The Regulation of Skin Immunity by CD109 with Relevance to Psoriatic-like Inflammation

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Contributions

The main thesis objectives were planned and directed by the laboratory's Principle Investigator, Dr. Irah King. The planning of experiments and techniques were a collaborative effort between Dr. King and Giustino Carnevale. All experimental animals were taken care of and managed by Dr. Ghislaine Fontés. The totality of each experiment was conducted by Giustino Carnevale, with the exception of some RT-qPCR assays and phospho-protein analysis performed by Émile Fortier and Dr. Ghislaine Fontés, respectively. Throughout various experiments, vital assistance was generously provided by Dr. Ghislaine Fontés, Alexandre Meli, Marilena Gentile and Émile Fortier. All data analysis was performed by Giustino Carnevale, with the exception of some RT-qPCR results analyzed by Émile Fortier. Funding for this MSc thesis project was provided by Canada Research Chair awarded to Dr. King, The Canadian Institutes of Health Research and the Faculty of Medicine, McGill University.

Abstract

Mammalian skin contains a complex network of immune and stromal cell types that serve an indispensable role in host defense against environmental insults and invasion by pathogenic microorganisms. In contrast, aberrant cutaneous immune responses may provoke the development of unwanted skin inflammation and disease. One of the most common inflammatory skin disorders, Psoriasis, is driven by interleukin (IL)-17-producing T lymphocytes. Specifically, a subset of innate-like γδT cells resident to the dermis that produce high levels of IL-17 (γδ17 cells) have recently been implicated in the progression of psoriatic disease. However, the pathways that regulate γδ17 cells have not been determined. The GPI-anchored surface protein CD109 promotes wound healing and regulates skin inflammation via inhibition of TGF-β and STAT-3-dependent signalling, pathways critical for γδ17 development and activation, respectively. However, the role of CD109 in cutaneous immunity has not been investigated. We hypothesized that CD109 acts as a negative regulator of skin γδ17 cells and psoriasiform-like inflammation. Examination of mice genetically deficient in CD109 ($CD109^{-/-}$) revealed spontaneous epidermal hyperplasia, transcriptional activation of the IL-23/IL-17 immune axis and enhanced T cell infiltration into both epidermal and dermal compartments compared to wild-type mice. In addition, $CD109^{-/-}$ mice harboured an increased number of γδ17 cells in the dermis and cutaneous lymph nodes under steady state conditions. Upon induction of psoriatic-like inflammation following topical imiquimod treatment, $CD109^{-/-}$ mice exhibited exacerbated skin pathology including epidermal thickening and increased accumulation of γδ17 cells compared to wildtype controls. Importantly, bone marrow chimera studies determined that expression of CD109 by a radio-resistant cell type was critical for controlling γδ17 cell responses and skin inflammation. Collectively, these results identify a previously unknown T cell-extrinsic pathway regulating γδ17 cells in the skin and highlight CD109 as a novel target for the control of IL-17 dependent skin immunopathology.

Résumé

La peau est un organe dédié à la protection des tissus internes contre la déshydration, les substances nocives et les agents pathogènes. Cette protection cutanée, dirigée par les cellules immunitaires et stromales, est indispensable pour maintenir une peau saine. Par contre, des réponses immunitaires aberrantes augmentent le risque de développer des maladies inflammatoires indésirable. Le psoriasis, une des maladies inflammatoires de la peau la plus commune, est principalement causée par la production d'interleukine-17 (IL-17) par les lymphocytes T. Plus précisément, une population de lymphocytes γδT localisée dans le derme, ayant la capacité de produire de fort taux d'IL-17 (γδ17), a été récemment impliquée dans la progression du psoriasis. Toutefois, les voies de signalisation qui régulent les cellules γδ17 restent à être identifiées. La molécule CD109, une protéine de surface ancrée par le GPI, contribue à la cicatrisation des blessures et à la régulation des réponses inflammatoires *via* l'inhibition de la signalisation médiée par TGF-β et STAT-3, qui sont respectivement impliquées dans la différentiation et l'activation des cellules γδ17. Toutefois, le rôle de CD109 dans la régulation de la réponse immunitaire cutanée n'a pas encore été exploré. Notre hypothèse est que CD109 agit comme régulateur négatif des cellules γδ17 dermales et sur l'inflammation liée au psoriasis. L'analyse de souris dont CD109 est génétiquement délité ($CD109^{-/-}$) révèle une hyperplasie épidermique, une infiltration augmentée de lymphocytes T dans la peau, ainsi qu'une augmentation de la transcription de l'axe IL-23/IL-17 comparativement aux souris sauvages. De plus, la peau et les ganglions lymphatiques cutanés des souris $CD109^{-/-}$ sont naturellement enrichis de cellules γδ17. En condition d'inflammation psoriasique, suite à des traitements topiques avec Imiquimod sur la peau, les souris $CD109^{-/-}$ développent une pathologie de la peau accrue, avec une augmentation de l'hyperplasie épidermique et une accumulation de cellules γδ17, comparativement aux souris sauvages. De plus, les études utilisant des souris chimériques de moelle osseuse montrent que l'expression de CD109 par les cellules cutanées radio-résistantes est critique pour contrôler les réponses des cellules γδ17 et l'inflammation de la peau. Collectivement, ces résultats identifient un mécanisme inédit indépendant des cellules T pour contrôler les cellules γδ17 cutanées et met en avant le rôle de CD109 comme une nouvelle cible pour le contrôle des immunopathologies dépendantes d'IL17.

