine detection by flow cytometry, the protein transport inhibitor Golgistop (BD bioscience) was added into the digestion buffer (4 µl Golgistop per 6ml buffer). Following the incubation, the tissue was pipetted up and down in the digestion buffer to generate a single cell suspension, passed through a 100 µm cell strainer and

collected into a 50 mL conical tube. Any residual tissue pieces were crushed using a syringe plunger before all cell strainers were rinsed with RPMI 1640 media containing 10% FBS and 15mM HEPES. Cells were then washed and resuspended in R10 buffer prior to counting.

Flow cytometry

For extracellular staining, single cell suspensions were incubated with fixable viability dye in 100 µl PBS (eBioscience), washe and incubated with anti-Fc receptor (clone 2.4G2, BD biosciences) for 10 minutes before adding fluorochrome-labelled antibodies at predetermined concentrations in 100 µl FACs buffer (PBS containing 2% FBS and 10mM HEPES) for 30 minutes on ice. For intracellular staining, cells were incubated with fixable viability dye and fixed with the Intracellular fixation/permeabilization buffer (eBioscience) according to the manufacturer's instructions, followed by antibody labelling in 100 µl permeabilization buffer. After intracellular labelling, cells were washed and resuspended in 300 µl FACS buffer. Data were acquired on a LSR Fortessa (BD Biosciences) and then analyzed with FlowJo™ software.

Skin-T cell co-cultures

Single cell suspensions from total skin tissue was generated as described above and re-suspended in 5 mL R10 buffer, then pipetted onto 5 mL Ficoll at room temperature in a 50 mL Falcon tube. Tubes were centrifuged for 20 min at room temperature. Live cells were isolated from the media-Ficoll interface and washed with 45 mL R10 buffer prior to suspension in culture media. Total CD3+ T cells were isolated from the skin-draining lymph nodes using T cell isolation kit (STEMCELL Technologies). 5×10^5 live skin cells and 1×10^6 T cells were mixed and added into a 24-well plate and cultured for 24 hours in various conditions. Anti-IL-23p19 antibodies (G23-8, BioXcell) or Rat IgG1 isotype control were added to some wells. Culture supernatants were collected and processed for IL-17A detection by ELISA (Invitrogen).

Tissue processing and confocal microscopy

In brief, ear samples were embedded and flash frozen in OCT, cut into 6-8 µm sections and fixed on superfrost slides with ice-cold fixation solution (75% Acetone+25% Ethanol), blocked by anti-Fc receptor for 1 hour, stained with fluorochrome-conjugated antibodies cocktail for 1 hour and nuclei staining (DRAQ5, eBiosciences) for 5 minutes at room temperature. For whole-mount

staining, mouse ears were depilated with Nair and transferred into the fixation solution for 20 minutes at room temperature. For epidermal whole-mount staining, mouse tail skin (~1cm^2) was depilated with Nair followed by a digestion in 5mM EDTA for 4 hours at 37˚C to separate the epidermis. Then the epidermis sheets were transferred into 4% Paraformaldehyde for 1 hour. Following fixation, tissue was incubated in PBS containing 25U of Collagenase IV at 37°C for 30 minutes prior to antibody labelling as described above.

Quantitative RT-PCR analyses

Ear skin from individual animals were separated into epidermal and dermal sheets as described above. Samples were flash frozen and total RNA was extracted by using the QIAGEN RNeasy Mini Kit as per the manufacturer's instructions. Next, equal amounts of RNA from each sample were reverse-transcribed by QuantiTect Reverse Transcription Kit (Thermofisher). Primers (*Ccl20, Il17, Ifng, Il4, Il5, Il3, Il23a, Il12a, IL12b, S100a7, S100A8, S100A9, RegIIIg* and *Cramp*) were designed specific for the target genes and tested by end-point PCR first. Relative expression of genes of interest was measured by real-time RT-PCR. The expression of the genes of interest from individual cDNA samples were normalized to the reference gene *Hprt* and expressed as a fold change using the $^{\Delta\Delta}$Ct formula.

Nanostring analysis

Samples were divided into 4 groups (epidermis_KO, epidermis_WT, dermis_KO and dermis_WT) and were analyzed in biological triplicates. Total RNA was isolated from the epidermis and dermis of CD109+/+ and CD109-/- ears and the gene expression profile is determined using a Nanostring codeset. Data were analyzed with nSolver® 4.0 software. Internal positive controls and housekeeping gene controls were selected by the nCounter Expression Data Analysis Guide. The geometric mean of internal postive controls and housekeeping genes were used to normalize the sample gene expression and variability control correspondingly. Normalized sample genes were analyzed and ranked by the magnitude of alteration. For pathway analysis, data were analyzed according to the nSolver® software advanced analysis tools.

Anti-IL-12/23p40 administration

Mice were intraperitoneally injected with 200μg anti-IL-12/23p40 antibody (C17.8) or isotype IgG2a (2A3) purchased from BioXCell every third day for two weeks. Frequency and number of IL-17-producing γδT cells from epidermis and dermis were detected by flow cytometric analysis.

Imiquimod-induced skin inflammation

Dorsal ears of 8-10 wk old mice were treated with 3-4mg 3.75% IMQ cream (Zyclara) for 7 consecutive days. Control mice were left untreated. Ear thickness was measured by a digital Vernier caliper (accuracy: 0.01mm, Proster).

Topical antibiotic treatment and 16S rRNA quantification

Neosporin® or the vehicle control Aquaphor was applied to the dorsal and ventral ear skin of mice daily for one week using a sterile microspoon.

ELISA

Cell supernatants were collected and tested for mouse IL-17A, IFN-γ and IL-13 mouse ELISA kit (Invitrogen) as per the manufacturer's instructions.

Bone marrow chimera generation

8-10 week old WT or CD109-/- mice were irradiated with 900 rads and subsequently i.v injected with 5×10^6 adult bone marrow cells and, in some cases, 1×10^6 neonatal thymocytes from 2-3 day old mice simultaneously. Donor and recipient cells were distinguished by CD45 alleles: WT (CD45.1)→WT (CD45.2), WT (CD45.1)→KO (CD45.2), and KO (CD45.2)→WT (CD45.1). After 12 weeks reconstitution, mice were sacrificed for flow cytometric analysis.

Percutaneous absorption studies

Sections of back skin (3 cm^2) from neonatal (postnatal day 1) or adult (>8 weeks old) was depilated and hypodermis was gently removed by scraping with a scalpel. Skin sections were placed over a 5 ml beaker containing 5 ml PBS (dermis down, exposed to the liquid surface). 200 μl of 1mM Lucifer yellow (452 Da, Invitrogen) was added onto the epidermis and incubated at 37 °C, 5% CO2. At 2 or 4 hours post-incubation, the optical density of the underlying saline

solution was read using a spectrophotometer (Infinite M1000, Tecan) with excitation and emission wavelengths at 425 and 550 nm, respectively.

16S rRNA sequencing of skin microbiota

Six and twelve week-old CD109+/+ and CD109-/- littermates were used for microbiome analysis. Back skin was shaved with hair clippers and cleaned with a Kimwipe. Under a sterile biosafety cabinet, FLOQSwabs from Copan Diagnostics were moistened with sterile PBS and firmly swabbed over the shaved back skin of each mouse for four minutes using a rotating movement. The tips of the swabs were cut and immediately placed in an Eppendorf tube containing 1ml of sterile PBS and frozen at -80°C for later analysis. Moistened swabs exposed to ambient air were used as negative controls. DNA was extracted from thawed swabs using the QIAGEN QIAamp DNA Microbiome Kit and quantified using the Picogreen assay. Frozen DNA samples were sent to The Children's Hospital of Philadelphia Microbiome Center for sequencing. The V1 to V3 hypervariable regions of the 16S ribosomal DNA segment was amplified using primers specific to this region. Each amplicon was sequenced and the sequencing data was processed and analyzed using the QIIME2 pipeline as described in [https://doi.org/10.7287/peerj.preprints.27295v2]. DADA2 [PMID 27214047], implemented as a QIIME2 plug-in, was used for sequence quality filtering. Taxonomic analysis was done using a Naïve Bayes classifier trained on the Greengenes 13_8 99% OTUs. For diversity metrics including the Faith phylogenetic diversity and UniFrac distances, a rooted phylogenetic tree was generated: first, a multiple sequence alignment was performed using MAFFT [PMID 23329690] and high variable positions were masked to reduce noise in a resulting phylogenetic tree. A mid-point rooted tree was then generated using FastTree [PMID 20224823]. Statistical analyses were carried out using R (version 3.6.0). Shannon index and richness were calculated using the package vegan (version 2.5-5). Plots were generated using the package ggplot2 (version 3.1.1).

Statistical analysis

Data were analyzed with Graphpad® Prism 7. Unpaired student t test or Two-way ANOVA followed by Sidak's multiple comparisons test were used as appropriate. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

2.6 Acknowledgements

We thank members of the King laboratory and collaborators for critical reading of the manuscript and the McGill Integrated Core for Animal Modeling for embryo cryorecovery of CD109-/- mice. We are also grateful to the Children's Hospital of Philadelphia Microbiome Center, especially Dr. Jung-Jin Lee, for their assistance with skin microbiome sequencing and analysis. H.Z. is a recipient of a Chinese Scholarship Council Award. N.K. is a recipient of a Fonds de recherche du Québec – Santé (FRQS) Postdoctoral Fellowship. I.L.K. is a Canada Research Chair in Barrier Immunity. This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (RGPIN-2015-05491) and a Canadian Institutes of Health Research Project Grant (PJT-148552) awarded to I.L.K.

2.7 Author contributions

H.Z and G.C. designed and performed experiments, analyzed the data and wrote the manuscript. B.P. performed the RNAscope experiments. N.K. performed tissue harvests and analyzed data. M.E.G. performed cell-sorting experiments and qPCR analysis of intestinal tissue. G.F. assisted with experiments, performed western blots and provided critical technical input. D.V. provided reagents and intellectual input. B.T. performed the qRT-PCR for inflammatory gene expression and skin microbiome analysis. M.S. performed percutaneous absorption assays. R.P. provided technical support and intellectual input for the percutaneous studies. I.L.K. conceptualized the study, designed and performed experiments and wrote the manuscript.

2.8 Figures

Figure 2.1

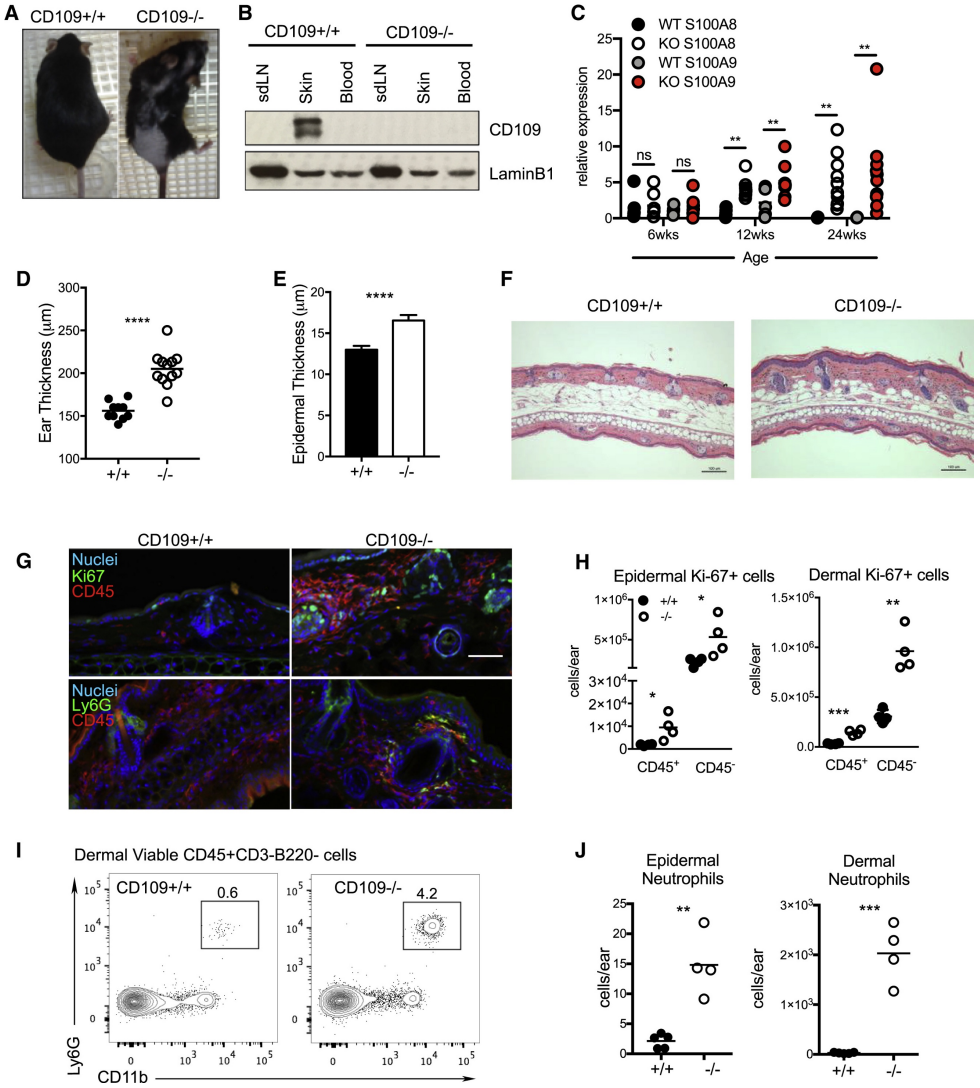

Figure 2.1. Spontaneous skin inflammation in CD109-/- mice.

(A) Phenotype of 12 week old CD109+/+ and CD109-/- mice. (B) Immunoblot of CD109 expression in skin-draining lymph nodes, skin and blood from CD109 and CD109-/- mice. (C) RT-qPCR analysis of *S100a8* and *S100a9* expression in the skin of CD109+/+ and CD109-/- mice at 6, 12 and 24 weeks of age. (D, E) Quantification of ear (D) and epidermal thickness (E). (F) Hematoxylin and eosin staining of ear sections from CD109+/+ and CD109-/- mice. Scale bars: 100µm. (G) Cross-section staining of CD45+Ki-67+ and CD45+Ly6G+ cells from CD109+/+ and CD109-/- ears. Scale bars: 50µm. (H) Total cell counts of epidermal and dermal CD45+Ki-67+ and CD45-Ki-67+ cells in CD109+/+ and CD109-/- mice. (I) Representative contour plots of dermal neutrophils. Numbers indicate the frequency of CD11b+Ly6G+ cells from the viable CD45+CD3-B220- population. (J) Cell counts of CD11b+Ly6G+ neutrophils. Data are representative of at least three independent experiments containing 4 mice per group. Each circle in the graphs represent individual mice. Error bars represent standard deviation. Mann-Whitney test (C, H, J), Student's t-test (D, E), *p<0.05, **p<0.01, ****p<0.0001.

Figure 2.2

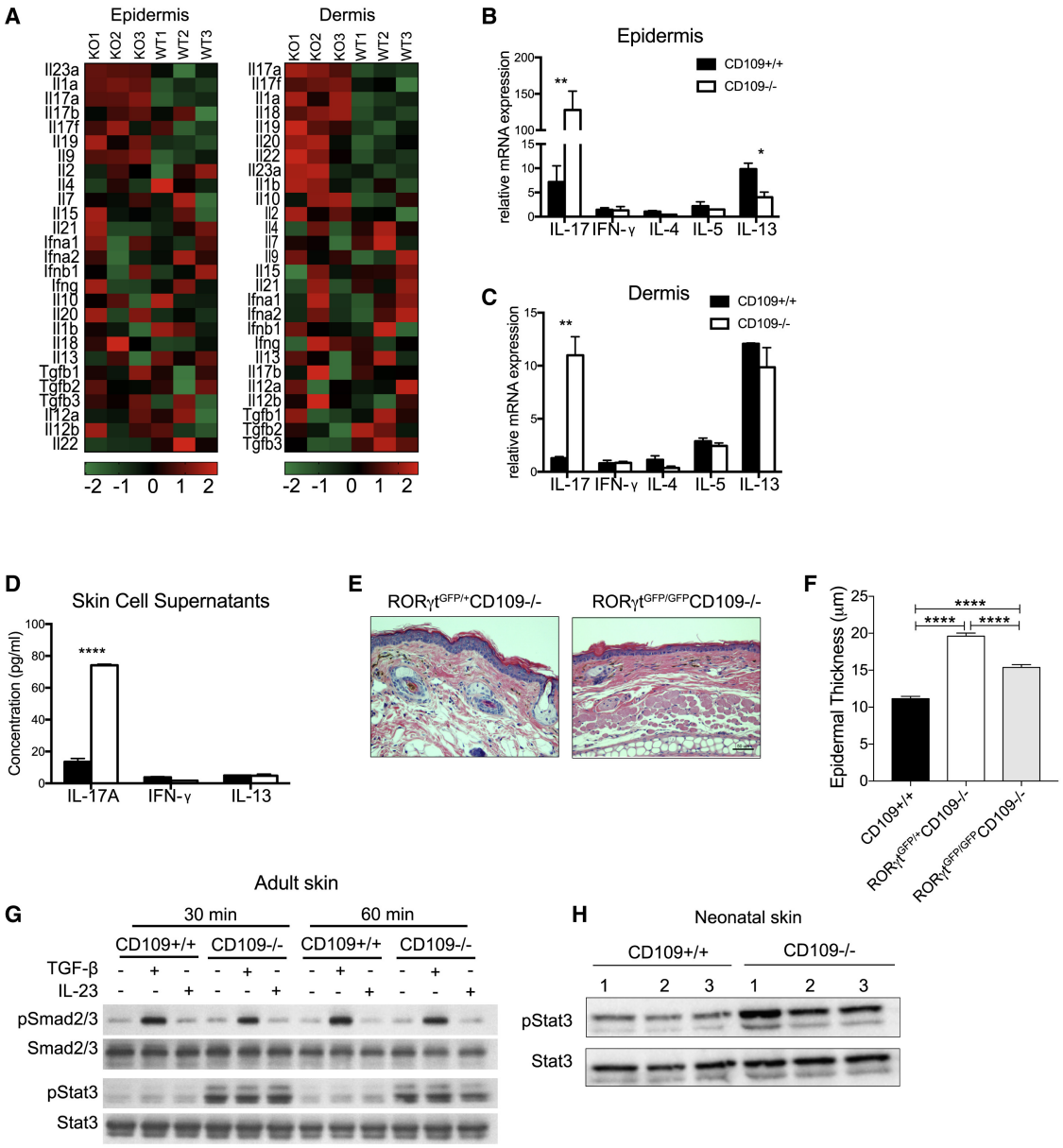

Figure 2.2. Loss of CD109 activates the cutaneous IL-23/IL-17 immune axis.
(A) A heatmap of epidermal and dermal cytokine mRNA comparing CD109+/+ (WT) and CD109-/- (KO) samples, three samples/genotype. (B and C) RT-qPCR analysis for mRNA expression of epidermal and dermal cytokines. (D) ELISA analysis of IL-17A, IFNγ and IL-13 secretion from cultured CD109+/+ and CD109-/- skin cell supernatants. (E) Hematoxylin and eosin staining of ear sections from RORγt+/-CD109-/- and RORγt-/-CD109-/- mice. Scale bars: 50μm. (F) Quantification of epidermal thickness. (G) Immunoblot of phospho-Smad2/3, Smad2/3, phospho-Stat3 and Stat3 in the whole skin lysates from skin cells in the context of TGF-β and IL-23 stimulation. (H) Immunoblot of phospho-Stat3 and Stat3 in the *ex vivo* whole skin lysates from neonatal mice. (B-F) Data are representative of three independent experiments containing 3-4 mice per group. Error bars represent standard deviation. Unpaired Student *t* test, **p*<0.05, ***p*<0.01, *****p*<0.0001.