Introduction

The skin is constantly exposed to chemical, physical and biological threats. Keratinocytes, the epithelial cells of the skin, provide an effective physical barrier, while leukocytes such as dendritic cells and T cells within and underlying the epidermis act as skin immune sentinels. Among other myeloid and lymphoid cells, they act as an immunosurveillance system and direct the inflammatory immune response following pathogen challenge or tissue damage. However, when the cutaneous immune responses are not properly controlled, it may result in aberrant inflammation such as that observed in psoriatic disease. Psoriasis is a chronic inflammatory disease of the skin currently affecting 2-3% of the world's population[2]. Symptoms are often manifested as red, dry, scaly plaques due to a dysregulation of keratinocyte proliferation and maturation with accumulation of infiltrating leukocytes into affected areas. This results in epidermal thickening, compromised barrier integrity and interleukin-17 (IL-17)-driven inflammation. While it remains uncertain what triggers disease onset, it is evident, that disease establishment occurs due to a loss of effective crosstalk between keratinocytes and cutaneous leukocytes and their ability to restore homeostatic conditions.

In psoriatic disease, IL-23 release by skin-resident dendritic cells and macrophages drives the activation or expansion of αβ and γδ T cell subsets that predominantly secrete IL-17 (referred to as Th17 or γδ17 cells) rather than quintessential Type 1 or Type 2 cytokines such as interferon-γ (IFNγ) or IL-4, respectively[3, 4, 5]. Previously, treatments for psoriasis have been limited to molecular inhibitors of IL-23 or TNF-α[3, 6, 7]. While these treatments were relatively successful in quelling psoriatic inflammation, they did not provide effective treatments to counter refractory psoriasis, and are often unsuccessful in patients suffering from moderate to severe forms of the disease[8]. As a result, many antibody-based drugs targeting IL-17 or its associated receptor, IL-17RA have been developed and recently FDA-approved for the treatment of moderate to severe psoriasis[7]. Although these therapies have shown great promise, they must be taken for the lifetime of the individual and may leave patients susceptible to invasive cutaneous infections. Thus, greater knowledge of regulatory pathways that limit the IL-23/IL-17 immune axis could lead to more effective strategies to alleviate psoriatic inflammation. Production of IL-17 by both Th17 and γδ17 cell types is dependent upon STAT-3 signaling following IL-23, IL-6 and IL-21 stimulation. In

addition, TGF-β signaling also plays a key role in the development and differentiation of IL-17 producing subsets[5]. Interestingly, CD109, a glycosylphosphatidylinositol (GPI)-anchored cell surface glycoprotein[9], negatively regulates TGF-β signaling and is suggested to control STAT-3 levels in keratinocytes[10, 11, 12]. In human psoriatic skin, CD109 expression is downregulated compared to uninvolved tissue suggesting a regulatory role for this protein in skin disease[11, 12, 13]. However, the role of CD109 in the immune system had not been investigated. We hypothesized that CD109 limits aberrant inflammation by regulating IL-17 producing T cells in the skin. The experiments in this thesis were designed to address two main aims. First, to determine whether CD109 regulates the IL-23/IL-17 immune axis under homeostasis conditions in the skin, and second, determine the impact of CD109 in skin immunity using a mouse model of psoriasiform-like inflammation. Uncovering mechanisms that regulate IL-17-producing cells is key to developing novel targeted therapies that alleviate the pathology associated with psoriasis and promote skin health.

Review of Relevant Literature

1. The skin: the gatekeeper of our bodies

The skin represents the most intimate association between a living organism and their immediate environment. It spans the entire surface of the body, moves with it, protects it, and regenerates upon injury. The skin is a dynamic interface at which living creatures interact and process information about their surroundings. In mammals, its functions include thermoregulation, hydration, sensory detection as well as providing effective barrier functions[14]. Serving as a physical, chemical, biological and immunological barrier, the skin constantly protects vital internal organs and tissues[15, 16].

1.1 Skin structure and function overview

Structurally, the skin is composed of two layers separated by a basement membrane: the outer epidermis and the inner dermis, both positioned atop an underlying subcutaneous fatty layer. The epidermis, comprised of structural cells called keratinocytes, is organized into four overlying layers. The deepest of these layers is the single-celled stratum basale composed of progenitor keratinocytes, which give rise to cells of the next layer, the stratum spinosum[15]. Keratinocytes entering this next layer differentiate and mature from a columnar to a more polygonal conformation or divide to replenish the stratum basale[15]. As the keratinocytes migrate upwards, they progressively lose their nuclei and replace their cytoplasm with keratin, a process known as cornification. This process begins in the next layer, known as the stratum granulosum and continues into the stratum corneum, the outermost layer of the skin composed of anuclear, flattened lipid-rich corneocytes[15]. Despite the rapid turnover of the mammalian epidermis (a tissue that is completely replaced every 5-7 days), the combination of tight junction proteins linking the stratum corneum to the stratum granulosum with the compact configuration of corneocytes provides an effective waterproof seal to the outside world[16].

In addition to the structural components of the skin, the epidermis is colonized by commensal microbes of bacterial, viral and fungal species, providing a natural biological barrier against damage-causing microbes[16, 17]. However, this microbial barrier does not exclude the possibility that normally harmless commensal microbes may become pathogenic upon loss of

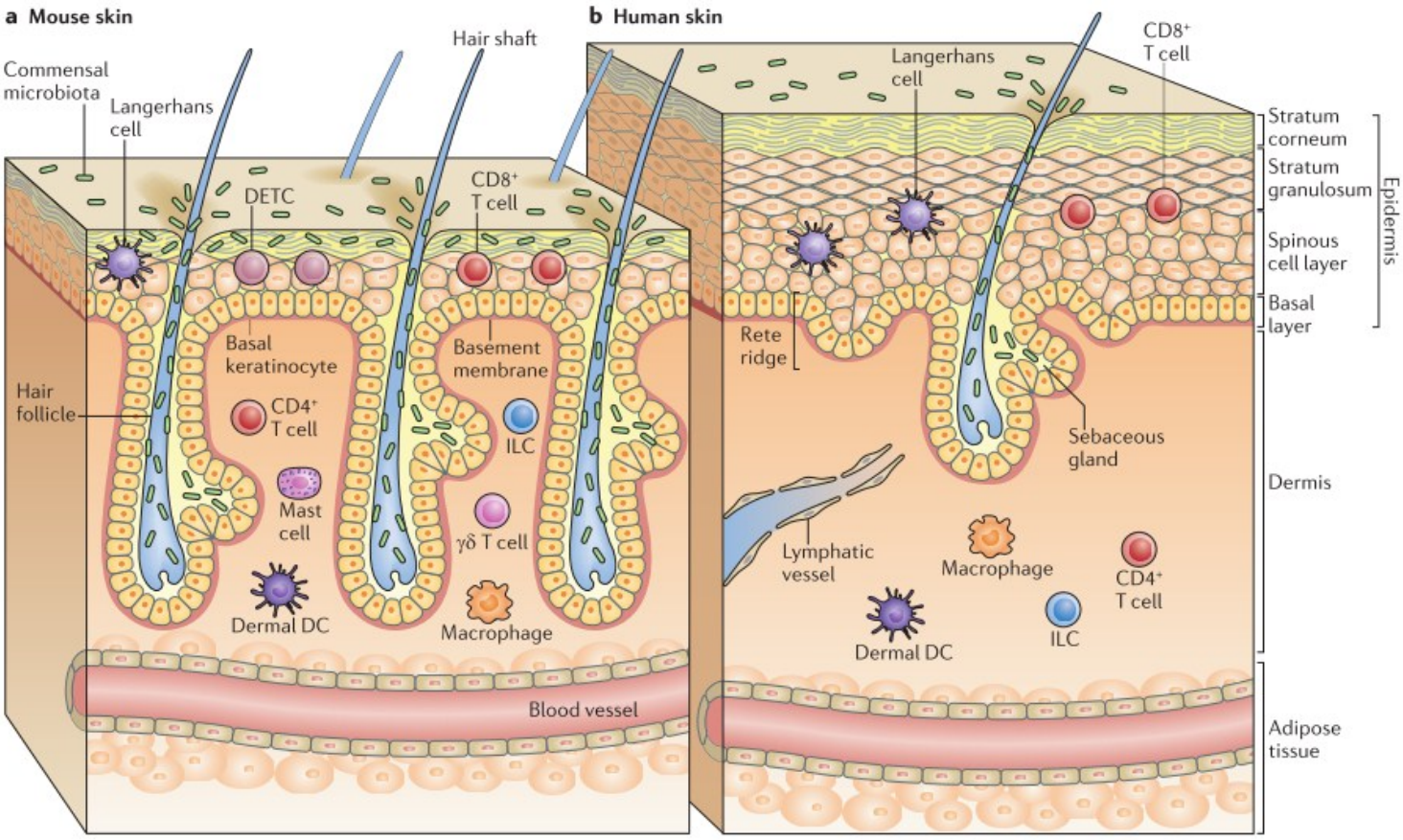


Image 1 – Overview of human and mouse skin. This image highlights some major differences and similarities in the structure and cellular composition of human and mouse skin. [1]

barrier integrity or microbial dysbiosis[18]. To maintain symbiosis with the microbial world, epidermal keratinocytes are equipped with the capacity to secrete chemical ammunition in the form of anti-microbial peptides (AMPs) and S100 proteins that limit the migration and/or survival of commensal and pathogenic microorganisms[16]. This epidermal barrier is buttressed by Langerhans cells (dendritic cells of the epidermis) and innate T cell subsets, while the dermis deploys a more diverse infantry involving both innate and adaptive arms of the immune system.

Specifically, the dermis is populated by dendritic cells, macrophages, mast cells, natural killer T cells, αβT cells, γδT cells and innate lymphoid cells (ILCs). Structurally, the dermis is composed of dense connective tissue, such as collagen and extracellular matrix secreted primarily by fibroblasts and vascularized by both blood and lymphatic vessels [1, 15]. Although the dermal immune and structural microenvironment differs substantially from the epidermis, their complementary features maximize the strength of the skin barrier.

1.2 The skin's defenses must protect from constant exposure to environmental threats

The skin must cope with and respond to constant exposure to potential pathogens, and at the same time avoid reacting towards self-antigens. As a result, both the epidermis and dermis are populated by various innate and adaptive immune cells which contribute to the extensive protective functions of the skin.

1.2.1 Keratinocyte innate immunity, leading by example

Keratinocytes do more than just provide a physical barrier to the immediate environment, they actively participate in innate immunity. They express pattern recognition receptors (PRRs), toll-like receptors (TLRs) and Nod-like receptors (NLRs) on their cell surface which recognize evolutionarily conserved molecular components, known as pathogen-associated molecular patterns (PAMPs), expressed by microbes[19]. In addition to PAMPs, they can also recognize danger/damage-associated molecular patterns (DAMPs)[20]. In response to pathogenic stimuli, keratinocytes produce and release anti-microbial peptides (AMPs) such as LL-37, β-defensins, S100 proteins and RNases which function as bactericides[20]. Keratinocytes not only provide direct assault to foreign invaders, but can recruit hematopoietic cells to the site of inflammation by secreting chemokines such as CXCL9, CXCL10, CXCL11 and CCL20[1]. Additionally, keratinocytes help modulate immune cell activation and effector function by producing cytokines such as tumor necrosis factor (TNF), IL-6, IL-10, and IL-1 family cytokines (IL-1α, β and IL-18)[1]. Overall, the epidermis functions as an effective innate barrier to protect underlying organs from environmental toxins and harmful biological agents. Thus, keratinocytes act as a natural innate defense system.