Figure 2.3

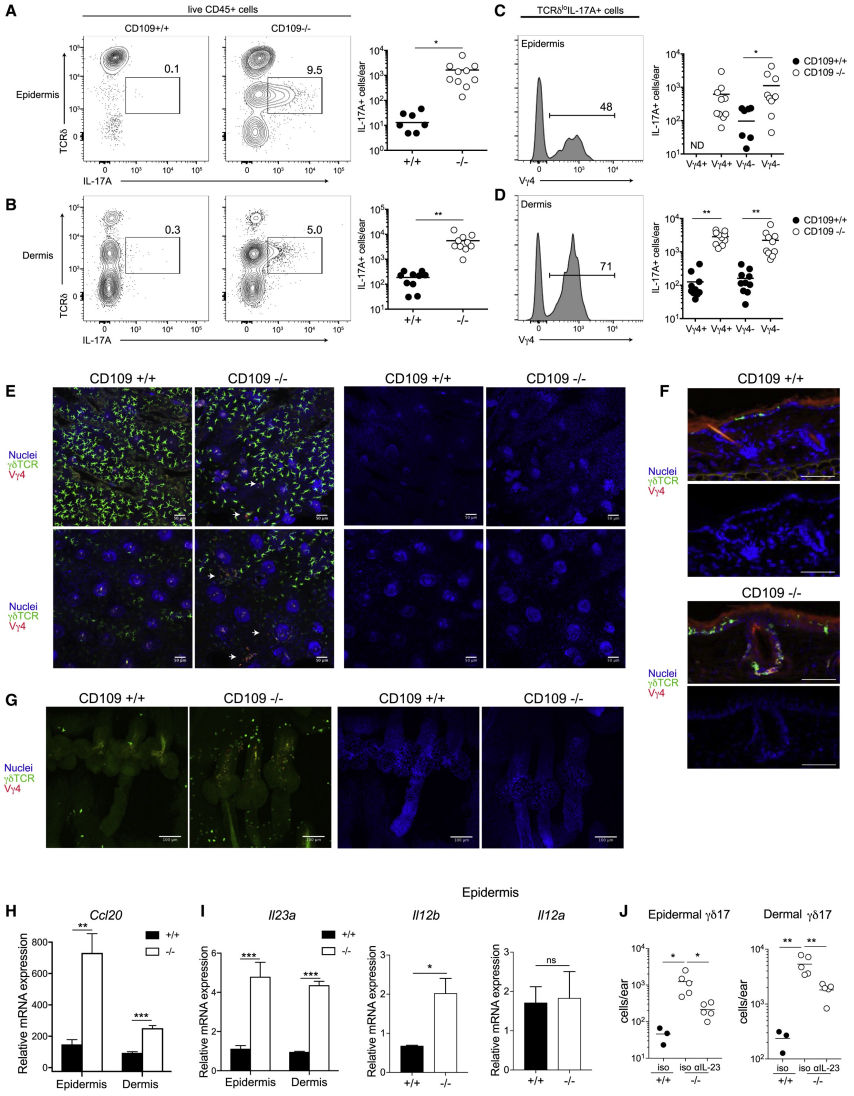

Figure 2.3. γδT cells accumulate in the epidermis and are the dominant source of IL-17A in CD109-/- skin. (A and B) Contour plots of live CD45+ epidermal or dermal cells showing the cellular source and frequency of IL-17A producing cells (left) in CD109+/+ and CD109-/- mice. Total cell counts of epidermal and dermal IL-17-producing cells (right). (C and D) Histograms showing the frequency of Vγ4+ cells within the IL-17A producing TCRδlo cell population from the epidermis and dermis of CD109-/- mice (left). Total cell counts of epidermal and dermal IL-17 producing cells Vγ4+ and Vγ4- γδ T cells (right). nd, not detected. (E, F) Confocal microscopy images of whole-mount (E) and cross-section (F) staining for Vγ4, TCRδ and nucleated cells in CD109+/+ and CD109-/- ears. Arrows in E denote Vγ4+TCRδ+ cells. Scale bar, 50μm. (G) Confocal microscopy images of epidermal whole-mount staining for Vγ4 and TCRδ in CD109+/+ and CD109-/- tail skin. (H) *Ccl20* mRNA expression in epidermal and dermal sheets from ear skin of CD109+/+ and CD109-/- mice. (I) *Il23a*, *Il12a* and *Il12b* mRNA expression in epidermal and dermal sheets from ear skin of CD109+/+ and CD109-/- mice. Error bars indicate standard deviation. (J) Total cell counts of epidermal and dermal γδ17 cells after two weeks of treatment with anti-IL-12/23p40 neutralizing antibody. Each circle represents an individual mouse. (A-D) Pooled data from two independent experiments with 5 mice/group. (E, F) Representative images from at least 5 mice per genotype. (H, I) Representative data from three independent experiments containing 3 mice/group. ns, not significant. (J and K) Representative data from 3 independent experiments with 4-5 mice/group. Unpaired Student *t* test, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 2.4

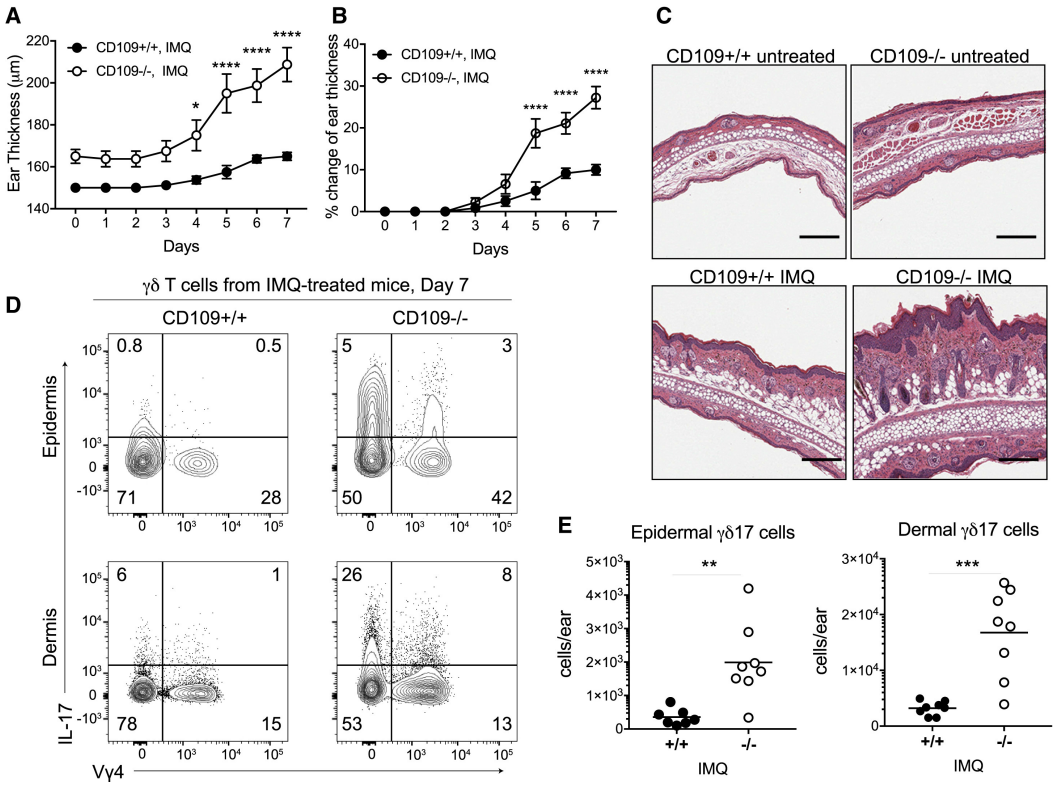

Figure 2.4. Loss of CD109 enhances the susceptibility of psoriasis. (A) Ear thickness measurements over seven days of daily IMQ administration to CD109+/+ and CD109-/- mice. (B) Same data from A shown as a change in ear thickness relative to untreated controls from each genotype. Two-way ANOVA with multiple comparisons, *p<0.05, ****p<0.0001. (C) Representative H&E stained cross-sections from untreated or IMQ-treated ears from CD109+/+ and CD109-/- mice. (D) Representative contour plots of epidermal and dermal γδT cells. Numbers indicate the frequency of corresponding gate from the viable CD45+CD3+B220-TCRβ-TCRδ^{lo} population. (E) Total cell counts of epidermal and dermal γδ17 cells in CD109+/+ and CD109-/- ears after 7 days of IMQ treatment. Each circle represents an individual mouse. Unpaired Student t test, **p<0.01 and ***p<0.001. Data shown is pooled from two independent experiments with 4 mice/group. Scale bar, 200μm.

Figure 2.5

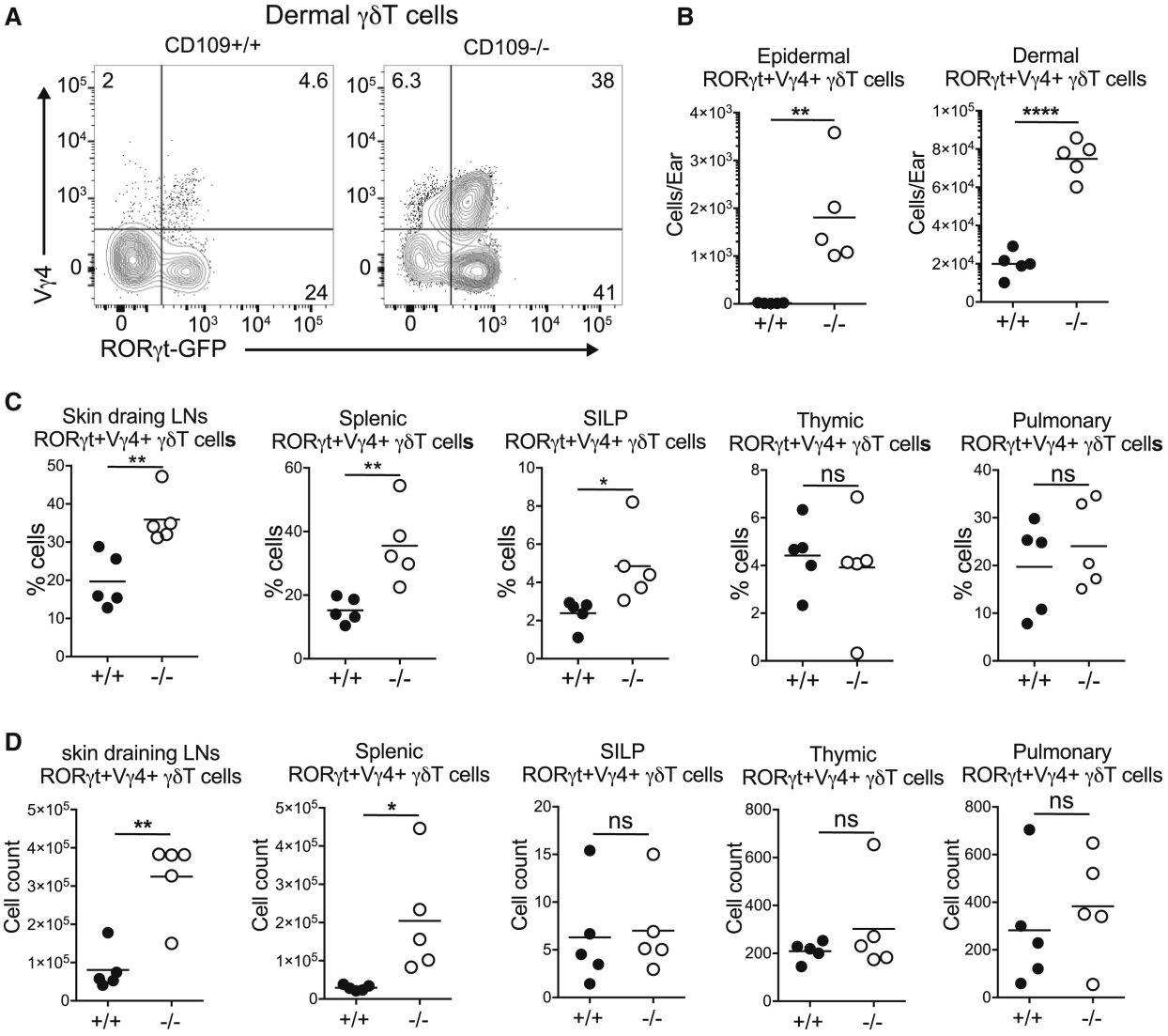

Figure 2.5. The activation of γδ17 cells in the absence of CD109 is tissue-specific. (A) Contour plots showing RORγt-GFP and Vγ4 expression by dermal γδT cells from CD109+/+ and CD109-/- ears. Data shown is gated from the dermal TCRβ-TCRδlo population. Numbers indicate the frequency of cells within each quadrant. (B) Total cell counts of epidermal and dermal RORγt+Vγ4+ γδT cells. (C and D) Percentage (C) and total cell counts (D) of RORγt+Vγ4+ cells in γδT cells of the skin-draining lymph nodes, spleen, small intestine lamina propria, thymus and lung tissue. Each circle represents an individual mouse. (A-D) Data is representative of two independent experiments with 4-5 mice/group. (B-D) Unpaired Student t-test, *p<0.5, **p<0.01, ****p<0.0001. ns, not significant.

Figure 2.6

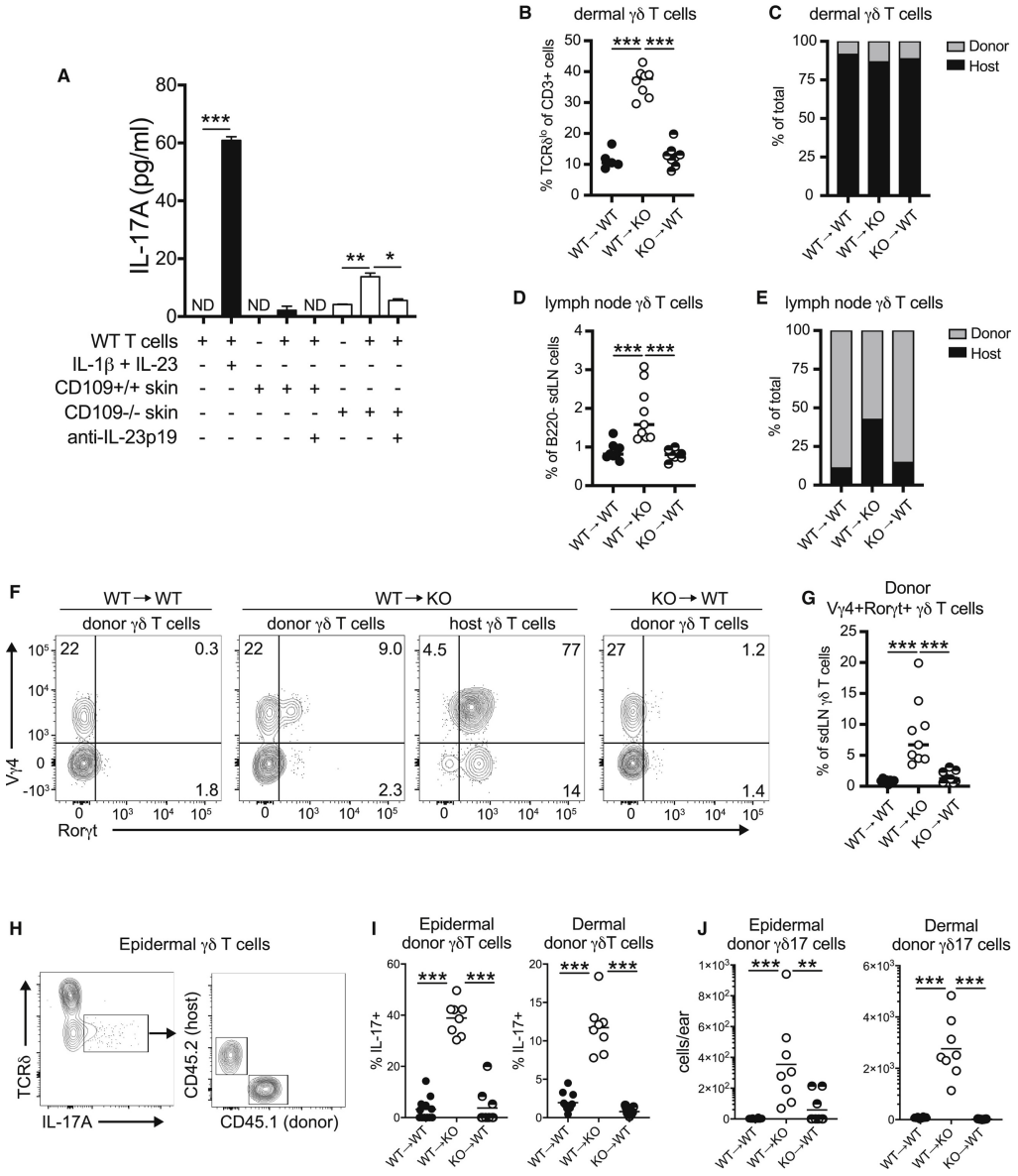

Figure 2.6. The activation of γδ17 cells in the absence of CD109 is cell-extrinsic. (A) Purified T cells from the skin-draining LNs were cultured with skin cells from WT or CD109-/- mice in the presence or absence of anti-IL-23p19 antibodies or isotype control. Stimulation of T cells with rIL-1β and rIL-23 was used as a positive control. Supernatants were collected 24 hours later and IL-17A secretion was detected by ELISA. (B-E) Bone marrow chimeras using either WT or CD109-/- donor and recipients were assessed by flow cytometry for (B) the frequency of total dermal γδ T cells, (C) the proportion of host vs donor dermal γδ T cells as determined by congenic CD45 expression, (D) the frequency of total sdLN γδ T cells and (E) the proportion of host vs donor sdLN γδ T cells as determined by congenic CD45 expression. (F) Representative contour plots of Vγ4 and Rorγt expression by donor or host γδ T cells from sdLN of chimeric mice. (G) The frequency of Vγ4+Rorγt+ sdLN γδ T cells from each group of chimeric mice. (H) Representative contour plots showing total γδ17 cells in reconstituted epidermis (left) containing donor (CD45.1+) and recipient (CD45.2) cells (right). (I and J) Frequency and total cell counts of epidermal and dermal donor γδ17 cells from the indicated groups. Each circle represents an individual mouse. (A) Data is representative of two independent experiments. (B-G) Data is pooled from two independent experiments with 3-5 mice/group. (H-J) Data shown is pooled from two independent experiments with 4-5 mice/group. (B-D) Unpaired Student t-test and (F) two-way ANOVA with multiple comparisons, *p<0.5, **p<0.01, ***p<0.001.