1.2.2 Skin immunocytes orchestrate and carry out skin inflammation and repair

Skin contains a diverse repertoire of leukocytes that are either constitutively present or recruited during immune challenge. Of particular relevance to our studies, are the myeloid and T cell lineage cells. In mice, three subsets of dendritic cells (DCs) have been identified: Langerhans cells, $CD103^{-}CD11b^{+}$ DCs and $CD103^{+}CD11b^{lo}$ DCs[20, 21]. Although humans and mice have comparable subsets of skin DCs, $CD11b^{+}$ equivalent cells found in human skin are either a $CD1c^{+}$ subset of pre-DC origin, or more mature $CD14^{+}$ monocyte-derived DCs[22]. In addition, human

$CD141^+$ DCs are analogous to $CD103^+$ DCs in mice, where they both function in self-tolerance by cross-presenting self-antigens to $CD8^+$ T cells in the skin, as well as being responsible for viral immunity[22].

In addition to skin DC subsets, the dermis is home to skin-resident macrophages as well as circulating monocytes. These populations have similar roles as skin DCs and contribute to immune surveillance as well as initiation of inflammatory responses, either locally or following migration to skin-draining lymph nodes[23]. There are two distinct subsets of macrophages in the skin. First, the classically-activated M1 macrophages which, following engagement of bacterial or viral antigens play a key role in resolving acute infections. Second, the regulatory M2 macrophages (a.k.a. wound-healing macrophages) that contribute to resolution of response and tissue repair by their production of IL-10 and TGF-β, respectively[1].

In terms of the adaptive immune compartment of the skin, there are nearly double the number of αβT cells in human skin than in the circulation, all of which memory or antigen-experienced phenotype[24]. In the epidermis, tissue-resident memory T cells are $CD8^+$ and are not found in periphery, whereas the dermis contains memory $CD4^+$ T cells in transit from the systemic circulation[24, 25] Roughly 10% of the $CD4^+$ T cell population express FoxP3, a transcription factor endowing them with immunosuppressive properties[25]. The epidermal $CD8^+$ T resident memory cells express CD103, the ligand for E-Cadherin expressed on epithelial cells, and enables their retention in the epidermis in a TGF-β signaling-dependent manner[26].

The skin of both mouse and humans is also populated by an innate-like subset of T cells expressing a non-conventional γδT cell receptor (TCR). In mice, they are the exclusive $CD3^+$ residents of the epidermis, while dermal γδT cells are found in cohabitation αβT cells in both species[27]. Dermal γδT cells have been shown to be the predominant source of IL-17 in the skin[28] and hence play important roles in protection to bacterial and fungal infections. They respond to pro-inflammatory cytokines produced by dendritic cells or keratinocytes and can be activated and provide effector response within hours[29]. In addition, γδT cells have been reported to enhance adaptive immune responses by enhancing the priming of αβT cells, such as during mycobacterial infection[30].

Overall, the mammalian skin is well adapted for a flexible texture to allow motility of complex organisms, yet is well equipped with a sophisticated cellular battalion to protect its host from infection and damage.

1.3 Psoriasis as a quintessentiel IL-23/IL-17 immune axis skin disease

Immune-mediated inflammatory skin diseases, such as psoriasis, atopic dermatitis, eczema and contact dermatitis are merely four examples of common skin disorders experienced by ~5% of the global population[31]. In general, skin diseases pose great economic, social and individual burdens worldwide[31]. Of these, psoriasis is one of the most widely studied and documented skin diseases, with extensive human data and several established mice models[15]. This can be attributed to the relevance of psoriasis research compared to many other inflammatory skin diseases, as well as its relevance to other chronic inflammatory diseases with unknown etiology, such as Rheumatoid Arthritis and Crohn's disease[15].

Psoriasis is a chronic inflammatory skin disease affecting 1-3% of the world's population, characterized by periods of relapse and remitting skin pathology[2, 32]. There are many different forms of psoriatic disease but it is generally characterized by the development of red, painful, scaly plaques, and depending on the severity, can cover all parts of the body. It is also a life-debilitating disease with symptoms extending far beyond skin pain and discomfort. For example, longevity of psoriasis patients is reduced, as a cohort of men afflicted by severe psoriasis had an average reduced lifespan of 3.5 years and women with psoriasis lived 4.4 years less than an unaffected control population[33]. Additionally, psoriasis commonly results in low self-esteem and loss of productivity, affecting the overall physical and mental well-being of patients[34]. On top of this, psoriasis patients can be more prone to developing secondary illnesses such as cardiovascular disease, depression or arthritis, known as psoriatic arthritis[2, 34] In 2012, it was estimated 1 million Canadians suffer from psoriasis, from which 85% are diagnosed with the most common form, plaque psoriasis[34]. Of these affected individuals, approximately 25% of them have experienced moderate to severe forms of psoriasis[34]. This latter form of the disease afflicts roughly 200 000 people in Canada, amounting to an estimated annual cost of 1.7 billion dollars, with about 40% of this being an indirect effect of loss in productivity[33].

The exact causes of psoriasis are currently unknown, but environmental, genetic or chemical triggers coupled with impaired barrier function and a dysfunction in the immune system may all contribute to disease progression. In fact, it is still debated whether the trigger disrupts or activates keratinocytes which then miscommunicate with cutaneous immune cells to initiate inflammation, or whether underlying immune dysfunction is the precipitating event. Genome-wide association studies have identified a correlation between the incidence of psoriasis and particular

genetic loci. For example, patients with psoriasis are more likely to express a particular HLA-Cw0602* allele of HLA-C allele that contains 10 genes termed the psoriasis susceptibility 1 (PSORS1) locus[35, 36]. Interestingly, a 1999 study found that as much as 60% of psoriasis patients express the HLA-Cw0602* allele, and this gene is present in approximately 15-20% of the population[37]. The precise role that this particular HLA molecule might play in the onset of psoriasis is currently not known, but it may affect the cellular and immunological behaviour of antigen-presenting cells that express it[2].

Histologically, psoriasis is characterized as epidermal hyper-proliferation and impartial differentiation of keratinocytes, immune cell infiltrates and altered cutaneous and systemic cytokine production[11]. Initially thought to be a Th1-driven inflammatory disease, the neutralizing antibody Ustekinumab, which targets the p40 subunit of both IL-12 and IL-23 cytokines, was effective in treatment therapy[38]. Around the same time, the Th17 lineage was discovered and given the beneficial effect of IL-17 blockade in psoriasis, the effect of Ustekinumab is now attributed to its ability to antagonize IL-23 signaling. These results corroborate other human studies in which elevated levels of both IL-23p19 and IL23p40 mRNA transcripts were detected in lesion biopsies in comparison to uninvolved skin tissue[39]. In addition, immunofluorescent microscopy studies have shown IL-23 to co-localize with $CD11c^+$ dendritic cells (DCs) and CD68+ macrophages in human psoriatic skin[28]. These clinical findings were largely informed by inducible mouse models that recapitulate many features of psoriatic-like inflammation. For example, intradermal injection of mice with recombinant IL-23 results in the development of psoriatic-like skin inflammation after 4 days of treatment[40]. In another mouse model of psoriasis, topical administration of imiquimod (a TLR-7 agonist) results in red, scaly, psoriatic-like plaque formation[41]. Wild-type mice treated with imiquimod also demonstrated IL-23p19 co-localization with DC and macrophages in lesional skin suggesting these cell types are the primary source of IL-23 in psoriatic skin[28].

A dominant effect of IL-23 is to promote the survival and/or expansion of IL-17 producing T cells[28]. Indeed, Cai et al. demonstrated that dermal γδT cells are the predominant source of IL-17 in the skin following *in vitro* stimulation by IL-23 and following IMQ treatment in lesioned skin. The relevance of IL-17 in psoriasis development was reinforced when recombinant IL-23 was injected into skin of $IL\text{-}17^{-/-}$ mice and failed to induce psoriatic-skin lesions[40]. In addition, $TCR\delta^{-/-}$ mice treated with IMQ had reduced ear thickness compared to wild-type mice following

7 days of treatment, providing supportive evidence that γδT cell-derived IL-17 is responsible for psoriatic plaque formation in mouse models of psoriasis[42]. Further data supporting these results is that γδ17 cells in IMQ treated mice require the expression of RORγt, a transcription factor deemed the master regulator of IL-17 production[42, 43]. In mice, the IL-17 producing γδT cells are enriched for cells expressing the Vγ4 subunit of the γδ TCR. A potential human equivalent to murine $V\gamma4^{+}ROR\gamma t^{+}$ IL-17 producing γδT cells, may be the $V\gamma9^{+}V\delta2^{+}$ expressing T cells. These γδT cells are enriched in human psoriatic lesions and depleted in the blood of patients with active disease. Importantly, this population could be restored in the peripheral blood following various systemic anti-psoriasis therapies[44]. The significance of this finding is that current mouse models of psoriasis are a relevant and effective method to study the activation, function and regulation of γδ17 cells, in order to apply basic knowledge of this cell type to human disease.

2. The best and worst of γδT cells

γδT cells are a unique lineage of T cells that have been evolutionarily conserved for 430 million years and continue to serve important roles in host defense[45]. Although their adaptive counterparts, αβT cells, provide a robust and tailored response upon immune challenge, they depend on interaction with cognate antigen presented in the context of major histocompatibility complexes (MHC). This results in a delayed response leaving a gap in time where invaders can take advantage of an adaptive immune response still in the chaos of setting up its troops. To compensate for this gap in defense, γδT cells act as first responders to tissue damage or pathogen invasion and can react independently of MHCI interactions. In addition, they are strategically localized at barrier surfaces such as the skin, lung and gut where they can rapidly respond to environmental threats. Although their functions are diverse, they primarily complement other mechanisms of the innate immune response by providing a potent source of effector cytokines such as IFN-γ and IL-17 that enhance neutrophil recruitment, support anti-tumor and anti-viral cytotoxicity, and drive secretion of epithelial cell growth factors to promote tissue repair [45, 46, 47, 48].

2.1 γδT cells develop in the thymus and populate barrier surfaces

T cell development in the thymus results in the generation of two distinct T cell lineages, known as the αβT cells and γδT cells[49]. These two cell types share a common CD4-CD8- stage 2 double-negative (DN2) lymphocyte progenitor and both undergo V(D)J recombination to generate their antigen-specific TCR[45, 50]. γδT cells complete their thymic development before αβT cells and although some take up residence in lymph nodes and spleen (red and white pulp), they primarily populate epithelial-associated sites, such as the skin, gastro-intestinal, uro-genital and pulmonary tissues[48, 51]. While some γδT cells can exit the thymus as mature T cells and can acquire effector function in the absence of antigen, a significant proportion of γδT cells also leave the thymus as immature cells, and rely on engagement of cognate antigen for their activation[45]. However, a key feature of γδT cells is that their effector fate is pre-determined in the thymus based on antigen exposure during embyonic development [52]**.**

γδT cell progenitors in the thymus can be detected by cell surface expression as early as embryonic day 14[53, 54], suggesting their importance in neonatal immunity[48]. Their contribution to neonatal immunity is further supported by studies showing that in athymic mice γδT cell development is retained in the fetal liver and gut[54]. Studies by Jensen et al, also demonstrated that some transgenic γδT cells that recognize the MHC-I β-microglubulin associated molecules T10 and T22 (two known ligands for γδT cells) still developed and exited the thymus in mice lacking β2m (β2m$^{-/-}$). This was also supported by two other independent groups, Schweighoffer/Fowlkes and Correa et al, suggesting that γδT cells also do not require positive or negative selection of their TCRs[55, 56]. Therefore, the requirement for antigen engagement in γδT cell development remains controversial. By contrast, thymic antigen encounter can contribute the ultimate effector fate of γδ T cells. Indeed, Jensen et al demonstrated that γδT cells that do encounter thymic ligands develop into IFN-γ producers, whereas those which do not encounter antigen during thymic development become programmed to produce IL-17[52].