Figure 2.7

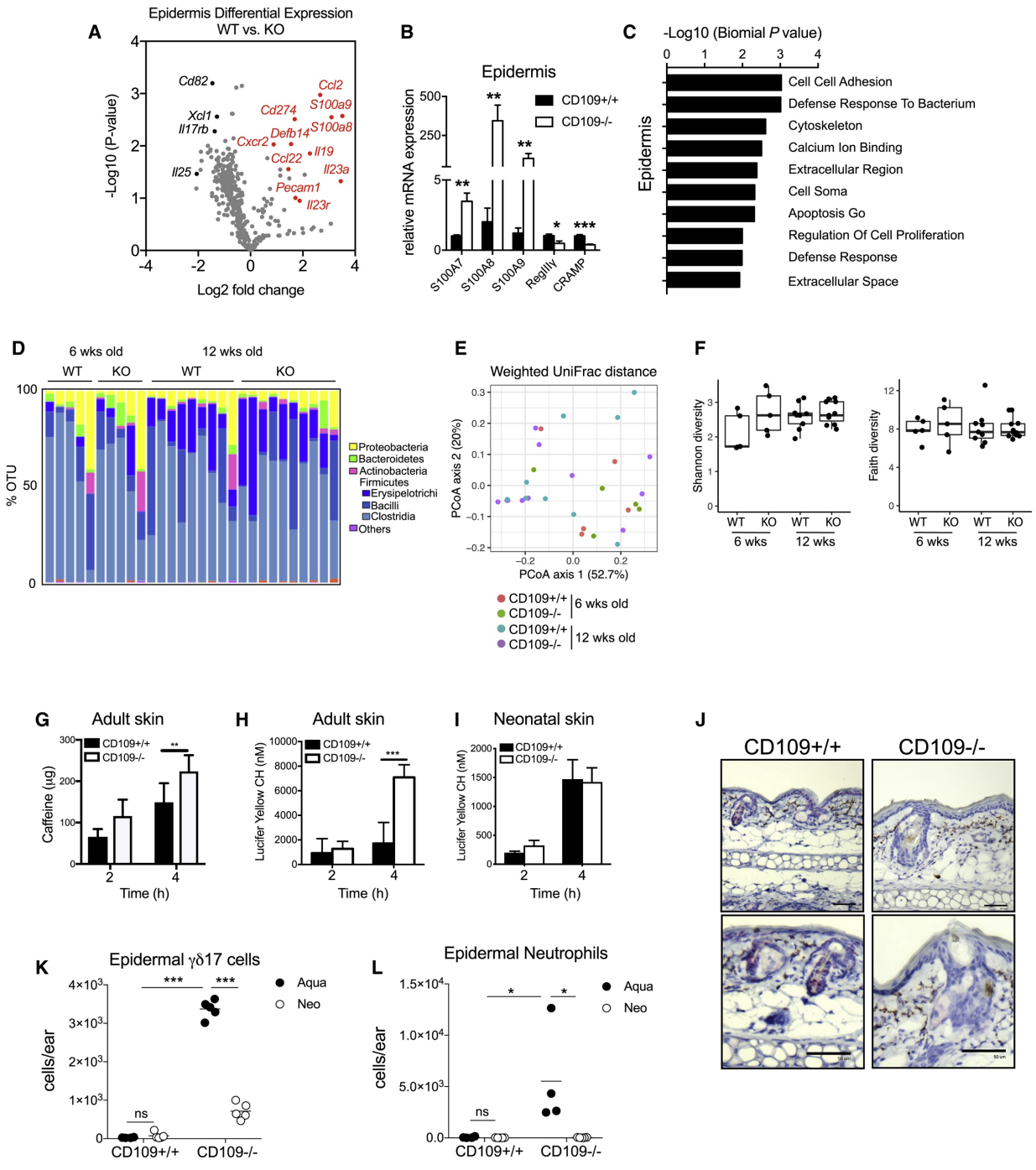

Figure 2.7. CD109 participates in barrier integrity maintenance and *γδ*17 cell activation via commensal microbiota. (A) Volcano plot of gene expression showing the significantly changed genes in epidermis comparing skin from CD109-/- vs CD109+/+. Selected gene names in red denote transcripts associated with anti-microbial function and the IL-23/IL-17 immune axis. (B) RT-qPCR expression of antimicrobial genes in

CD109+/+ and CD109-/- epidermis. (C) Top 10 significantly increased pathways comparing CD109-/- vs CD109+/+ mice based on advanced nCounter software analysis of epidermal tissue gene expression. (D-F) 16S rRNA sequencing results from skin swabs of WT and CD109-/- littermates at 6 and 12 weeks of age. (D) Represents bacterial phyla composition based on operational taxonomic unit (OTU) annotation. (E) Principal component analysis using weighted UniFrac analysis. (F) Box plots showing beta diversity of all groups using Shannon and Faith indices. (G-I) *Ex vivo* percutaneous absorption of caffeine (G) and lucifer yellow (H, I) by adult (G, H) and neonatal (I) skin at 2 and 4 hours post-exposure. (J) RNAScope of CD109 transcripts in CD109+/+ and CD109-/- ear skin. Red dots indicate *Cd109* mRNA. Scale bar, 50 μm. (K, L) Total cell counts of epidermal γδ17 cells and neutrophils following 7 days of Aquaphor or Neosporin treatment. Numbers in red indicate the proportion of mice in which neutrophils were examined, but not detected. Error bars indicate standard deviation. Data shown represents two (B) or three (K, L) independent experiments. Nanostring data (A, C) is from one experiment containing 3 mice/group. Each circle in scatter plots represents an individual mouse. Aqua: Aquaphor; Neo: Neosporin. ND: undetected. Unpaired Student *t* test, $^{**}p<0.01$, $^{****}p<0.001$. Two-way ANOVA with multiple comparisons, $^{*}p<0.05$ and $^{**}p<0.01$.

Figure S2.1

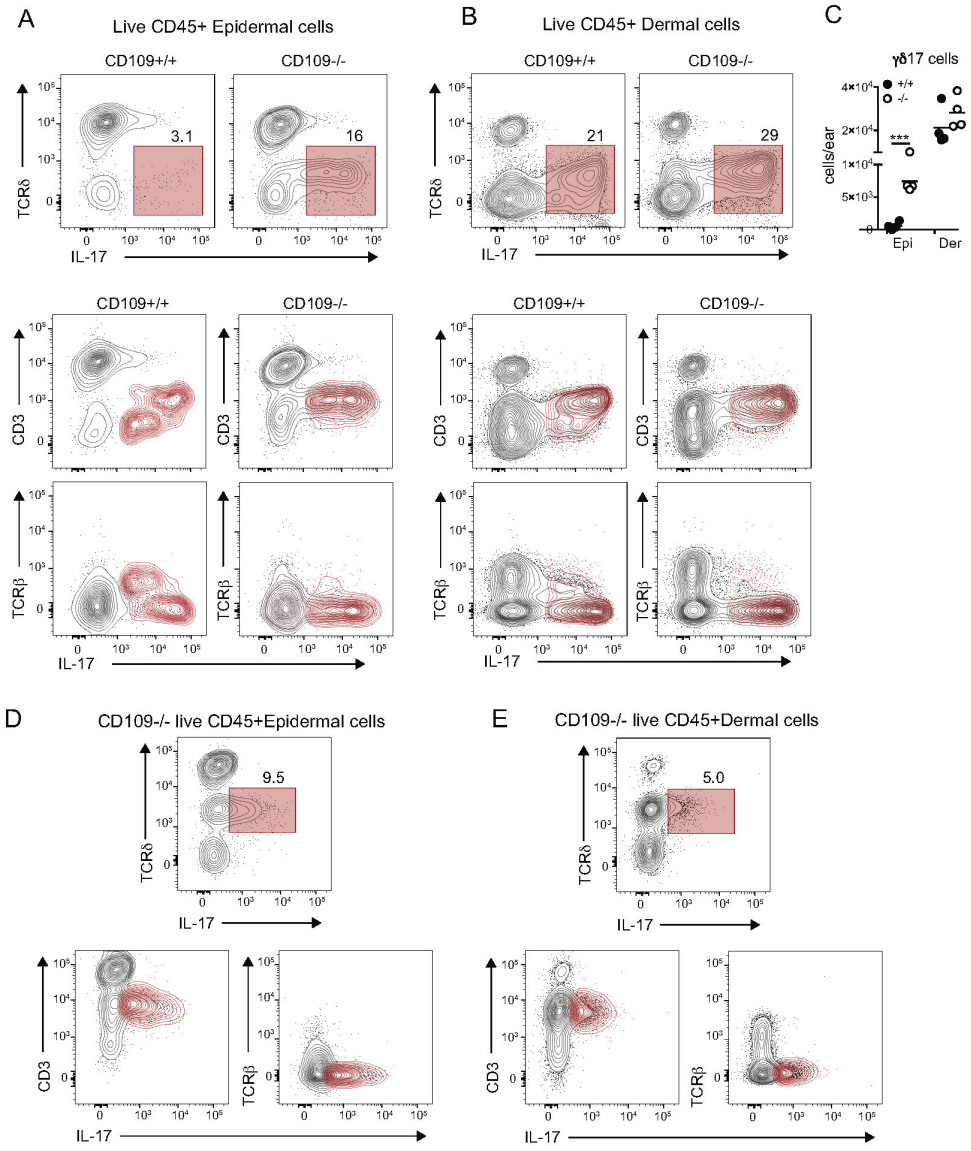

Figure S2.1 related to Figure 2.3. Immunophenotyping and IL-17 production by cutaneous γδT cells. Representative contour plots showing the frequency of (A) epidermal and (B) dermal IL-17 producing cells from the total CD45+ cell compartment following *in vitro* Ionomycin and PMA stimulation for 4 hours. The IL-17+ population gated in the red box is plotted directly below in the context of total CD45+ compartment to determine expression of CD3 and TCRβ. (C) Shows the total number of IL-17 producing γδT cells in each group. Unpaired Student t test, ***$p<0.001$. (D, E) shows further characterization of the population shown in Figure 2.3A and B that is spontaneously producing IL-17 from the epidermis and dermis of CD109-/- mice. Data shown is representative of three independent experiments.

Figure S2.2

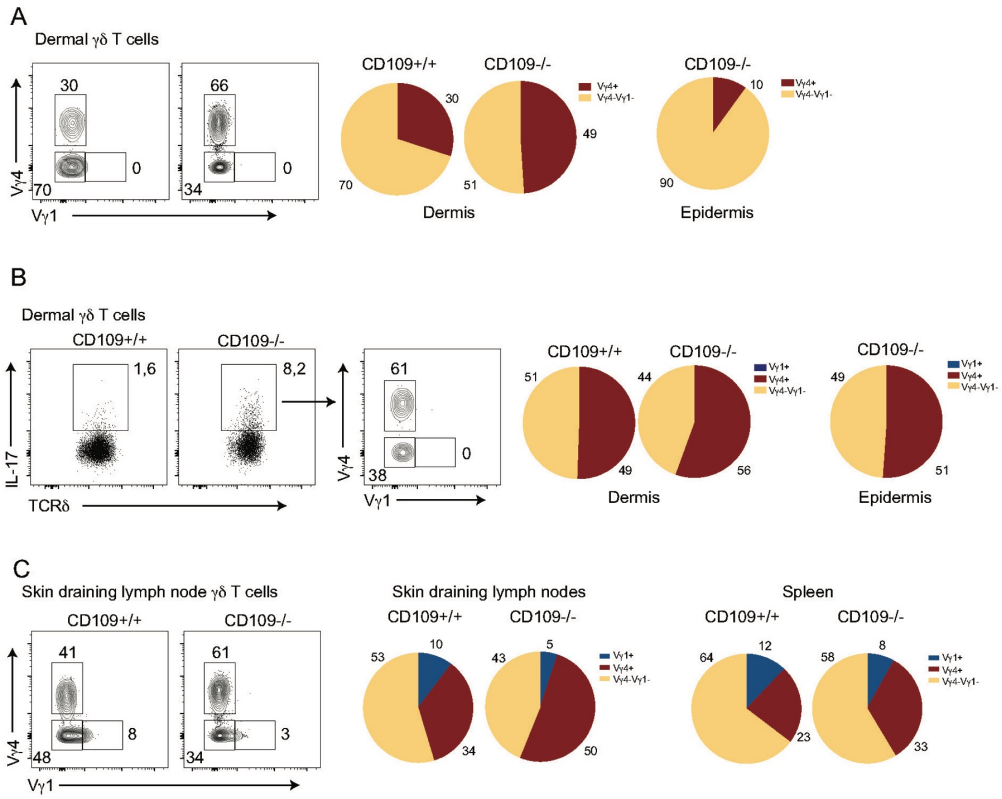

Figure S2.2 related to Figure 2.3. Dermal γδ17 cells from CD109-/- mice are composed of Vγ4+ and Vγ4-Vγ1- subsets. (A, B) Contour plots and pie charts showing the frequency of (A) total and (B) IL-17 producing epidermal and dermal γδ T cells expressing the Vγ4 and Vγ1 TCR subunits. (C) Contour plots and pie charts showing the frequency of skin-draining and splenic γδT cells expressing the Vγ4 and Vγ1 TCR subunits.

Figure S2.3

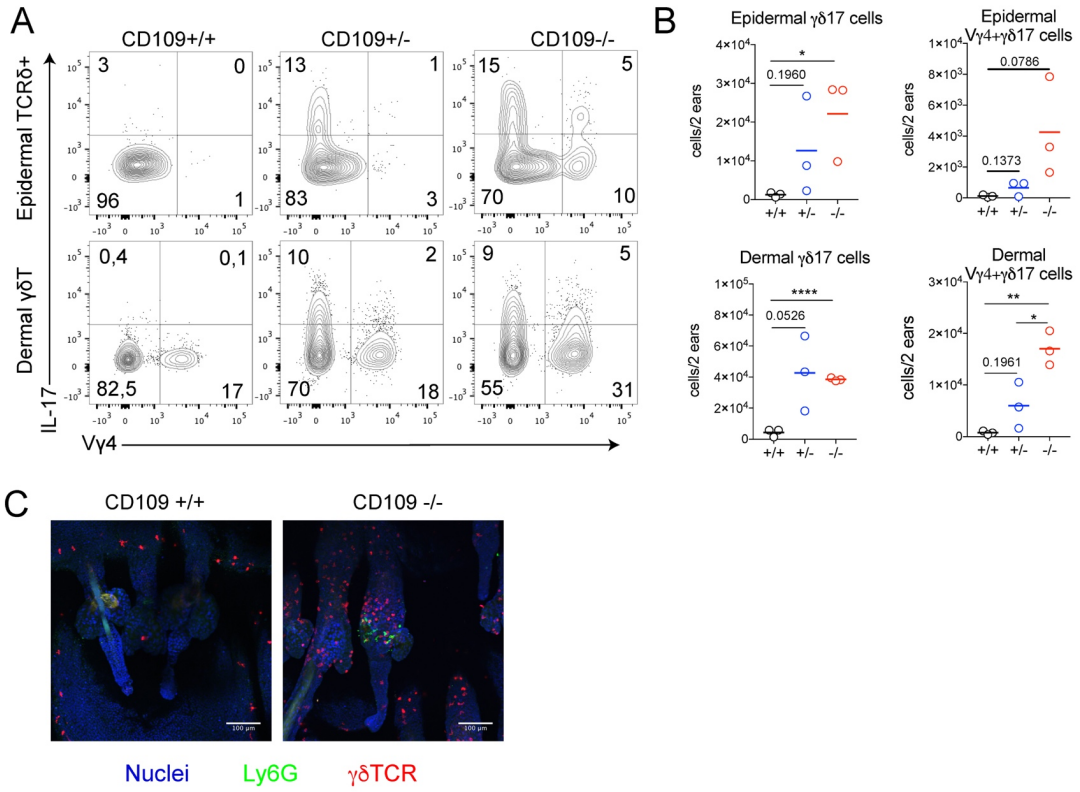

Figure S2.3 related to Figure 2.3. CD109 has a gene dosage effect on the number of cutaneous γδ17 cells. (A and B) Representative contour plots and cell counts of total and Vg4+ epidermal and dermal γδ17 cells from ear skin of CD109+/+, CD109+/- and CD109-/- littermates at 12 weeks of age. (C) Confocal microscopy images of epidermal whole-mount staining TCRδ+ and Ly6G+ cells in CD109+/+ and CD109-/- tail skin. Two-way ANOVA with multiple comparisons, *p<0.05, **p<0. 01 and ****p<0. 0001. Data shown is representative of two independent experiments.

Figure S2.4

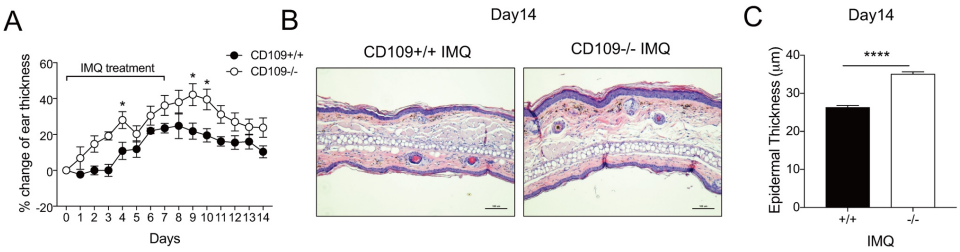

Figure S2.4 related to Fig2.4. CD109-/- mice exhibit delayed resolution following IMQ treatment. (A) Percent of ear thickness change after the IMQ treatment of CD109+/+ and CD109-/- mice. IMQ treatment was stated at day 0 and stopped at day 7. (B) Hematoxylin and eosin staining of ear sections from at day 14. Scale bars: 100µm. (C) Epidermal thickness measurement from the H&E staining. ****$p < 0.0001$. Error bars, SEM. Data shows one experiment with 4 mice/group.