Finally, upon γδT cell egress from the thymus, these cells localize to organized secondary lymphoid organs and mucosa-associated lymphoid tissues (MALT). In adult humans γδT cells comprise roughly 4% of total CD3$^+$ T cells in all anatomical locations, such as peripheral blood, lymph nodes, tonsils as well as mucosal epithelium[57]. Whereas in adult mice γδT cells are biased towards populating MALT, including the reproductive tract (10-20% of total T cells) intestinal epithelium (20-40%), but also the skin [30, 57].

2.2 Cutaneous γδT cell types and function

In mouse epidermis, γδT cells are known as dendritic epidermal T cells (DETC). These DETCs express Vγ5Vδ1 TCR and represent a monoclonal population in the epidermal layer of the skin[45]. They are the first population of γδT cells found in the fetal thymus and migrate to the epidermis where they make up 100% of the T cells in that tissue[30, 45, 53]. To date, however, there is no known ligand for this γδT cells subset, representing a current and relevant challenge in the field to study these DETCs. It is thought that these cells bind to self-antigens expressed by keratinocytes during epidermal damage or stress, where they take part in inflammation, tumour killing and wound healing[46, 47]. Although in mice, DETCs are the only T cells populating the epidermis, both γδ and αβ T cells are found in human epidermal tissue[27].

Murine dermal populations of γδT cells represent 60% of total $CD3^+$ T cells and are a more heterogenous population whereby ~30% of γδT cells express Vγ4 receptors and the remaining are neither $V\gamma4^+$ nor $V\gamma5^{+}$[30]. Both dermal and epidermal γδT cells are largely radio-resistant and undergo homeostatic proliferation within the skin compartment[58]. During inflammation however, a subset of Vγ4-expressing T cells has been shown to migrate to skin-draining lymph nodes where they undergo expansion before returning to the skin[43]. Therefore contrary to their epidermal counterparts, dermal γδT cells represent a more motile population able to circulate through the blood and lymphatic vessels, whereas DETCs remain constrained to the epidermis[59].

As mentioned above, DETCs express monoclonal TCRs and are therefore likely to recognize specific pathogen-associated antigens[48]. They are also known to primarily produce IFN-γ, and have a cell surface phenotype of $CD27^+CD62L^+CD44^-CCR6^-$. In addition, DETCs have cytolytic properties by producing and secreting Granzyme B and perforin, therefore contributing to anti-tumor responses[47, 51 45]. On the other hand, dermal γδT cells can be characterized as $CCR6^+CD27^-CD62L^-CD44^+$ cells and both IL-17 or IFNγ-producing subsets have been identified[45]. Most ligands for γδT cells still remain unknown, including the DETC population. While some ligands for γδT cells, such as phosphoantigens and non-classical major histocompatibility complex (MHC) molecules, are currently known, current research aims at identifying novel ligands for γδT cells, particularly those which activate the monoclonal epidermal subtype[48].

2.3 Contributions of γδT cells to health and disease

Contrary to αβT cells, which bind processed antigen presented on an MHC molecule and require clonal selection before acquiring effector function, γδT cells can respond and secrete cytokines in a much quicker time frame[27, 52]. This property of γδT cells make them the ideal cell type to provide a rapid and early start to the adaptive immune response upon tissue damage or pathogen challenge, until further re-inforcements arrive from the adaptive immune compartment. γδT cells have an important relationship with their surrounding epithelial cells. For example, following cutaneous tissue damage, DETCs sense and respond to keratinocyte-derived stress antigens, in a TCR-dependent manner[46]. Specifically, DETCs produce keratinocyte growth factor (KGF) also known as, fibroblast growth factor-7 (FGF-7), and FGF-10, which bind to their respective receptors on keratinocytes to promote proliferation and tissue repair[46]. This important property in skin regeneration was highlited in TCR$\delta^{-/-}$ mice, which had a 2-3 day delay in would closure compared to wild-type mice, as well as reduced keratinocyte hyperproliferation, a feature associated with γδT cell-derived keratinocyte growth factors[46]. The role of γδT cells in maintening epithelial cell integrity is also mirrored by the intra-epithelial lymphocyte (IEL) population of γδT cells in the gut, which can also secrete KGF and maintain the regeneration of intestinal epithelial cells (IECs)[27]. To understand the role of γδT cells function in the intestinal epithelieum, both TCR$\delta^{-/-}$ and TCR$\beta^{-/-}$ mice were compared in a DSS-induced model of colitis. Mice lacking γδT cells developed enhanced pathology compared to mice lacking αβ-T cells, and upon removal of DSS a delay in the recovery from colitis was seen in the mice lacking γδT cells[27]. Both Jameson and Paul demonstrated consistent roles for γδT cells in maintaining tissue homeostasis, most prominently at epithelial surfaces of mucosal sites, as well as providing early responses upon tissue damage as well as the crucial functions of wound repair.