Figure S2.5

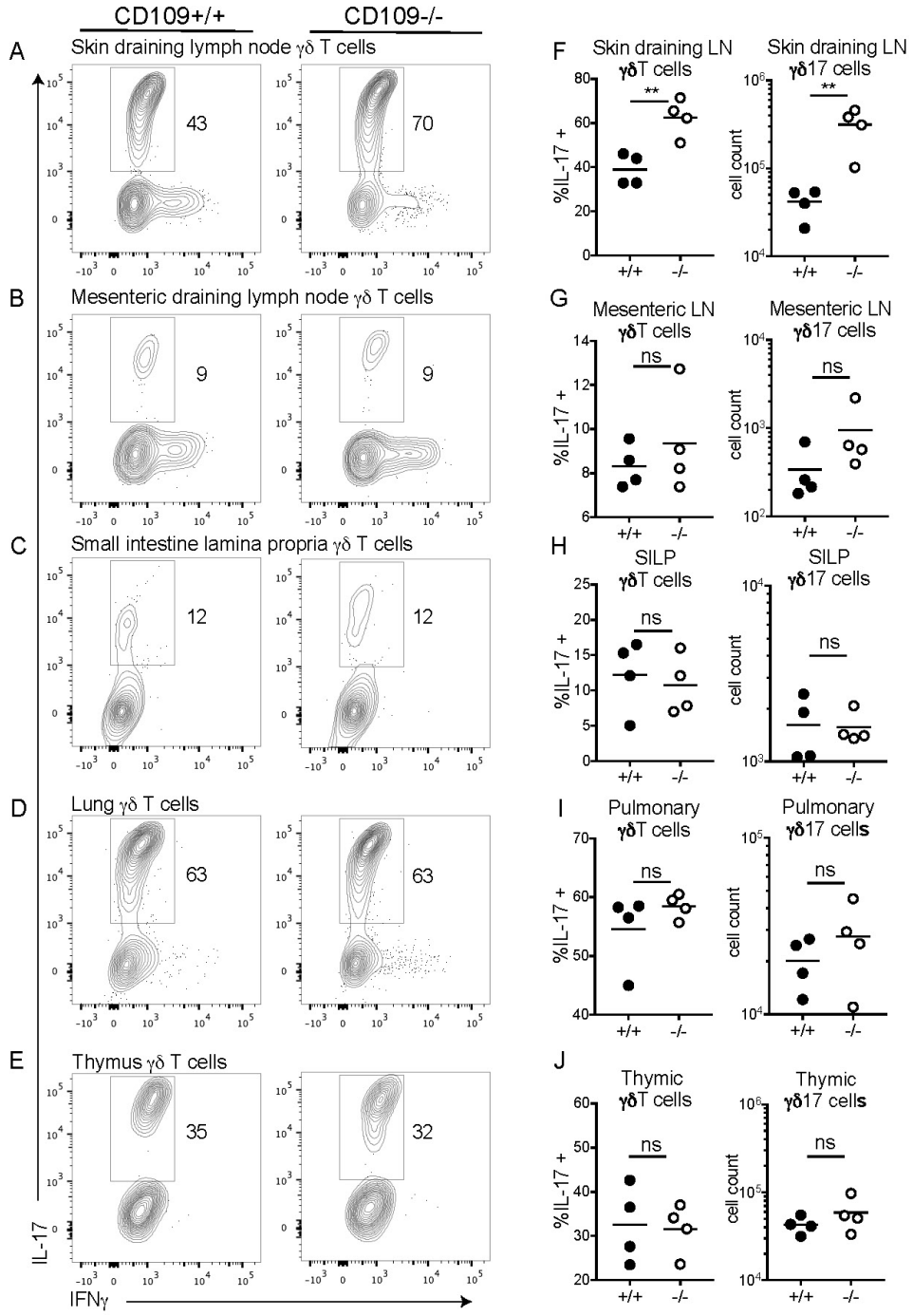

Figure S2.5 related to Figure 2.5. Loss of CD109 enhances IL-17 production by γδT cells in a tissue-specific manner. (A-E) Representative contour plots showing the frequency of IL-17A+IFNγ- γδ T cells in (A) skin draining lymph nodes, (B) mesenteric draining lymph nodes, (C) small intestinal lamina propria, (D) lung and (E) thymus upon in vitro Ionomycin and PMA stimulation for 4 hours. (F-J) The frequency and total cell counts of γδ17 cells from the indicated organs. SILP, small intestinal lamina propria. ns, not significant. Unpaired Student t test, $**p<0.01$. Data shown is representative of two independent experiments with 4 mice/group.

Figure S2.6

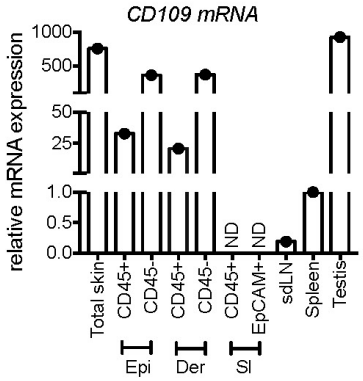

Figure S2.6 related to Figure 2.5. CD109 has a tissue-specific expression profile. RT-qPCR analysis of CD109 mRNA expression from total skin, FACS-sorted CD45+ and CD45- cells from the epidermis and dermis of CD109+/+ ear skin, FACS-sorted CD45+ and EpCAM+ cells from the small intestine epithelium, total skin draining lymph node cells, splenocytes and testis. Data shown is representative of two independent experiments with at least two mice/group. Epi, epidermis; Der, dermis; SI, small intestine. ND, not detectable.

Figure S2.7

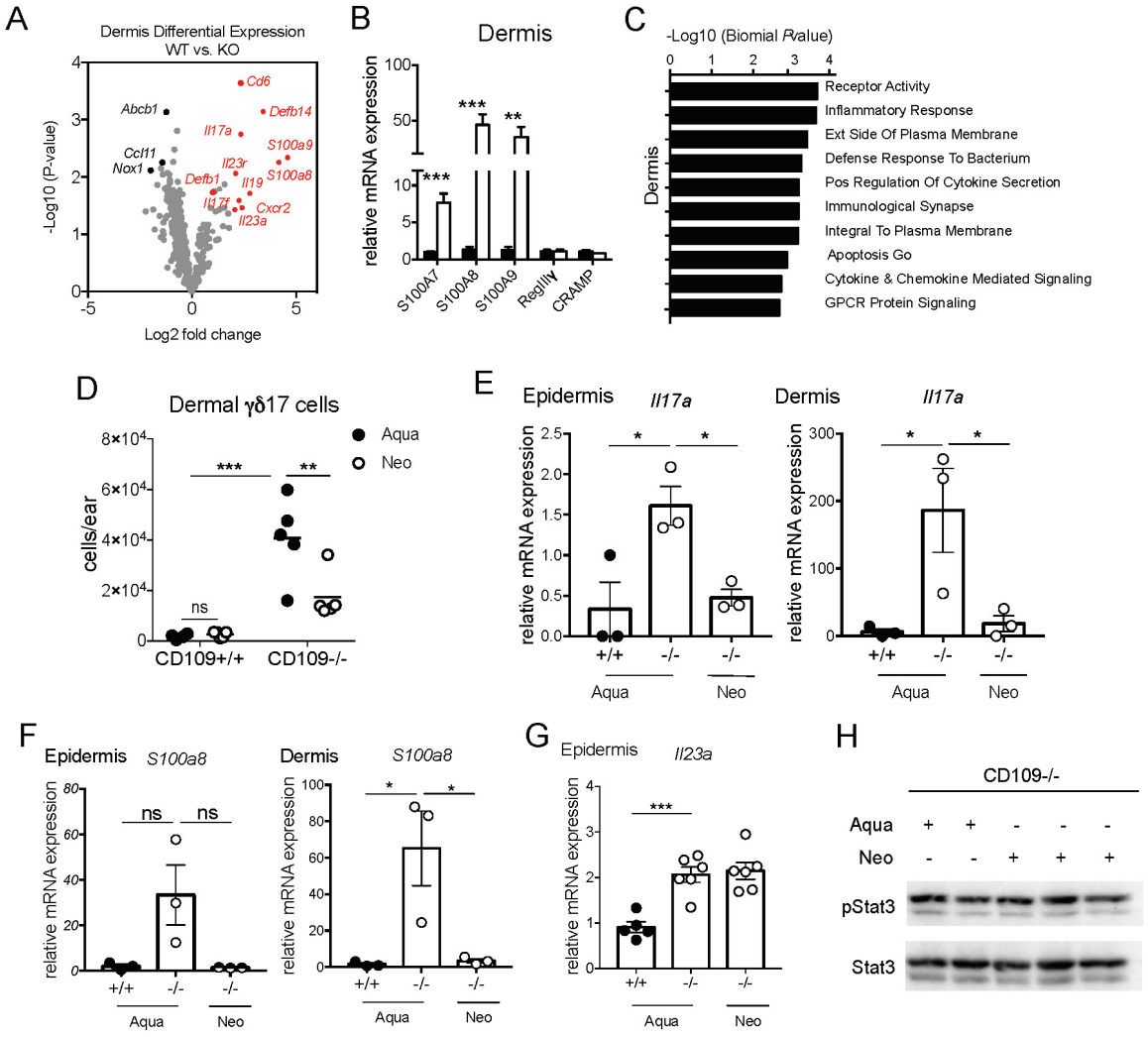

Figure S2.7 related to Figure 2.7. Topical antibiotic treatment alters dermal γδ17 cell accumulation and decreases epidermal neutrophil recruitment. (A) Volcano plot of gene expression showing the significantly changed genes in dermis comparing skin from CD109-/- *vs* CD109+/+. Selected gene names in red denote transcripts associated with anti-microbial function and the IL-23/IL-17 immune axis. (B) Top 10 significantly increased pathways comparing CD109-/- *vs* CD109+/+ mice based on advanced nCounter software analysis of dermal tissue gene expression. (C) RT-qPCR expression of antimicrobial genes in CD109+/+ and CD109-/- dermis. (D) Total cell counts of dermal γδ17 cells following one week of topical antibiotic or vehicle treatment of ears from CD109+/+ and CD109-/- mice. Aqua: Aquaphor; Neo: Neosporin. (E-F) RT-qPCR analysis of epidermal and dermal (E) IL-17, (F) S100A8 and (G) IL-23p19 mRNA expression following one week of topical antibiotic or vehicle treatment of ears. (H) Immunoblot of phospho-STAT3 and total STAT3 from whole ear skin lysates of CD109-/- mice 7 days of Aquaphor or Neosporin treatment. Data shown is representative of one (A, C, H) or two (B, D-G) two experiments. Two-way ANOVA with multiple comparisons, *p<0. 05, **p<0. 01, ***p<0. 001. ns, not significant.

Preface to Chapter 3

"Human geneticists were choosing their disease and laboriously slogging their way through the genome to find the genetic mutation that causes the disease—once found, it was often unclear how the protein contributed to the disease. Instead, I wanted to start with my protein. What was it doing in cells? To me that was the fun part."

Dr. Elaine Fuchs

In Chapter 2 of this thesis, we identified CD109 as an T-cell extrinsic regulator of IL-23/γδ17 immune axis in the skin via the commensal microbiome. Moreover, loss of CD109 will lead to disturbed skin barrier integrity. However, the events that mediate the activation of cutaneous IL-23/IL-17 axis are still unknown. Keratinocytes are either potent producers of IL-23 nor IL-17, so, what CD109 is doing in keratinocyte? By looking back on our Nanostring analysis data, we found that the expression level of IL-1α is significantly increased in the absence of CD109. IL-1α is abundantly expressed in keratinocyte at steady state conditions [11]. It has been shown to be crucial for both skin-resident commensals and *S.aureus* -derived IL-17 production of dermal T cells [193, 309]. Furthermore, the calpains, which are the cysteine proteases for IL-1α, together with CD109, have been reported to be components of a special organelle of keratinocyte called lamellar bodies. Based on those hints, is it possible that CD109 regulates the IL-23/IL-17 immune axis through the calpain/IL-1α machinery? The goal of Chapter 3 is to identify the role of CD109 in keratinocyte, thus to broaden our understanding of basic function of keratinocytes.

Chapter 3. The role of CD109 in regulating the cutaneous IL-1α/IL-17 Immune Axis

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Unpublished.

3.1 Summary

In Chapter 2, I described our studies demonstrating that CD109 is a negative regulator of the cutaneous IL-23/IL-17 immune axis. Furthermore, our data indicated that CD109 is predominantly, if not exclusively, expressed on keratinocytes (KCs) to control IL-17 producing γδT cell reactivity to the commensal microbiota in a cell-extrinsic manner. However, the specific mechanism by which CD109 impacts KC-directed immune responses remains unclear. Here we report that deletion of CD109 results in aberrant production of IL-1α in KCs. In addition, KC-derived IL-1α is necessary to drive γδ17 cell activation *in vitro*. Consistently, *in vivo* blockade of IL-1α reduced γδ17 cytokine production and accumulation in the epidermis in CD109-/-, but not littermate control animals. Using immunoprecipitation and mass spectrometry technology, we demonstrated that CD109 binds to the molecule Calpain, which controls the cleavage and secretion of IL-1α. Calpain activity assay showed that loss of CD109 in the skin leads to an enhanced Calpain activity *in vitro*. Collectively, our data suggest that CD109 expression in KCs may regulate calpain-mediated activation of IL-1a to limit pathogenic cutaneous γδ17 T cell activation and maintain homeostasis of this important barrier site.

Key words: Psoriasis, keratinocyte, IL-1α, IL-17-producing γδT cell.

3.2 Introduction

The skin is the initial interface between the body and environment to provide defense against environmental stress and infectious agents [1]. As the largest organ, it contains several skin-resident immune cells, which act as sentinels to maintain tissue homeostasis. However, under pathological conditions, those cells also drive uncontrolled skin inflammation, as observed in psoriasis [2]. Psoriasis is a chronic inflammatory disease of the skin, characterized by epithelial cell hyper-proliferation (hyperplasia), immune cell infiltration, aberrant dermal angiogenesis and enhanced production of anti-microbial peptides (AMPs) [199, 200].

Animal models of psoriatic-like inflammation have highlighted the IL-23/IL-17 immune axis in the development of the disease. The production of IL-23 by skin-resident myeloid cells has been identified to drive the production of IL-17 family members by T cells that are positive for the transcriptional factor RORγt. IL-17 production, in turn, enhances keratinocyte (KC) proliferation and chemokine production, leading to the recruitment of cytotoxic neutrophils, that induces tissue thickening, enhances cell death, and causes scaling of the inflamed skin [217]. It has also been shown that IL-17 plasma levels in psoriatic patients correlate with disease severity, and administration of neutralizing antibodies against IL-17 leads to resolution of disease [158, 310]. Apart from Th17 cells, the innate lymphoid cells and γδT cells also produce IL-17 during infection and chronic inflammation such as psoriasis. Either intradermal injection of IL-23 or the topical administration of IMQ, a TLR7/8 agonist, successfully induce features of psoriasiform inflammation in mice and lack of IL-17-producing γδT cells (γδ17 cells) results in decreased inflammation in both models [70, 126].

IL-1 signaling has also been shown to promote psoriasis by the induction of IL-17. It has been reported that IL-1 and IL-23 could induce innate IL-17 production from γδT cells, amplifying Th17 responses and autoimmunity[118]. Blocking IL-1R3, a co-receptor for IL-1 family members, significantly attenuated the severity of psoriasis [220]. There are two IL-1 family members, IL-1α and IL-1β. Compared to IL-1β, which is not constitutively expressed and produced only by myeloid cells, the IL-1α precursor is present in mesenchymal cells of healthy humans and mice, including keratinocytes. There is no requirement of caspase-1 cleavage of the IL-1α precursor to process and release the active cytokine, but the matured IL-1α has greater biological activity [143]. To note, various enzymes have been reported to cleave pro-IL-1α, such as calcium-activated membrane calpains, thrombin and granzyme B [11, 144, 145]. IL-1α has dual functions,

extracellularly and intracellularly. Extracellularly, like IL-1β, it binds to the IL-1R1 on the cell surface, which recruit the co-receptor IL-1R3 and initiates the pro-inflammatory signal. Intracellularly, the precursor of IL-1α functions as a transcriptional factor in the nucleus [142]. During apoptosis, IL-1α leaves the cytosol and binds tightly to chromatin. In apoptotic cells, IL-1α bind to chromatin, but not its cell surface receptor, causing no inflammation by the cell contents. In contrast, in necrotic cells, IL-1α leaves the nucleus and could be found in the cytosol. The IL-1α precursor could be released with necrotic cell contents, where it binds to IL-1R1 on adjacent live cells [146]. Thus, IL-1α works as an "alarmin" during inflammation.

CD109 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein highly expressed in healthy skin and malignant tumor cells such as lung adenocarcinoma [261, 263, 264]. It has been shown to be decreased in lesioned skin of psoriatic patients [287]. Mice lacking CD109 exhibit a delay in hair development, persistent epidermal hyperplasia, and high bone turnover [263, 264]. We previously reported that CD109 as a selective regulator of the skin IL-23/IL-17 immune axis and γδ17 cells. A deficiency of CD109 in mice resulted in spontaneous inflammation of the skin, including epidermal thickening, hyper-proliferation of keratinocytes, neutrophil accumulation and aberrant IL-23-dependent activation of dermal γδ17 cells. Consistent with a dysregulated IL-23/IL-17 pathway, the absence of CD109 led to enhanced psoriasiform inflammation following imiquimod application. We also demonstrated that CD109 acts in a skin-specific and cell-extrinsic manner to limit IL-23-dependent γδ17 activation by the commensal microbiota [311]. However, in the skin, CD109 is dominantly expressed in the epidermis, especially keratinocytes. What are the mechanisms that cutaneous immune cells activated by CD109-deficient keratinocytes are unknown. Here we show that CD109 binds to proteolytic protease Calpain, that can cleave pro-IL-1α. Loss of CD109 in the skin leads to enhanced Calpain activity and IL-1α production. We further found that, like IL-1β, IL-1α with IL-23 could also induce IL-17 production of the γδT cells. Compared with conditioned media (CM) from wildtype keratinocytes, CM from CD109-deficient keratinocytes significantly enhanced IL-17 production of γδT cells. Although the genetic deletion of the IL-1R1, the receptor required for both IL-1α and IL-1β signaling, did not decrease cutaneous γδ17 cells in CD109-deficient mice, *in vivo* anti-IL-1α antibody blocking reduced IL-17 production by cutaneous γδT cells. These findings suggest that CD109 controls γδ17 cell activation by regulating a calpain- IL-1α axis in keratinocytes.

3.3 Results

Loss of CD109 leads to increased IL-1α levels in the skin.

Our previous study showed that CD109 negatively regulates the cutaneous IL-23/IL-17 immune axis. However, CD109 is rarely detectable in the blood and skin-draining lymph node, and the dominant source of CD109 in the skin is keratinocytes, indicating that CD109 regulates the IL-23/IL-17 in an immune cell-extrinsic way [311]. By using Nanostring® technology, we noticed that IL-1α, rather than IL-1β transcripts were significantly increased in both CD109-/- epidermis and dermis compared with wild-type skin (Figure 3.1A and B). Consistent with these data, we detected elevated IL-1α mRNA levels in the CD109-/- epidermis via qPCR analysis (Figure 3.1C). To determine if IL-1 is increased at the protein level in CD109-/- skin, we isolated the live skin cells and cultured for 24 hours. IL-1β was not detected in the culture supernatants at the basal level, which is consistent with previous reports indicating that IL-1β is not constitutively expressed in the skin [312]. IL-1α was also undetectable at steady state in the wild-type skin cell culture supernatants, while it was detectable in the CD109-/- skin cell culture supernatants. After LPS stimulation, both IL-1α and IL-1β production increased, however, only the IL-1α in CD109-/- skin culture showed a significant increase than the wild-type (Figure 3.1D). Since IL-1α has been reported to be mainly detected in the keratinocytes of the skin [11], we isolated keratinocytes from the skin of adult mouse tails and measured IL-1α transcription and secretion. We confirmed the CD109 expression at mRNA level by wild-type keratinocyte, and CD109-/- keratinocytes showed a complete absence of *Cd109* expression (Figure 3.1E). Furthermore, there was no difference of *Keratin5*, *Keratin10* and *Involucrin* expression, indicating the differentiation of CD109+/+ and CD109-/- keratinocytes is similar (Figure 3.1E). Although IL-23 has been reported to be expressed in the keratinocytes[308], we were unable to detect transcription for this cytokine (data not shown). Next, we detected the mRNA levels of IL-1α and IL-1β in the keratinocytes. Compare with wild-type keratinocytes, CD109-/- keratinocytes showed significant increased expression of both IL-1α and IL-1β (Figure 3.1F). Consistent with the RT-qPCR results, both IL-1α and IL-1β were elevated in the culture supernatants of CD109-/- keratinocytes compare with the wild-type, however, IL-1α is about 10-fold higher at the basal level than IL-1β (Figure 3.1G). We also observed IL-1α expression in primary keratinocytes by confocal imaging (Figure 3.1H). Interestingly, the keratinocytes from CD109-/- skin looked smaller when compared with the wild-type keratinocytes, suggesting that CD109-deficent keratinocytes have defaults on spreading

compared with wild-type keratinocytes (Figure 3.1H). Altogether, these results indicate that CD109 regulates the expression and release of IL-1α from keratinocytes.