In contrast to the beneficial roles for γδ T cells in tissue homeostasis and repair, γδT cells have been identified as the primary producers of IL-17 in mouse models of psoriasis and are present in high numbers in human psoriatic lesions[28]. In mice lacking γδT cells or the IL-17 receptor, reduced neutrophil recruitment to the skin, as well as reduced thickening of the epidermis was observed compared to wild type mice, confirming the negative contribution of γδT cells in skin immunopathology[28]. Consistent with these data, mice that contain a mutation in their gene encoding the transcription factor Sox13 are deficient in IL-17-producing V$\gamma4^{+}$ γδT cells and protected from mouse model of psoriasis-like dermatitis[43, 60]. In addition to colitis and psoriasis,

γδT cells have also been implicated in the pathogenesis of other auto-immune diseases such as rheumatoid arthitis, multiple sclerosis and Sjogren's syndrome[27]. However, factors that control the function of γδ17 cells in health and disease remains relatively unexplored.

3. CD109: The guardian molecule of the skin?

3.1 Getting to know CD109

Cluster of Differentiation 109 (CD109) is a glycosylphosphatidylinositol (GPI)-linked surface glycoprotein and a member of the α_2 macroglobulin/C3, C4, C5 family of thioester-containing proteins[61 12, 62]. The protein's GPI-anchor region makes CD109 susceptible to phosphoinositol-phospholipase C (PI-PLC), and hence can be released in solution as a soluble glycoprotein[9, 62]. It has been reported to be expressed on a subset of fetal and adult $CD34^+$ bone marrow cells which constitute the majority of hematopoietic stem cells and progenitor cells of the myeloid lineage[63]. CD109 protein was also described to be expressed by activated T cells, thrombin activated platelets, leukemic megakaryoblasts, keratinocytes, testis, basal cells of the prostate, epithelium and myoepithelial cells of mammary, lacrimal, salivary and bronchial glands[12]. In addition, CD109 mRNA expression is detected at very high levels in tumour cell lines as well as in human tumour tissues, in particular squamous cell carcinomas in various barrier tissues, including malignant melanoma of the skin[12, 64, 65]. Not only is CD109 up-regulated in tumour cells, but is highly expressed on a subset of circulating endothelial cells enriched in cancer patients and is being considered a molecular target for cancer therapies[65, 66].

Among these tissues and cell types, the function of CD109 has primarily been studied in human keratinocyte HaCaT and N/TERT-1 cells lines by the group led by Dr. Anie Philip. Their data demonstrate that CD109 binds to the transforming growth factor-β receptor (TGFβR) and negatively regulates TGF-β signaling[10]. More recently, a second independent research group led by Dr. Takahashi also demonstrated CD109 negatively regulates TGF-β signaling in the SK-MG-1 glioblastoma cell line[67]. Indeed, transgenic mice overexpressing CD109 specifically in the epidermis (K14 promoter-driven) display lower levels of phosphorylated Smad2 (pSmad2) and

Smad3 (pSmad3), two key transcription factors downstream of canonical TGF-β signaling, during skin wound healing[68]. On the other hand, immunostaining for phosphorylated-Smad2 in mice genetically deficient in CD109 ($CD109^{-/-}$) showed no difference in epidermal staining compared to wild-type mice[12]. Despite these seemingly contradictory results, more dermal cells of $CD109^{-/-}$ mice appeared to be stained with anti-pSmad2 compared to wild-type skin, however the authors did not quantify this data nor make reference to it in their results. Taken together, these results indicate that CD109 is a co-receptor and negative regulator of TGF-β signaling in vitro, but evidence that it acts in a similar manner *in vivo* remains to be confirmed.

As an additional observation made by Mii et al. when examining $CD109^{-/-}$ mice under steady-state conditions was the spontaneous development of epidermal hyperplasia compared to wild-type mice, quantified from histological measurements of interfollicular epidermal thickness. By selective immunohistochemistry staining of keratinocytes in the four layers of the epidermis, it was determined that thickening was greater in basal and suprabasal keratinocytes, while spinous and granulomous keratinocytes were similar between wild-type and $CD109^{-/-}$ mice[12]. Consistently, the epidermis of $CD109^{-/-}$ mice had more BrdU-positive cells and an equal frequency of caspase-3^{+} cells compared to control animals, implying that $CD109^{-/-}$ epidermal thickness is due to keratinocyte hyper-proliferation without a concomitant increase in cell death[12].

In addition to its role in TGF-β signalling, CD109 has also been reported to regulate STAT-3 activation in human keratinocyte cell lines[11]. The authors showed that addition of recombinant CD109 to cultured N/TERT-1 cells increased p-STAT-3 protein and that this activation was inhibited in the presence of TGF-β. By contrast, however, epidermal tissue of $CD109^{-/-}$ mice had elevated p-STAT-3^{+} cells by immunostaining compared to wild-type mice[12]. While these results appear contradictory, it may be a result of differential effects of soluble versus membrane-bound CD109 protein. Nevertheless, several studies implicate CD109 in regulating keratinocyte functions via TGF-β and STAT3-dependent pathways.

3.2 Post-translational modifications and processing of CD109

The CD109 gene is translated and processed into a 155 kD core polypeptide in the endoplasmic reticulum and subsequently linked to a GPI anchor to create pre-mature CD109 protein, which then becomes glycosylated (high-mannose form) and shuttled to the Golgi apparatus[9]. CD109 enters the Golgi as a 190 kD glycoprotein, where further glycosylation results

in a mature 205 kD form[9]. Finally, during its transit through the trans-Golgi network, CD109 is cleaved by furin into two 25 and 180 kD forms[9]. Furin is a member of the furinase family of endoproteases, which function in the Golgi apparatus to process immature precursor proteins into their active form[69]. CD109 is then expressed on the cell membrane as a 205kD complex containing its 25 and 180 kD units, and localizes to lipid rafts. On the cell surface, CD109 is prone to PI-PLC cleavage releasing the 180 kDa form of soluble CD109. Furin pre-treatment is vital for its function, since a mutated form of CD109 resistant to furinase cleavage failed to associate with and alter TGF-βRI signaling. Interestingly, CD109 expressed on some cells is resistant to PI-PLC treatment, but is susceptible to GPI-associated phospholipase D cleavage[62]. The heterogeneity of CD109 post-translational modifications may represent different lipid raft solubility and ultimately cell surface function. In particular, solubility of CD109 in non-ionic detergent was greater in T cells compared to other GPI-anchored proteins[70], suggesting cell-type specific regulation of this membrane protein.