CD109-deficient keratinocytes undergo enhanced cell death *in vitro*.

IL-1α is constitutively expressed as a 31kDa precursor by keratinocytes [11]. Unlike IL-1β, it exhibits a basal amount of activity in its immature unprocessed form. It can be released from necrotic cells as pro-IL-1α, or intracellularly processed and released in a cleaved, mature form [313]. To test if elevated IL-1α is due to more cell death in the CD109-/- keratinocytes, supernatants of cultured keratinocytes were collected. Indeed, the O.D. values of LDH assay from CD109-/- keratinocytes was significantly higher than that of the CD109+/+ (Figure 3.2A). Consistent with figure 3.1G, CD109-/- cells had increased concentrations of IL-1α when compared with CD109+/+ group (Figure 3.2B). Interestingly, IL-1α secretion positively correlated with LDH release (Figure 3.2C), indicating that increased IL-1α from CD109-/- keratinocytes might be released from necrotic cells.

Keratinocyte-derived IL-1α, together with IL-23, can induce IL-17 production by γδT cells *in vitro*.

IL-1α shares similarities with IL-1β, and *S.aureus* infection can induce keratinocyte derived IL-1α production to facilitate the IL-17 response [11, 309]. Since neither IL-1β nor IL-23 alone but IL-1β combined with IL-23 could induce a robust IL-17 production of γδT cells [118], we hypothesized that, when combined with IL-23, IL-1α could function like IL-1β, to help the IL-17 production of γδT cells. As expected, like IL-1β [118], IL-1α alone is not able to induce a robust IL-17 production of γδT cells, and IL-1α combined with IL-23 can successfully induce the IL-17 production of γδT cells to the similar level of IL-1β with IL-23 (Figure 3.3A). Using the same setting as Figure 3.3A, we also observed similar results of IL-17 production of skin-draining lymph node cells by ELISA (Figure 3.3B). Interestingly, skin-draining lymph node cells from CD109-/- mice other than CD109+/+ mice seemed to have higher IL-17 production after 24-hour stimulation, indicating that those γδT cells are more potentiated to produce IL-17 (Figure 3.3C).

Next, we wanted to know if IL-1α from the culture supernatants of primary keratinocytes could induce IL-17 production. To address this, we used supernatants from day 3-cultured keratinocytes, combined with T cell media to prepare conditioned media, and stimulated skin-draining lymph

node cells with IL-23 for 4 hours. Compared with the wild-type, supernatants from CD109-/- KCs induced a significant increase of IL-17+γδT cells (Figure 3.3D). Additionally, this induction is dose-dependent (Figure 3.3E). Notably, conditioned media alone was able to induce IL-17 production of γδT cells in taken from CD109-/-, but not WT, KCs (Figure 3.3E). To confirm that IL-1α in the conditioned media was responsible for the increased IL-17 production, we blocked IL-1α. IL-1β neutralization was used as a control since IL-1α and IL-1β share the same receptor [314]. Compared with isotype control treated cells, IL-1α blockade successfully decreased the IL-17 production of γδT cells stimulated by the CD109-/- CM plus IL-23, while anti-IL-1β blocking did not (Figure 3.3F), a result consistent with IL-17 produced from skin-draining lymph node cells (Figure 3.3G). Altogether, keratinocyte-derived IL-1α has similar potential as IL-1β to activate the γδ17 cells and is required for aberrant IL-17 production *in vitro*.

Blockade of IL-1α *in vivo* decreases IL-17 production by cutaneous γδT cells.

To address whether acute blockade of IL-1α could decrease the IL-17 production by γδT cells *in vivo*, we administered anti-IL-1α blocking antibodies or isotype controls to either wild-type or CD109-deficient mice for two weeks. Compared with the isotype control treated group, there was a significant reduction in the frequency and numbers of epidermal γδ17 cells in CD109-/- mice that received the anti-IL-1α compared to WT treated mice, whereas the frequency of dermal γδ17 cells was unchanged (Figure 3.4A and B). However, neither frequency and numbers of RORγt+ γδT cells in the sdLNs were different between treated and untreated groups (Figure 3.4C). Consistent with these results, the frequency and numbers of epidermal neutrophils from CD109-/- mice significantly decreased after the IL-1α blockade (Figure 3.4D).

IL-1α and IL-1β both bind to the IL-1R1 to trigger intracellular signaling in T cells [315]. To investigate whether IL-1 signaling drives skin inflammation in the CD109-deficient mice, we crossed the IL-1R1-null mice with CD109-deficient mice. Consistent with what we have observed in figure 3.2, and in comparison with WT cells, keratinocytes deficient in CD109 had increased levels of both IL-1α and LDH, including the IL-1R1-/-CD109-/- keratinocytes (Figure 3.5A). To our surprise, loss of IL-1 signaling did not reduce the number of epidermal or dermal γδ17 cells compared to CD109-/- mice (Figure 3.5B). Rather, the frequency of epidermal and dermal IL-17+γδT cells from IL-1R1-/-CD109-/- mice was trending higher than that of IL-1R1+/+CD109-/- (CD109-/- in figures) mice (Figure 3.5 C and D). Consistent with the γδ17 cell numbers, neutrophil

numbers were also significantly increased in both epidermis and dermis of CD109-/- and IL-1R1-/-CD109-/- compared to WT mice (Figure 3.5F). Even though the frequency of RORγt+γδT cells from the skin-draining lymph node of CD109-/- mice increased when compared with the wild-type mice, there was no difference between the CD109-/- and IL-1R1-/- mice (Figure 3.5E). Collectively, deletion of IL-1R1 did not reverse the activation of γδ17 cells in the CD109-/- skin. All these data indicate that a short-term blocking of IL-1α could partially reverse the cutaneous inflammation in the CD109-/- mice, however, genetically knock-out of IL-1R1 could not.

CD109 binds to cysteine protease Calpains to regulate their activity.

Since pro-IL-1α can be cleaved by various proteases, including Calpains, Granzyme B and thrombin [11, 144, 145], we wanted to know if CD109 directly regulates this activity or not. To this end, we conducted the tandem mass spectrometry (LC-MS/MS) analysis by CD109 immunoprecipitated fractions from WT mice. Sixteen peptide sequences were shown to be selectively elevated from WT fractions including Calpain 1 and Calpain 2 (Figure 3.6 A and B). Interestingly, it has been previously reported that CD109 and Calpains are enriched in the epidermal organelle lamellar bodies [17], indicating that the possible binding of CD109 and Calpains. We used the co-immunoprecipitation to confirm that CD109 can binds to Calpain 2 (Figure 3.6C). We also detected Calpain 2 protein by immunoblot, showing that wild-type and CD109-/- skin have similar Calpain 2 expression (Figure 3.6C). Since protein expression levels of Calpain 2 are similar in CD109+/+ and CD109-/- skin, we tested Calpain activity in skin-draining lymph node cells and skin cells. Compare with wild-type skin-draining lymph node cells, CD109-/- cells showed similar levels of Calpain activity. By contrast, skin cells from CD109-/- mice had significant increased Calpain activity than than WT controls (Figure 3.6D). All these data above suggest that CD109 binds to Calpains to regulate their activity.

3.4 Discussion

Inflammation occurs after the disruption of tissue homeostasis including cell stress, injury or infection. IL-1α and IL-1β are potent components of the inflammatory process and dysregulation of either cytokine cause devastating disease. Although hematopoietic cell-derived IL-1β has drawn much attention in understanding its biological functions, the distinct role of IL-1α in tissue inflammation remains poorly understood. IL-1α is constitutively expressed in many tissues in steady state conditions, such as skin, and its expression can be elevated in response to pro-inflammatory or stress-associated stimuli [316]. Consistent with previously reported that IL-1α is abundantly expressed in the epidermis, in our study, we could successfully detect IL-1α from either epidermis or primary keratinocytes from the wild-type skin. Furthermore, we identified that loss of CD109 leads to up-regulated IL-1α in both epidermis and primary keratinocytes (Figure 3.1), indicating the potential role of CD109 in regulating IL-1α in the keratinocytes. In addition, IL-1α expression could be rapidly induced by a variety of physiological components, such as oxidative stress [317], lipid overload [318], exposure to cytokines (including IL-1α itself), and microbial originated TLR agonists [319]. Since CD109-deficient mice has been reported to have appendage abnormalities, and commensals could contribute to the skin inflammation of the CD109-/- skin [263, 311], the mechanisms of CD109 in regulating IL-1α expression should be further explored.

Pro-IL-1α contains the functional nucleus localization signal (NLS) [320], and it is localized to the nucleus upon LPS stimulation in macrophage. However, in keratinocytes, pro-IL-1α is localized to the nucleus in steady state conditions without any cell stimulation [321]. It can be released from the damaged cells, for example, necrotic cells, and exhibits a basal amount of activity [322]. In addition, pro-IL-1α can also be processed intracellularly, and both full-length and processed IL-1α can bind the IL-1R, being biologically active [145]. We have identified that increased IL-1α from CD109-/- primary keratinocytes strongly correlated with cell death, indicating that IL-1α could be from the necrotic cells (Figure 3.2). However, we need to further confirm our observation by the other techniques such as immunoblot.

It has been reported that IL-1β plus IL-23 could successfully induce the IL-17 production of cutaneous γδT cells [228]. In our study, we found that IL-1α and IL-1β had similar potential for IL-17 production in skin-draining lymph node γδT cells (Figure 3.3). Like IL-1α, cell supernatants of CD109-/- keratinocytes with IL-23 could also successfully increase the IL-17 production of γδT cells, in a dose-dependent manner (Figure 3.3). We further discovered that, CD109-/- keratinocyte-

derived IL-1α, rather than IL-1β, is the main source of IL-1 to active the γδT cells by *in vitro* stimulation of conditioned media (Figure 3.3). These results are expected, because IL-1β is more hematopoietic cell-derived, whereas barrier epithelial cells such as keratinocytes routinely express IL-1α at steady state. Similarly, *in vivo* two-week blockade of IL-1α in CD109-/- mice, could significantly reduce the number of epidermal and dermal γδ17 cells, as well as the neutrophils in epidermis (Figure 3.4). Unexpectedly, CD109-deficient mice crossed with IL-1R1-knockout mice showed similar numbers of γδT cells in epidermis and dermis (Figure 3.5), indicating that CD109 could regulate the activation of γδ17 cells bypass the IL-1R1 signaling. This inconsistency between IL-1R1-/-CD109-/- mice and IL-1α blockade mice, could be due to several reasons. First, there are multiple ways to activate γδT cells. For example, lipid antigen presented by CD1d could activate the γδ17 cells [197]. It could be one reason because CD109 deficient mice exhibit abnormally high lipids levels in the hair follicle (Data not shown). Additionally, microbial products could activate γδ17 cells through the cell-surface TLR2 [323]. We previously found that the loss of CD109 will result in a disturbed skin barrier, which facilitate the invasion of commensals [311]. In this case, microbial products from the commensal microbiota may directly activate the γδ17 cells in CD109-/- skin, which induces the subsequent skin inflammation. Regarding the importance of IL-1 at barrier site to fight against the pathogens, the activation of γδ17 cells might be increased to compensate the loss of IL-1 signaling in IL-1R1-/-CD109-/- skin. Secondly, nuclear location of IL-1α enables it to interact directly histone acetyltransferases such as p300, PCAF and GCN5, and stimulate transcription of genes, including those encoding pro-inflammatory chemokines, independently of IL-1R1 signaling [313]. IL-1α from the IL-1R1-/-CD109-/- keratinocytes, might induce other pro-inflammatory cytokines such as IL-33, to induce the activation of γδ17 cells.

Calpain belongs to a family of proteases involved in cellular proliferation, differentiation and apoptosis. The calpain-mediated proteolysis of pro-IL-1α resulted in several-fold increase of the bioactivity of IL-1α, which is consistent with that the pro-IL-1α had 50-fold lower affinity for IL-1R [145]. It suggests that calpain might play a role in regulating inflammatory response through converting IL-1α into its more potent form. In human, loss-of-function mutations in calpastatin (CAST), the endogenous specific inhibitor of calpain, could cause PLACK syndrome characterized by generalized peeling skin, leukonychia, acral punctate keratoses, cheilitis, and knuckle pads [324]. Conversely, transgenic mice overexpressing calpastatin could delay the wound healing [325]. Additionally, calpain-1 inhibitor treatment could decrease the epidermal thickness

in an IMQ-induced psoriasis mouse model [326]. In our study, we identified that calpain could directly bind to CD109, and loss of CD109 in the skin leads to increased calpain activity of the skin cells (Figure 3.6). Considering that calpain could cleave IL-1α to increase its bioactivity, we could deduce that CD109 regulate the activity of IL-1α in a calpain-dependent way. However, further research need to be explored.

Our data find CD109 potentially bind to calpain, being a central component in regulating the cutaneous IL-17-producing γδT cells through keratinocyte-derived IL-1α. It broadens our insight on how one molecule expressed on structural cells could regulate the cutaneous immune response, thus to maintain the homeostasis of the skin.

3.5 Materials and Methods.

Animals

The CD109+/+, CD109-/- and IL-1R1-/-CD109-/- (8-12 weeks old) on a C57BL/6 background were bred and maintained under specific pathogen-free conditions at the Animal Resources Centre at McGill University Health Centre. All experiments involving CD109-/- mice used littermate controls or mice originally derived from CD109+/- breeders. CD109-/- and IL-1R1-/- mice were provided by Dr. Masahide Takahashi (Nagoya University, Japan) and Dr. C.A. Piccirillo (McGill University, Canada). All animal studies were approved by the McGill University Animal Care Committee.

Isolation of skin cells

To prepare single cell suspensions from skin tissue, ears were cut across the hairline and manually partitioned into rostral and caudal sides using fine scalpels. To partition the epidermis from the dermis, each side was incubated dermal side down in separation buffer (RPMI 1640 containing 1mg/ml Collagenase/Dispase) for 1.5 hours in a 35mm petri dish at 37°C. The epidermis and dermis were then rinsed in cold PBS, cut into ~2mm^2 pieces and incubated with digestion buffer (RPMI 1640 containing 154 U/ml Collagenase IV, 200 U/ml Dnase I and 2% FBS) for 2 hours in 24 well plates at 37°C. For spontaneous cytokine detection by flow cytometry, the protein transport inhibitor Golgistop (BD bioscience) was added into the digestion buffer (4 µl Golgistop per 6ml buffer). Following the incubation, the tissue was pipetted up and down in the digestion buffer to generate a single cell suspension, passed through a 100 µm cell strainer and collected into a 50mL conical tube. Any residual tissue pieces were crushed using a syringe plunger before all cell strainers were rinsed with RPMI 1640 media containing 10% FBS and 15mM HEPES. Cells were then washed and resuspended in R10 buffer prior to counting.

Flow cytometry

For extracellular staining, single cell suspensions were incubated with fixable viability dye in 100µl PBS (eBioscience), washe and incubated with anti-Fc receptor (clone 2.4G2, BD biosciences) for 10 minutes before adding fluorochrome-labelled antibodies at predetermined concentrations in 100 µl FACs buffer (PBS containing 2% FBS and 10mM HEPES) for 30 minutes on ice. For intracellular staining, cells were incubated with fixable viability dye and fixed with the

Intracellular fixation/permeabilization buffer (eBioscience) according to the manufacturer's instructions, followed by antibody labelling in 100 µl permeabilization buffer. After intracellular labelling, cells were washed and resuspended in 300 µl FACS buffer. Data were acquired on a LSR Fortessa (BD Biosciences) and then analyzed with FlowJo™ software.

Primary keratinocyte isolation from adult tail skin for *in vitro* short-time culture

Mice tails were removed from killed mice that have been washed with alcohol. Skin was pulled from tail at the base toward the tip. Then the tube of the skin was opened by a longitudinal cut, followed by other cross-sectional cut into 3 pieces. Flattened skin pieces were transferred into dishes with dermis-side-down on the surface of recently thawed 1% trypsin in PBS for 1 hour at 37ºC. Then, dermis was removed, and epidermis was transferred into high-calcium (HiCa) media and chopped into tiny pieces with scissors. Re-suspended cells were filtered through a 100-mm cell strainer into 50 mL conical tube. Cell strainer was rinsed with HiCa media, and cells were centrifuged at 150g for 5 minutes at 4ºC. For culture, cells were washed with calcium-free PBS and re-suspended in the low-calcium (LoCa) media and plated on fibronectin/collagen-coated plastic culture dishes.

Confocal microscopy and imaging processing

In brief, primary keratinocytes were fixed with ice-cold fixation solution (75% Acetone+25% Ethanol), blocked by anti-Fc receptor for 1 hour, stained with fluorochrome-conjugated antibodies cocktail for 1 hour and nuclei staining (DAPI) for 5 minutes at room temperature.

Quantitative RT-PCR analyses

Ear skin from individual animals were separated into epidermal and dermal sheets as described above. Primary keratinocytes were collected as described above. Samples were flash frozen and total RNA was extracted by using the QIAGEN RNeasy Mini Kit as per the manufacturer's instructions. Next, equal amounts of RNA from each sample were reverse-transcribed by QuantiTect Reverse Transcription Kit (Thermofisher). Primers (*il1a*, *il1b*, *cd109*, *keratin5*, *keratin10* and *involucrin*) were designed specific for the target genes and tested by end-point PCR first. Relative expression of genes of interest were measured by real-time RT-PCR. The expression

of the genes of interest from individual cDNA samples were normalized to the reference gene *Hprt* and expressed as a fold change using the $^{\Delta\Delta}$Ct formula.

Anti-IL-1α administration

Mice were intraperitoneally injected with 200μg anti-IL-1α antibody (ALF161) or isotype IgG purchased from BioXCell every third day for two weeks. Frequency and number of IL-17-producing γδT cells from epidermis and dermis were detected by flow cytometric analysis.

Immunoprecipitation and LC-MS/MS analysis

Total protein concentration of ultrasonicated samples was assessed by protein quantification assay under the manufacturer's instructions. For immunoprecipitation, cell lysates were then incubated with anti-CD109 antibody overnight at 4°C. Protein A/G magnetic beads were subsequently added to bind the antigen/antibody complex for 1 hour at room temperature. Beads were washed with 3 times with IP-MS wash buffer A and wash buffer B in order, then samples were eluted with IP-MS elution buffer for 10 min at room temperature. Elution was dried in vacuum concentrator and proceeded to in-solution digestion followed by MS analysis. The liquid Chromatography with tandem mass spectrometry (LS-MS/MS) analysis was conducted with the help of McGill University Health Centre Proteomics Platform.

For immunoblot, after the immunoprecipitation procedure, samples were then diluted in loading buffer, and incubated at 100 °C for 5 min. Next, samples were separated by SDS-PAGE and transferred on polyvinylidene difluoride membranes by wet blotting. The membrane was blocked by 5% milk powder in 1×TBS/0.1 % Tween-20 (TBST) at room temperature for 1 hour and incubated in primary antibody overnight at 4 °C. Membrane was washed 3 times and incubated with secondary antibody for 1 hour at room temperature. The blots were detected by the chemiluminescence light-detecting kit.

ELISA

Cell supernatants were collected and tested for mouse IL-17A mouse ELISA kit (Invitrogen) as per the manufacturer's instructions.

Calpain activity test

Skin draining lymph nodes (sdLN) cells and live skin cells were isolated for the measurement of *in vitro* Calpain activity. Freshly isolated sdLN cells or 1 day culture keratinocytes (2.10^6 cells) were washed twice with PBS 1X and incubated for 1 hour with the cell permeable fluorogenic Calpain Substrate IV (Millipore Sigma) at a final concentration of 20 µM. The cells were then washed 3 times with PBS 1X and resuspended in freshly prepared Calpain buffer (50 mM Tris, 150 mM NaCl). Basal activity was measured 3 times, every 2 min at 37 °C using the Cytation 5 multi-mode plate reader (BioTek, Winooski), with the settings for Excitation/Emission at 320 nm/480 nm. Calpain activity was then measured every 2 min, after the addition of 10 mM CaCl2, for 1 hour at 37 °C.

Statistical analysis

Data were analyzed with Graphpad® Prism 7. Unpaired student t test or Two-way ANOVA followed by Sidak's multiple comparisons test were used as appropriate. *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

3.6 Author Contributions

H.Z. designed and performed experiments, analyzed data and wrote the manuscript. G.F. and B.T. performed experiments. I.L.K designed the project, analyzed data, and wrote the manuscript.

3.7 Acknowledgement

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3.8 Figures

Figure 3.1

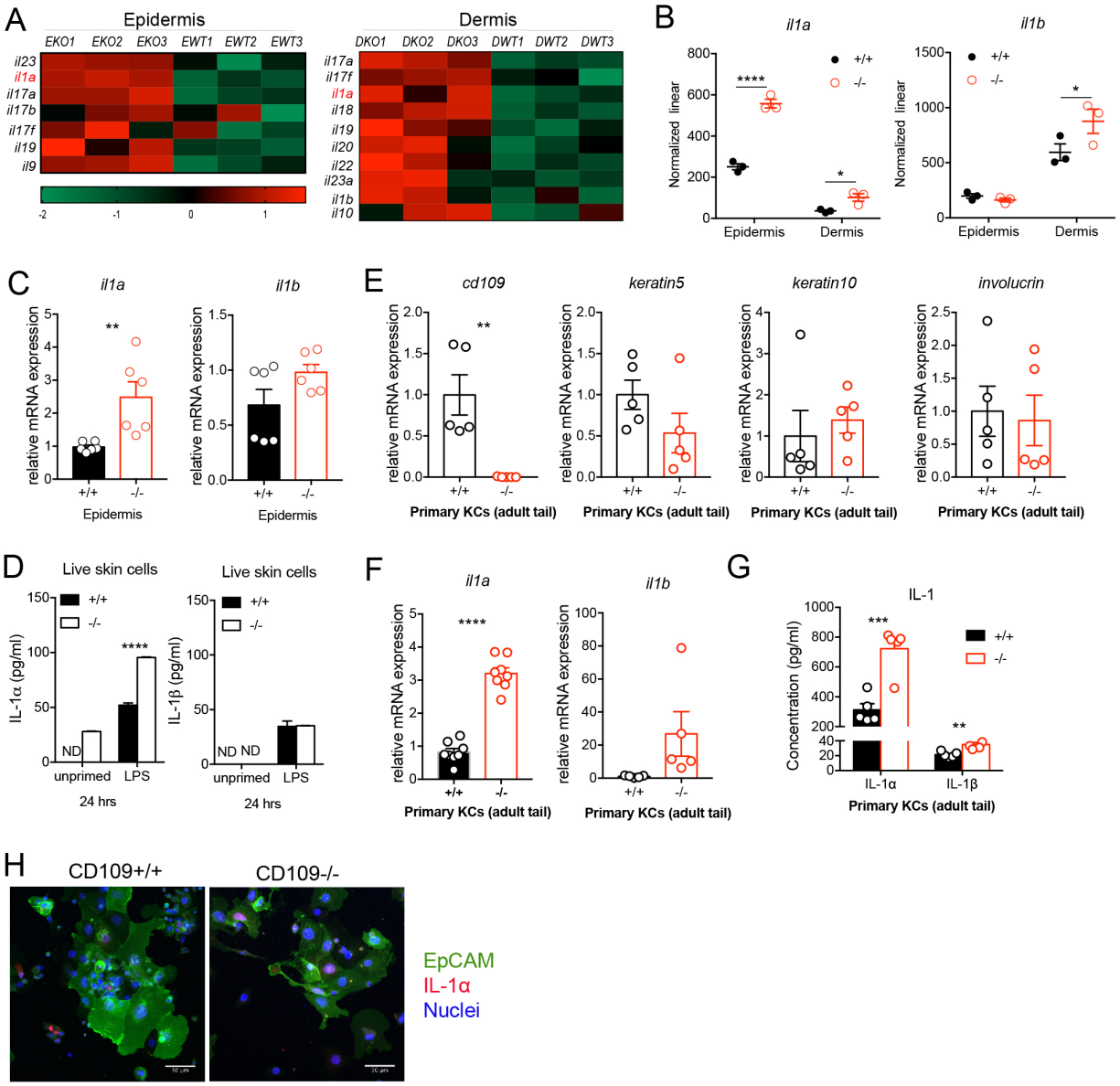

Fig.3.1. CD109 depletion result in increased IL-1α expression in the skin.
(A) Heat-map of nanostring analysis between CD109+/+ and CD109-/- epidermis and dermis. (B) The epidermal and dermal IL-1α and IL-1β transcripts, nCounter Analysis. One-way ANOVA, *p<0.05, ****p<0.0001. (C) RT-qPCR analysis for mRNA expression of IL-1α and IL-1β in CD109+/+ and CD109-/- epidermis relative to *Hprt*. Unpaired t test, **p<0.01. (D) ELISA analysis of IL-1α and IL-1β production of skin cells after 24-hours LPS stimulation. One-way ANOVA, ****p<0.0001. (E) RT-qPCR analysis for mRNA of CD109, Keratin5, Keratin 10 and Ivolucrin relative to *Hprt* in CD109+/+ and CD109-/- primary keratinocytes. (F) RT-qPCR analysis for mRNA of IL-1α and IL-1β from CD109+/+ and CD109-/- mouse adult primary keratinocytes. Unpaired t test, **p<0.01. (G) ELISA analysis of IL-1α and IL-1β production of CD109+/+ and CD109-/- primary keratinocytes after 3-day culture. One-way ANOVA, *p<0.05, ***p<0.001. (H) Staining of EpCAM, IL-1α and nuclei of CD109+/+ and CD109-/- primary keratinocytes. Scale bars: 50μm.

Figure 3.2

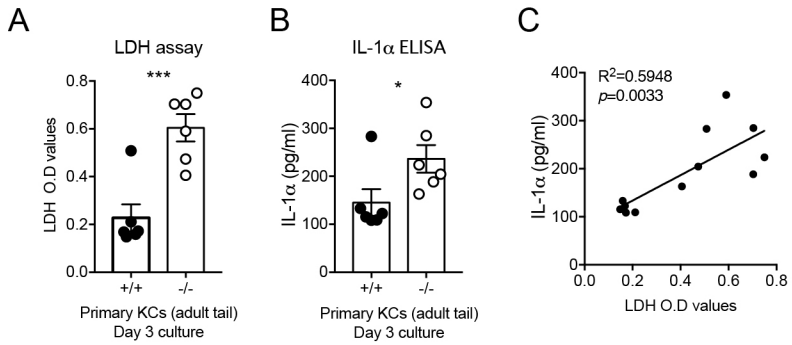

Fig.3.2 IL-1α release is related to cell death levels of the primary keratinocytes.
(A) LDH assay of the supernatants of cultured primary keratinocytes. Unpaired t test, ***$p<0.001$.
(B) Concentration of IL-1α. Unpaired t test, *$p<0.05$.
(C) Scatterplot of IL-1α concentration and LDH O.D. values.

Figure 3.3

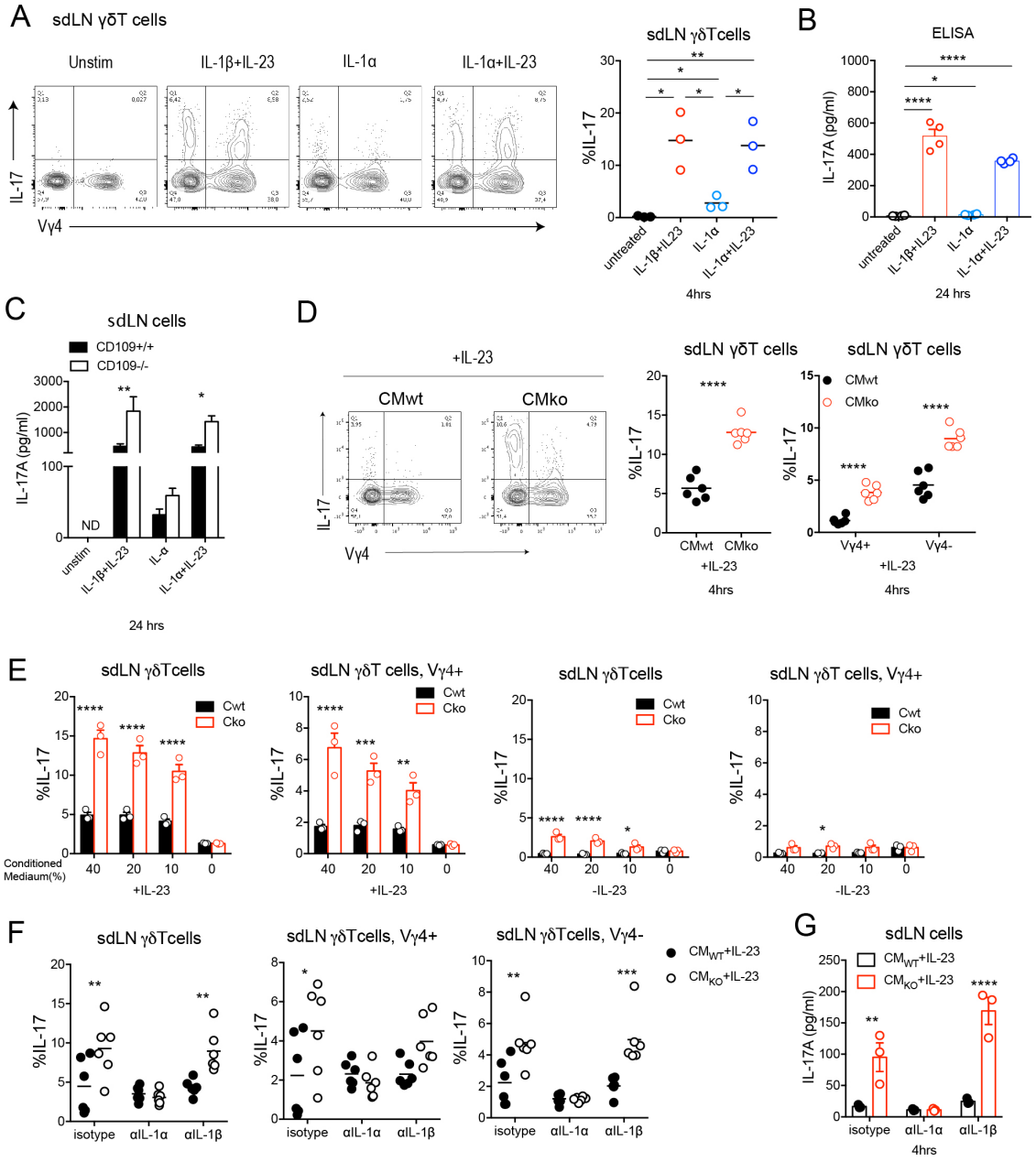

Fig.3.3. IL-1α equals the potential of IL-1β to induce IL-17 production of γδT cells *in vitro*.
(A) FACS plot and analysis showing the IL-17 production of sdLN γδT cells under the stimulation of IL-1α and IL-1β plus IL-23 for 4 hours. Unpaired t test, *p<0.05, **p<0.01. (B) ELISA analysis of IL-17 production of sdLN cells with the same stimulation as (A) for 24 hours. Unpaired t test, *p<0.05, ****p<0.0001. (C) ELISA analysis of IL-17 production of CD109+/+ and CD109-/- sdLN cells with the same stimulation as (A) for 24 hours. One-way ANOVA, *p<0.05, **p<0.01. (D) FACS plot and analysis showing the IL-17 production of sdLN γδT cells under the conditioned media (CM) stimulation for 4 hrs. One-way ANOVA, ****p<0.0001. (E) FACs analysis showing the IL-17 production of sdLN γδT cells under the gradient CM stimulation for 4 hours. One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (F) FACS analysis of IL-17+ γδT cells under the CM stimulation for 4 hrs with IL-1α and IL-1β blockade. One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001. (G) ELISA analysis of IL-17 production of sdLN cells under the CM stimulation for 4 hrs with IL-1α and IL-1β blockade. One-way ANOVA, **p<0.01, ****p<0.0001.

Figure 3.4

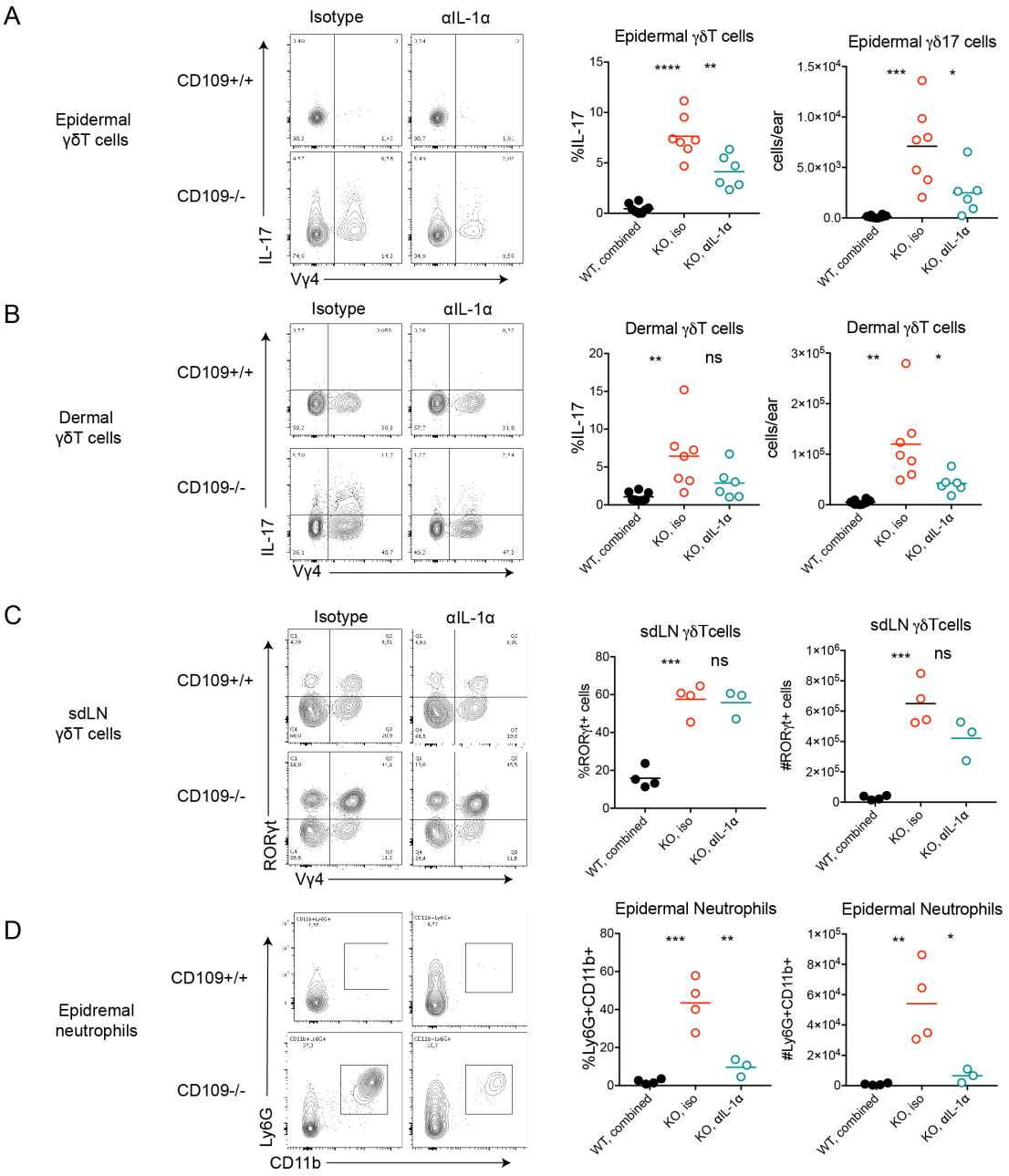

Fig.3.4. Blockade of IL-1α *in vivo* decreases the IL-17 production of γδT cells.
(A) FACS plot and analysis showing the frequency and number of epidermal IL-17++ γδT cells in CD109+/+ and CD109-/- mice with αIL-1α blockade. Unpaired t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (B) FACS plot and analysis showing the frequency and number of dermal IL-17++ γδT cells in CD109+/+ and CD109-/- mice with αIL-1α blockade. Unpaired t test, *p<0.05, **p<0.01. (C) FACS plot and analysis showing the frequency and number of sdLN RORgt+ γδT cells in CD109+/+ and CD109-/- mice with αIL-1α blockade. Unpaired t test, ***p<0.001. (D) FACS plot and analysis showing the frequency and number of epidermal neutrophils in CD109+/+ and CD109-/- mice with αIL-1α blockade. Gate: Viable CD45+CD3-. Unpaired t test, *p<0.05, **p<0.01, ***p<0.001.

Figure 3.5

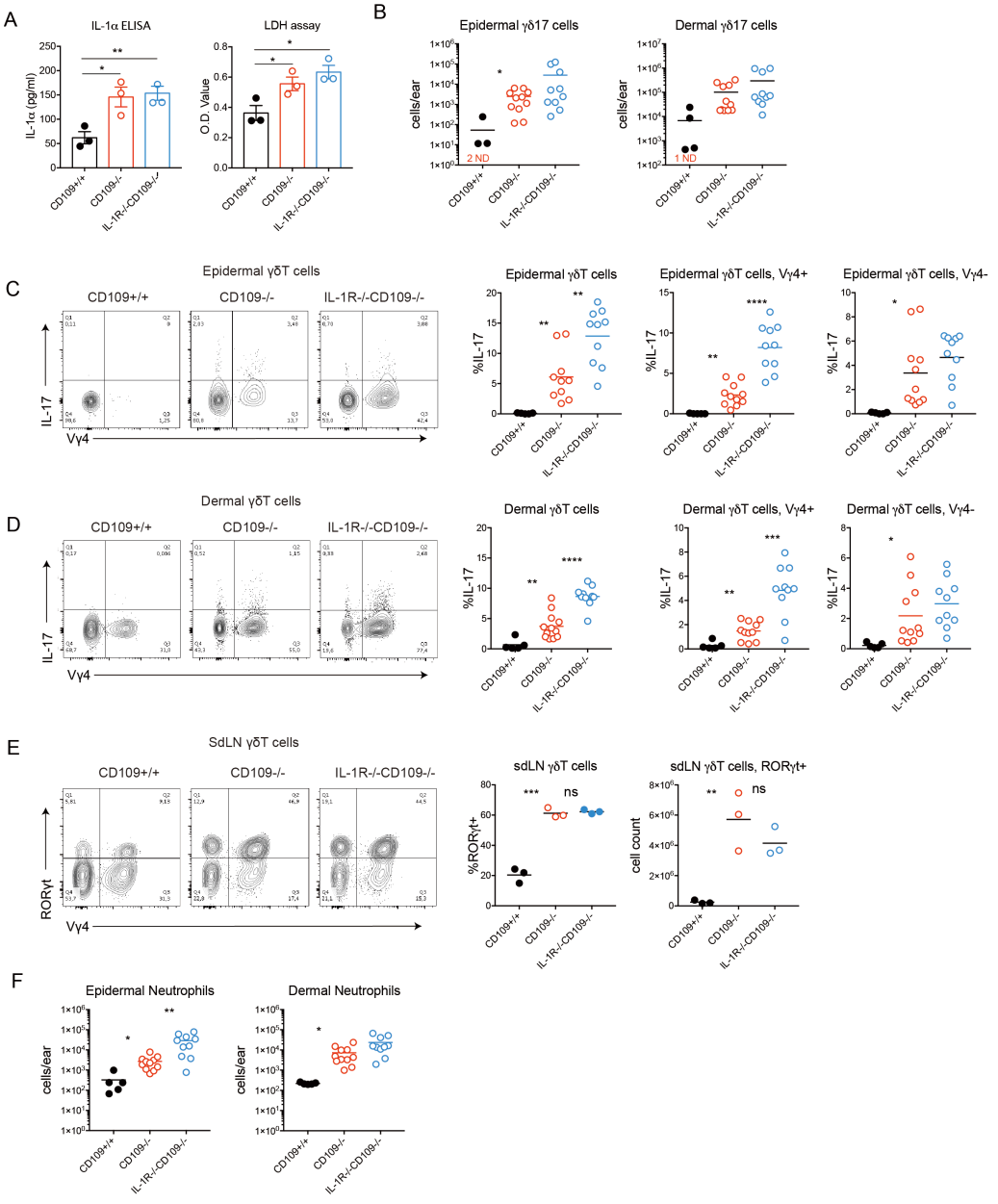

Fig.3.5. IL-1R1 signaling deficient does not block the IL-17 production of γδT cells in CD109-/- skin.

(A) IL-1α ELISA and LDH assay of the supernatants of cultured primary keratinocytes. Unpaired t test, *p<0.05, **p<0.01. (B) FACS analysis showing numbers of the epidermal and dermal γδ17 cells in CD109+/+, CD109-/- and IL-1R1-/-CD109-/- skin. Unpaired t test, *p<0.05, **p<0.01. (C) FACS plot and analysis of the frequency of epidermal IL-17+ γδ17 in CD109+/+, CD109-/- and IL-1R1-/-CD109-/- skin. Unpaired t test, *p<0.05, **p<0.01, ***p<0.001. (D) FACS plot and analysis of the frequency of dermal IL-17+ γδT in CD109+/+, CD109-/- and IL-1R1-/-CD109-/- skin. Unpaired t test, *p<0.05, **p<0.01, ****p<0.0001. (D) FACS plot and analysis of the frequency of sdLNs RORγt+ γδT in CD109+/+, CD109-/- and IL-1R1-/-CD109-/- skin. Unpaired t test, **p<0.01, ***p<0.001. (E) FACS analysis showing numbers of the epidermal and dermal neutrophils in CD109+/+, CD109-/- and IL-1R1-/-CD109-/- skin. Unpaired t test, *p<0.05, **p<0.01.

Figure 3.6

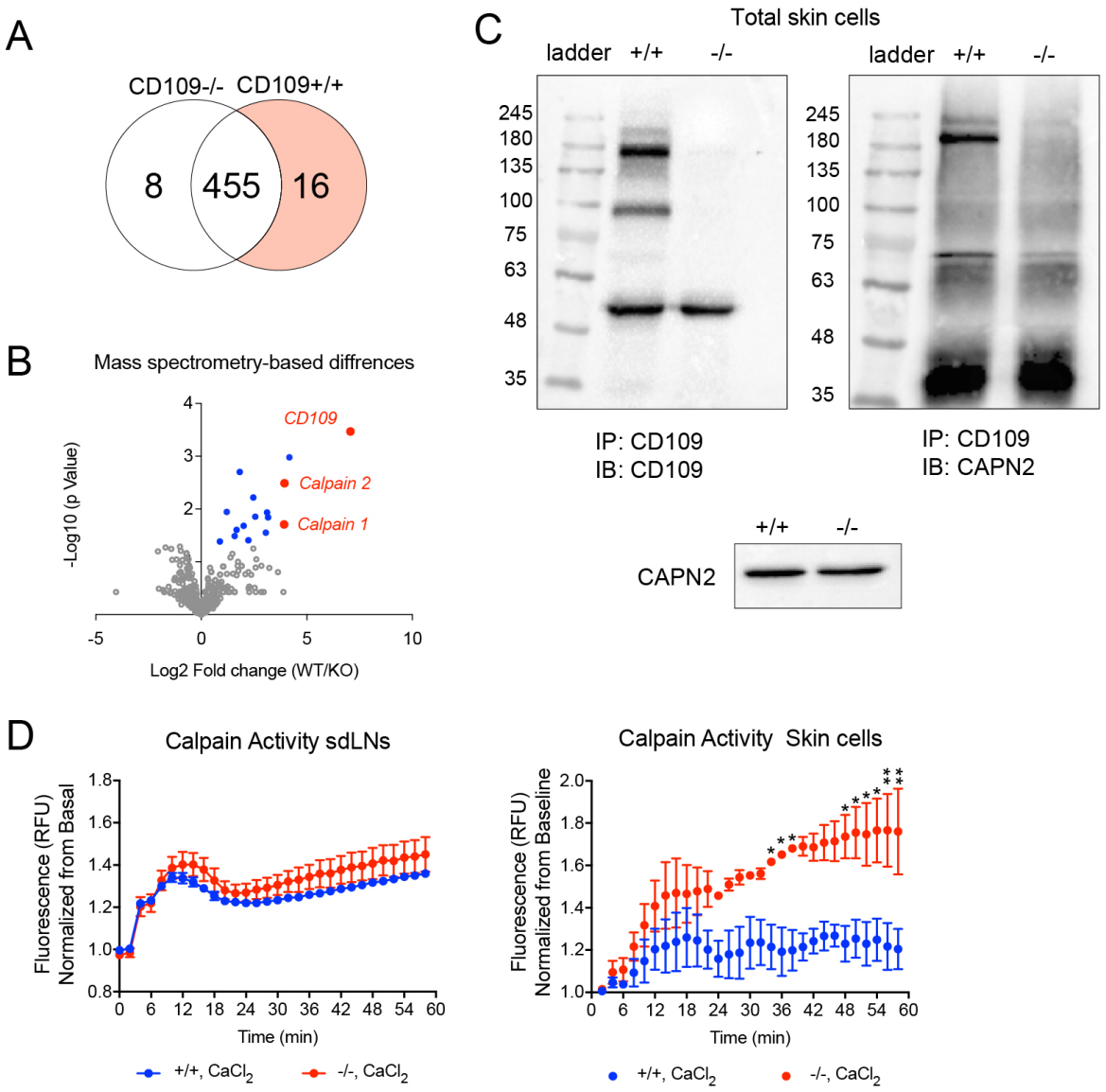

Fig.3.6. CD109 binds to cysteine protease Calpains to regulate their activity.
(A) Venn diagram, showing the number of overlapped proteins and proteins only elevated in the CD109+/+ skin after LC-MS/MS analysis. (B) Volcano plot (left), showing the proteins only elevated in the CD109+/+ skin compare to the CD109-/- skin. (C) Upper panel: immunoprecipitation blot to confirm that Calpain 2 combines to CD109; Bottom panel: immunoblot, showing the Calpain protein expression. (D) Calpain activity test of CD109+/+ and CD109-/- sdLN and live skin cells. Two-way ANOVA, *p<0.05, **p<0.01.

Chapter 4. Conclusions

"Never assume you know all things; always push that last mile. And be inspired by the knowledge that exists at the time you enter research, but be irreverent toward this knowledge, for this is the road to true understanding."

Charles A. Janeway, Jr.

4.1 Integrating CD109 into the arsenal of Keratinocyte-regulated immune-microbiota crosstalk.

The most superficial layer of mammalian skin, the epidermis, is a stratified epithelial layer predominantly composed of keratinocytes in varying differentiation states interspersed with specialized immune cells including Langerhans cells and DETCs [327]. The epidermis creates a robust physical and immunological barrier, in part, by harboring a vast collection of microorganisms including bacteria, fungi and viruses, collectively termed the microbiome [328]. The microbial communities of the skin reside on the tissue surface and in the associated epidermal appendages such as hair follicles and sebaceous glands [241]. This microbial shield ensures resistance to pathogen invasion. For example, *S. epidermidis* secretes bacteriocins that directly kill pathogens [329, 330]. However, their protective function is dependent on the barrier integrity of the skin. For example, *S. epidermidis* could successfully protect the skin colonization of *S. aureus*, but the effect was reversed by the tape-stripping induced barrier disruption, which generates an inflammatory environment to suppress the growth of commensals [331]. In our study, loss of CD109, resulted in a disturbed skin barrier (Figure 2.7 and S2.7). Although we were not able to identify differences in the overall composition or diversity of the skin microbiota between the wild-type and CD109-deficient mice (Figure 2.7), an interesting possibility is that a compromised barrier in the absence of CD109 may lead to aberrant immune reactivity to bacterial species that are normally symbiotic with the host.

In addition to the cutaneous microbiota, the keratinocyte maturation process involves a consistent cycling of increasingly differentiated cells from the basal to the outmost layer, enabling a physical 'escalator' of pathogen exclusion. As we observed an increase in the proliferation and cell death of CD109-deficient keratinocytes, we speculate that an imbalance in epithelial turnover

may compromise pathogen exclusion from immune detection. In addition, keratinocytes express a consortium of pattern recognition receptors (PRRs) that mediate direct microbicidal activity [7, 8, 332]. These PRRs sense microorganisms by recognizing the conserved microbial structures or so-called PAMPs, and transduce these signals to intracellular components, resulting in a cascade of signaling events translating the intracellular adaptor, usually NF-κB, to effectively induce pro-inflammatory cytokines, chemokines and AMP expression [7, 8, 332]. Keratinocytes are also important producers of AMPs, that show a broad spectrum of anti-microbial activity against a wide range of pathogens, including bacteria, fungi and enveloped viruses [333]. They are also a source of pro-inflammatory cytokines and chemokines, such as IL-1 family members, TNFα, CCL20, CXCL9 and CXCL10 [1]. IL-1 family members are shown to play important skin barrier functions, and the expression, cleavage and secretion of these cytokines are induced upon the stimulation of PRRs [314]. Additionally, keratinocytes store cytosolic IL-1α, that requires no further cleavage and allows for rapid activation of adjacent immune cells when cell death occurs [334]. These endogenous danger signals such as AMPs and IL-1 family members, are called alarmins. They are not only secreted by keratinocytes, but also by innate and adaptive immune cells when they are activated by PAMPs [335]. Upon sensing of the alarmins and microbial motifs, the keratinocyte can release cytokines such as TNFα, IL-1α and IL-18. IL-1 acts in an autocrine manner on KC, by inducing the expression of IL-1R and the IL-1R antagonist; the latter acts as a competitive inhibitor of IL-1α and is crucial in balancing/resolving inflammatory responses. Additionally, IL-1α stimulates the production of other pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α and stimulates the expression of various adhesion molecules on endothelial cells. CD109 negatively regulated keratinocyte activation, as indicated by the expression of AMPs such as S100 family members, CCL20 and IL-1α were significantly upregulated in the absence of CD109 (Figure 2.1, 2.3 and 3.1). This dysregulation of inflammatory mediators ultimately led to the aberrant recruitment of activated neutrophils and innate lymphocytes that exacerbated tissue pathology. Collectively, our results indicate that CD109 plays a key regulatory role in keratinocyte-mediated skin homeostasis.

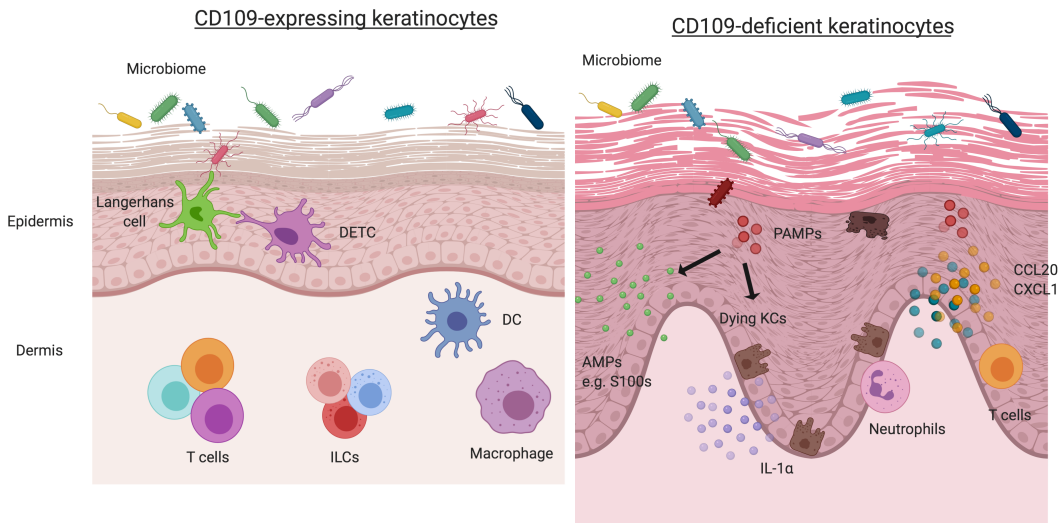

Figure 4.1. CD109-dependent mechanisms that regulate immune-microbe crosstalk.
CD109 is a key regulator of skin homeostasis. Loss of CD109 leads to a disturbed skin barrier, characterized as increased reactivity to commensal microbiome, including 1) elevated AMP production, 2) increased IL-1 levels, 3) increased chemokine production and neutrophil recruitment.

4.2 CD109 as a cell-extrinsic regulator of the IL-23/IL-17 immune axis.

The IL-23/IL-17 immune axis has been identified as a key pathway in host defense but also skin inflammatory disease. Following secretion by skin-resident dendritic cells and macrophages, IL-23 stimulates the differentiation of IL-17-expressing cells, through expression of the transcription factors RORγt and STAT3 [132, 137, 163, 336]. In addition, IL-23 promotes IL-22 expression by RORγt+ cells, including αβT, γδT, natural killer T and ILCs. Collectively, these cytokines support recruitment of microbicidal phagocytes and fortify the epidermal barrier against bacterial and fungal infections such as *S. aureus* and *C. albicans* [131, 140]. Thus, the IL-23/IL-17 immune axis serves as the central mediator of protective immunity. However, unchecked or chronic activation of this axis will lead to skin disease, the most common of which is psoriasis [199]. Psoriasis is a chronic autoimmune disease of the skin that is characterized by abnormal keratinocyte proliferation/differentiation and excessive immune cell infiltration in both epidermis and dermis [29, 199]. Over the past two decades, immune cells have been regarded as the drivers of psoriasis, and neutralizing antibodies targeting IL-23 and IL-17 signaling have achieved great efficacy in disease remission [29, 199, 337]. Additionally, it is well-defined that T cells have a key role in keratinocyte turnover and epidermal hyperplasia, characteristic of psoriatic plaques [337]. However, the etiology of psoriasis may not only be explained as a T cell-initiated disease. Indeed,

intrinsic alterations of keratinocytes have also been shown to play an important role in initiating, sustaining and amplifying the disease progression. For example, mice overexpressing TGF-β and STAT3 or lacking the inhibitor of NF-*k*B kinase 2 (IKK2) in keratinocytes developed skin lesions that closely resembled human psoriasis [214, 338]. Is it possible that keratinocyte is an initiator rather than simply a responder in the context of psoriasis? If so, what are the molecular mechanisms driving disease? CD109 has been reported to be a TGF-β co-receptor on keratinocytes, that negatively regulates canonical TGF-β signaling and was shown to be down-regulated in human psoriatic lesions [276, 287]. Consistent with the skin phenotype of CD109-/- mice (Figure 2.1), transgenic mice with TGF-β overexpression in basal keratinocytes also exhibited progressively hair loss with age [338]. However, by further comparing Smad2/3 activation of CD109-deficient total skin cells with wild-type, we were unable to detect differences (Figure 2.2). Furthermore, we found constitutive activation of Stat3 signaling in CD109-/- total skin cells (Figure 2.2), indicating that CD109 regulates Stat3 rather than the TGF-β signaling. Notably, it has been reported that the IL-10 family members IL-19, IL-20 and IL-24 were increased in psoriatic lesions and specifically activate Stat3 signaling in keratinocytes, but not immune cells, through the IL-20R [339]. Consistently, we found that the IL-20R-dependent cytokine IL-19 showed a significant increased expression in CD109-deficient than the wild-type epidermis (Data not shown), so a comprehensive analysis of Stat3 signaling and its related cytokines in CD109-deficient keratinocytes should be addressed in the future.

As mentioned above, the dysregulation of Stat3 signaling in keratinocytes results in a psoriasis-like phenotype [214]. Moreover, the skin inflammation of K5.Stat3 transgenic mice was associated with activation of the IL-23/IL-17 immune axis [214]. Although mice deficient in CD109 were reported to exhibit leukocyte accumulation in the skin, the mechanisms that drove the skin inflammation in CD109-/- mice were not examined [263]. We observed that CD109 depletion in mice skin led to dysregulated keratinocyte activation, as well as spontaneous activation of the IL-23/IL-17 immune axis, including the neutrophil accumulation, increased anti-microbial peptides expression and cytokine production (Figure 2.1 and 2.2).

Lymphoid sources of IL-17 includes innate and adaptive immune cells including CD4+ T cells, CD8+ T cells, γδT cells, ILCs and NKT cells [152]. As an early important source of IL-17, dermal γδ17 cells, which are mainly composed of Vγ4+ and Vγ6+ subsets, contribute as the first line of cutaneous host defense by their anti-bacterial and anti-fungal activity [133, 134, 194]. In IMQ-

induced psoriatic-like inflammation, dermal γδT cells were found aberrantly distributed in the epidermis, which participated in the pathological inflammation of the skin [126, 228]. We observed similar aberrant distribution of both Vγ4+ and Vγ4- IL-17 producing dermal-derived γδT cells in the epidermis of CD109 deficient skin (Figure 2.3), and the CD109-/- skin was pre-disposed to the psoriasiform inflammation (Figure 2.4).

As we mentioned before, transcriptional factor RORγt is cardinal for IL-17-producing cells [86], including γδT cells [116], it is efficient to identify the IL-17-producing γδT cells based on the RORγt expression. We found that, the accumulation of RORγt+ γδT cells in CD109-/- epidermis is tissue-specific, which was limited in the skin and its draining lymphoid organs (Figure 2.5). Furthermore, genetically depletion of RORγt in CD109-deficient skin could reverse the swelling of epidermis (Figure 2.2), indicating that γδ17 cells are critical for the CD109-depletion-induced skin inflammation.

IFN-γ vs IL-17-producing γδT cells could also be segregated by the expression of CD27 and CCR6 respectively [110, 111, 160]. The chemokine CCL20 and its receptor CCR6 have been shown to be increased in the psoriatic skin [340, 341]. In the IL-23-injection-induced psoriatic-like mouse model, CCL20/CCR6 has also shown their key role in psoriatic inflammation by regulating the dendritic and T cell migration [342]. Moreover, elevated CCL20 could recruit more CCR6+ γδT cells into the murine epidermis, participating in the development of IMQ-induced and IL-23-associated psoriasiform dermatitis [103, 124, 129, 343, 344]. We found that the loss of CD109 increased the CCL20 expression in the epidermal sheets (Figure 2.3), indicating that CD109 might regulate the CCL20-CCR6 axis to maintain the tissue homeostasis of the skin. It has been shown that hair follicle could elevate the CCL20 expression in response to external stress [35], which results in a migration of CCR6+ dendritic cells into the hair follicles [35]. Interesting, CCR6 is highly expressed on the dermal γδT cells at steady state conditions, and we found that the localization of Vγ4+ γδT cells, a subset of IL-17-potent population, were preferentially localized around the hair follicle (Figure 2.3). So, what are the driving-factor of the migration of dermal γδT cells from dermis to epidermis, or hair follicles, in our CD109-deficient mice? The combined use of bone marrow chimeras and neonatal thymocyte transfer revealed that a non-hematopoietic source of CD109 was required for dysregulated cutaneous immune cell activation (Figure 2.6). One possible explanation could be the skin flora, as microbiome-derived CCL20 was critical for the migration of Treg cells to the hair follicles [299]. Consistent with these data, CD109 expression

is enriched in the hair follicles (Figure 2.7), and topical application of antibiotics on the CD109-/- skin could significantly reduce the γδ17 cells (Figure 2.7). Thus, CD109 regulated the IL-23/IL-17 immune axis in a cell-extrinsic manner by commensal microbiota.

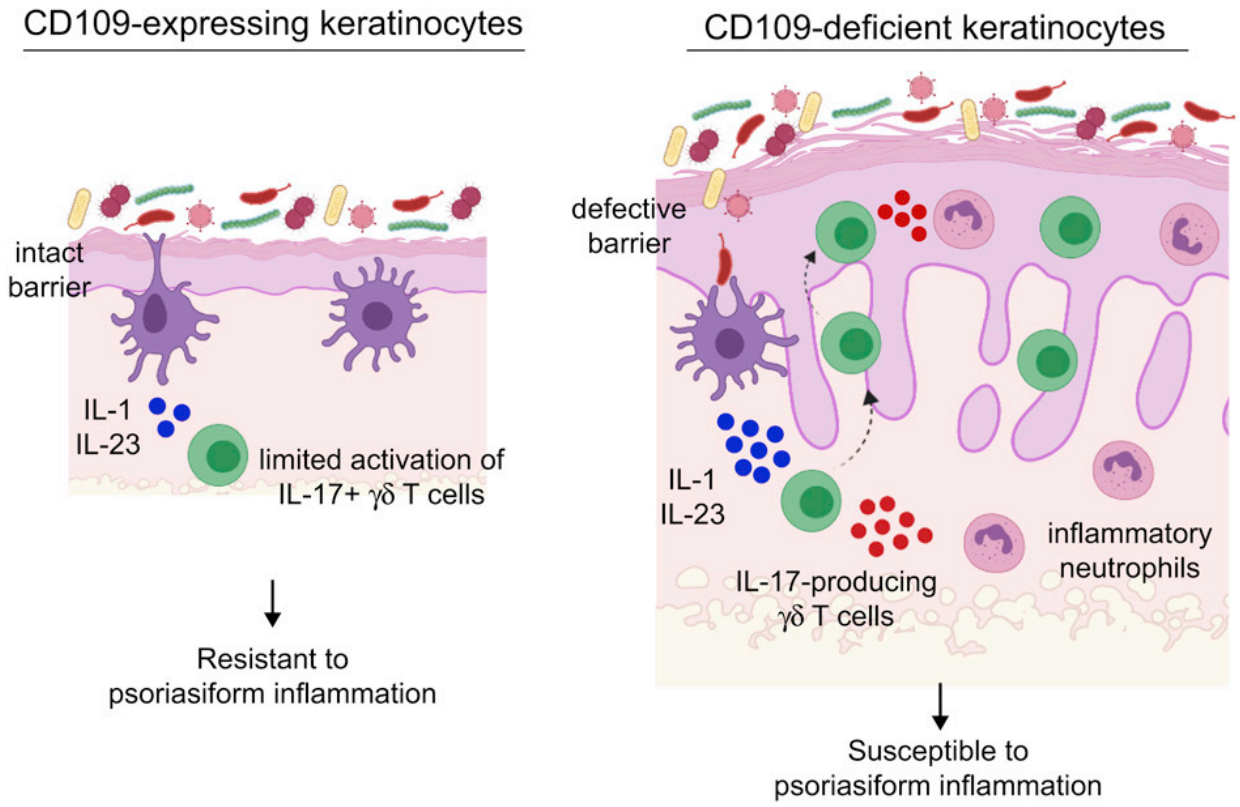

Figure 4.2. Summary of Chapter 2.
CD109 is a negative regulator of the cutaneous IL-23/IL-17 immune axis. When it is expressed by keratinocytes, CD109 maintains intact skin barrier, which limit the activation of IL-17-producing γδT cells, making skin be resistant to psoriasiform inflammation. Deletion of CD109 amplifies IL-17 production by cutaneous γδT cells, disturbs the skin barrier integrity and increases the reactivity to commensal microbiota, leading skin to be susceptible to psoriasiform inflammation.

4.3 Keratinocyte cell death and skin inflammation.

Epidermal keratinocytes undergo a unique form of terminal differentiation and programmed cell death known as cornification. The undifferentiated and mitotic keratinocytes from the basal layer of the epidermis become detached from the basement membrane and move to the spinous layer, where they start to undergo differentiation. In the spinous layer, differentiation-specific proteins (such as Keratins 1 and 10) are expressed, cytoskeleton rearrangement and proliferation arrest occurs and desmosomes (adhesive junctions between keratinocytes) form [345]. In the granular layer of the epidermis, the keratin filaments are cross-linked, DNA is degraded, organelles

are destroyed. Finally, the plasma membrane is replaced by the cornified envelope and ceramide is exuded from lipid-rich lamellar bodies, effectively making a waterproof seal [345]. The whole process of cornification is similar to apoptosis; however, the dead cells are not be removed by phagocytic cells such as Langerhans cells, but rather shed into the environment during desquamation [345]. As desquamation occurs, the cornified layer is filled with keratinocyte corpses [345]. Importantly, during terminal differentiation, keratinocytes activate anti-apoptotic and anti-necrotic mechanisms to prevent the premature cell death [346]. Incomplete cornification, which is commonly found in premature cornifying keratinocytes of psoriatic lesions, could inhibit the expression of late differentiation markers needed to execute full cornification [347]. In this situation, the premature death of the keratinocytes, through either apoptotic or necrotic machinery, will break the mechanical resilience and interconnection of the coordinated keratinocytes, to form extracellular or intracellular stress. These disorganized apoptotic or necrotic keratinocytes will subsequently release DAMPs to induce inflammation and disturb the differentiation process of adjacent keratinocytes [346]. We found that loss of CD109 in the mouse skin results in psoriasis-like symptoms *in vivo* (Figure 2.1 and 2.4), and increased cell death of the primary keratinocytes *in vitro* (Figure3.2), indicating that CD109 may participate in the regulation of keratinocyte terminal cornification. But how is CD109 enrolled in keratinocyte cornification? It has been reported that CD109 was enriched in a specific organelle of the epidermis called lamellar bodies [17], which participate in the process of terminal differentiation of keratinocytes by releasing the contents of themselves at the time point that keratinocytes transit from the granular to the cornified layer [346]. Moreover, altered LB structure has been detected in the lesional skin of psoriasis patients by electron microscopy [19]. It is possible that the absence of CD109 in the cutaneous lamellar bodies blocks LB extrusion, which subsequently leads to the premature cell death of keratinocytes. To comprehensively understand this process, the electron microscopy of wild-type and CD109-deficient murine skin is needed.

Protease activity is also indispensable during both the cornification as well as the cell death of keratinocytes. However, different enzymes are implicated in different processes. In epidermis, there is an increasing calcium gradient towards the surface of the skin and different cleavage events are calcium dependent. Calpains are cytosolic cysteine proteases activated in the presence of calcium. Even though it has been shown that calpains are present in the suprabasal layers of the epidermis, whether calpains are involved in the keratinocyte cornification remains controversial

[348, 349]. Interestingly, calpain activation seems to be involved in epithelial cell death, as it has been suggested to be vital in the destabilization of lysosomes [350, 351] and DNA fragmentation [352]. In addition, calpain activation also participates in plasma membrane permeabilization which leads to the secondary necrosis [353]. In humans, Calpain II expression was upregulated in psoriasis skin lesions [354]. In our study, we found that CD109 binds to calpain in the skin and regulates calpain activity (Figure 3.6), indicating that CD109 may regulate cell death via sequestration of calpain. Further studies should focus on the mechanisms by which CD109-Calpain interactions regulate keratinocyte differentiation and/or survival.

As one of the substrates of calpains [313], IL-1α is a component of keratinocytes that has been reported to participate in the development of pathological cornification, another important feature of psoriasis [326]. IL-1α is constitutively expressed in keratinocytes [11] and its expression is increased during stress and inflammation in other cell types [355]. Moreover, mice over-expressing either IL-1α or IL-1R1 suffer from spontaneous skin inflammation [13, 356]. In apoptotic cells, pro-IL-1α concentrates in dense nuclear foci, being retained in the chromatin fraction and not released from the cytoplasm [313]. Necrosis leads to the release of pro-IL-1α from the cells, and loss-of plasma membrane integrity could activate Calpain, to subsequently cleave pro-IL-1α into mature IL-1α, with elevated affinity to IL-1R1 [313, 357]. Once released into the extracellular milieu, IL-1α could bind to the IL-1R1 and stimulate a number of inflammatory events including cytokine secretion, neutrophil recruitment and antigen-presenting cell activation [142]. IL-1α is important for adaptive immunity as well, as a powerful cytokine to enhance the expansion, survival and differentiation of T cells [358]. We found that the depletion of CD109 resulted in an increased expression and secretion of IL-1α in primary keratinocytes (Figure 3.1), indicating that CD109 is a keratinocyte-intrinsic regulator of the IL-1α. IL-1α subsequently promotes IL-17 production by γδT cells *in vivo* and *in vitro* (Figure 3.3 and 3.4). However, genetically depletion of IL-1R1 in CD109 deficient mice did not reverse the activation of γδ17 cells (Figure 3.5), which is needed to be further explored. Studies are currently underway to dissect the mechanisms by which CD109 could shift the focus from an IL-23/IL-17 axis to a IL-1α/γδ17 cell axis.

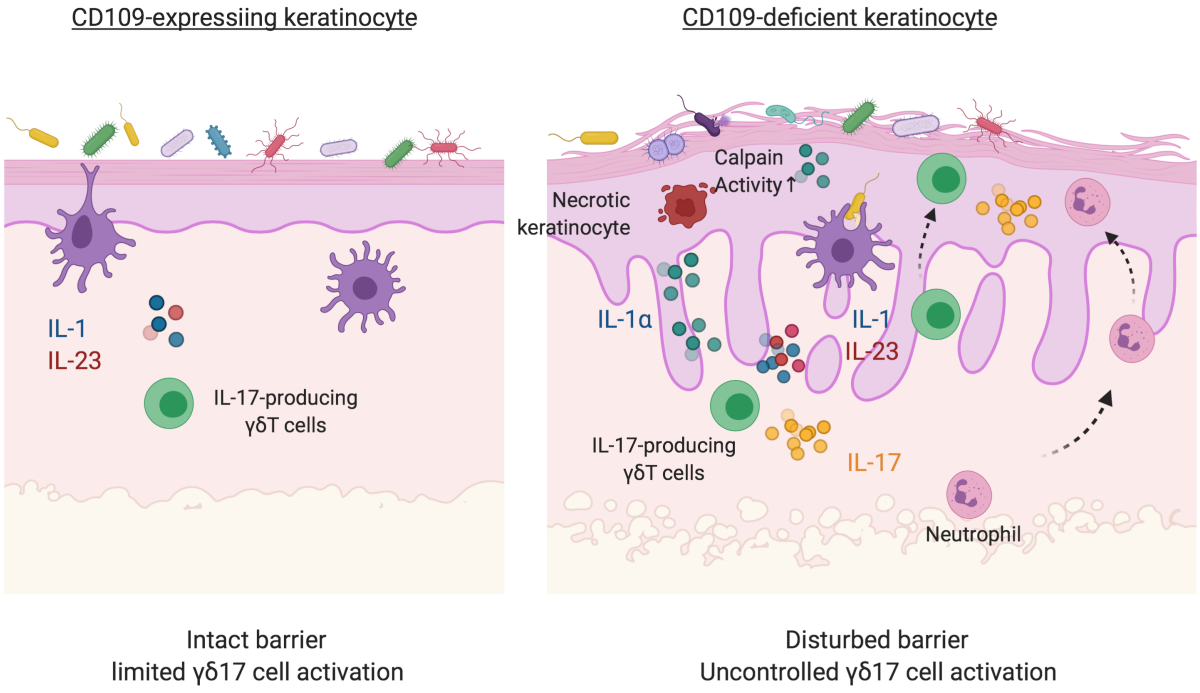

Figure 4.3. Summary of Chapter 3.
At steady state, keratinocytes could form an intact skin barrier, which limit the γδ17 cell activation. CD109-deficient in keratinocytes could result in increased Calpain activity and keratinocyte necrosis. Necrotic cells release more IL-1α, together with IL-23, could induce uncontrolled activation of γδ17 cells.

4.4 Concluding remarks

Skin is a complex coordinated system where stromal cells and immune cells ensure a controlled immune response against physical injury, toxins and pathogens while maintaining tissue homeostasis with the help of commensal microbiome. However, the mechanisms by which keratinocytes orchestrate skin immunity is poorly understood. In Chapter 2, I identified that CD109 regulates the cutaneous IL-23/IL-17 immune axis in a T cell-extrinsic manner. In addition, loss of CD109 led to disturbed barrier integrity and pathological reactivity to commensal microbiota. In Chapter 3, IL-1α was defined as keratinocyte-derived cytokine dysregulated in the absence of CD109 that led to spontaneous IL-17 production and cutaneous inflammation. We provide data suggesting that CD109 regulates IL-1a by limiting the activity of Calpain, an event that is associated with increased keratinocyte necrosis. This collective work adds depth and understanding to how keratinocytes act as immune sentinels in controlling skin health.

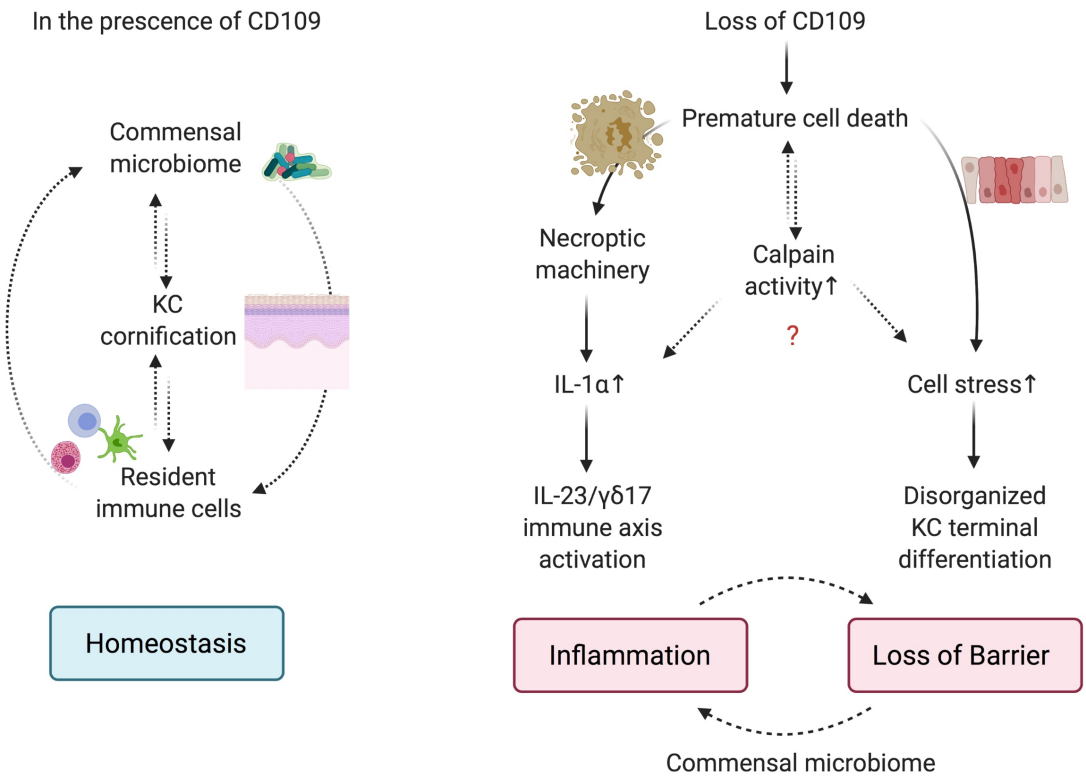

Figure 4.4 Working model.
At steady state, the commensal microbiota, keratinocyte and resident immune cells work together to maintain the homeostasis of skin. However, CD109-deficiency disturbs the keratinocyte cornification, inducing the premature cell death of keratinocyte in either necrotic machinery or cell stress. Calpain activity is also increased when loss of CD109. Necrotic cells release IL-1α, which subsequently activate the IL-23/γδ17 immune axis, resulting in skin inflammation. Elevated cell stress causes disorganized keratinocyte terminal differentiation and the loss of cutaneous barrier integrity. Commensal microbiome is also involved in this process, by accelerating the inflammation and barrier dysfunction.

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