3.3 TGF-β signaling pathway and skin homeostasis

The mammalian TGF-β receptor (TGF-βR) is composed of two subunits, TGFβRI and TGFβRII, both containing a single transmembrane portion with serine/threonine kinase domains in their cytoplasmic region[9, 71]. TGFβRII is constitutively active, and upon binding of TGFβ−1, −2 or −3, results in heterodimerization of the two receptor subunits and transphosphorylation of TGFβRI by TGFβRII. Phosphorylated TGFβRI then phosphorylates cytoplasmic receptor-regulated Smad2 or Smad3 (R-Smads). R-Smads then associate with the coSmad, Smad4, forming a R-Smad/CoSmad complex which can translocate to the nucleus and act as a transcriptional activator of TGF-β target genes. One dominant target gene of TGF-β is the inhibitory Smad, Smad7, which competes with R-Smads for binding to the activated TGFβRI, thereby acting as a mechanism of autoregulation[72]. Additional key TGF-β target genes include inhibitors of cyclin-dependent kinases, which promote cell-cycle arrest and inhibit cell division.

TGF-β signaling in keratinocytes has important implications in differentiation and proliferation of these skin cells. Normally, TGF-β signaling in keratinocytes results in reduced cell division and aberrant TGF-β signaling in the skin can lead to impaired wound healing, increased scarring, cancer and psoriasis[10]. In TGF-β1 null transgenic mice, whose keratinocytes produce no

TGF-β, basement membrane cells underwent hyper-proliferation[73]. In contrast, transgenic mice models overexpressing wild-type TGF-β1 using K1 promoter had greatly reduced keratinocyte growth[74]. This is consistent with established roles of *in vivo* TGF-β signaling in mice skin. However, different studies using transgenic mice overexpressing TGF-β1 using K10 or K6 promoter showed the opposite effect, whereby skin of transgenic mice underwent considerably greater keratinocyte proliferation[75]. In addition, inducible over-expression of TGF-β1 using a K5 promoter, which overexpressed TGF-β1 specifically in basal keratinocytes and in hair follicle keratinocytes revealed that this elevated expression of TGF-β1 only inhibited keratinocyte growth in neonatal mice, but not in adult skin[76 58]. In fact, Liu et al described an increase in apoptotic keratinocytes in these animals, as well as greater fibrosis and inflammation of the skin. Therefore, while current reports demonstrate variations in keratinocyte TGF-β over-expression in the skin, the effect TGF-β may exert on keratinocyte proliferation and skin homeostasis is dependent on a multitude of factors, including expression by keratinocytes at different stages of maturation.

TGF-β also has many important roles on hematopoietic cells, in particular those of the skin-associated immune system. Keratinocyte derived-TGF-β signaling controls epidermal residence of Langerhans cells and $CD8^+$ resident memory T cells, as well as DC migration to the skin-draining lymph nodes and their capacity to initiate inflammatory responses[26, 77]. TGF-β signaling also drives FoxP3 expression in naïve $CD4^+$ T cells to promote their differentiation into T regulatory (Treg) cells, while naïve $CD4^+$ T cells require TGF-β signaling, along with IL-6, to become Th17 cells. During thymic development, γδT cells become programmed to commit to an IL-17 or IFNγ producing subset. While γδT cell development was not impaired in TGF-$\beta1^{-/-}$ or Smad3-deficient mice, their capacity to produce IL-17 was significantly reduced[78].

3.4 STAT3-dependent signalling pathways in skin health and disease

Signaling through receptor tyrosine kinases at the surface of cells, can activate intracellular JAK molecules which then phosphorylate and activate cytoplasmic STAT3[19]. p-STAT3 molecules then dimerize and translocate into the nucleus where they can act as transcriptional activators of target genes[19]. Cytokines which signal through STAT3 include IL-6, IL-23, IL-10, EGF and G-CSF and others[79]. In particular, IL-6 and IL-23 signaling promotes the differentiation and activation of IL-17-producing T cells[80], whereas activation of STAT3 signaling in keratinocytes is

implicated in cell division, differentiation and wound-healing responses of the skin[79]. It is of little surprise then that skin sections from human psoriasis patients had activated STAT3 expression in the epidermis, and this expression was enriched in the nuclei of cells[81]. Consistent with this observation, transgenic mice expressing constitutively activated STAT-3 under the Keratin 5 (K5) promoter developed skin lesions similar to human psoriasis[81]. Of further note, Sano et al demonstrated the importance of active STAT3 signaling in both keratinocytes and activated T cells in the development of psoriasis-like disease[81].

In conclusion, psoriasis is one of the most common skin diseases and is characterized by dysregulated keratinocyte differentiation and hyper-activation of the IL-23/IL-17 immune axis. Within this context, both TGF-β and STAT3 dependent signalling are likely dysregulated and contribute to disease progression. Given that CD109 appears to negatively regulate both of these signalling pathways and is downregulated in human psoriatic skin lesions[11, 13], it stands to reason that CD109 may be a critical nexus point balancing skin homeostasis and IL-23/IL-17-driven inflammation. The following studies support this position and set the foundation for future studies into how CD109 regulates the cutaneous immune system in health and disease.

Results

Deletion of CD109 results in spontaneous skin abnormalities

To study the role of CD109 on skin immunity we began by characterizing the skin of wild-type (WT) and CD109-deficient ($CD109^{-/-}$) mice. Following H&E staining, some areas of the epidermis of $CD109^{-/-}$ mice displayed a mild thickening during steady state conditions compared to WT mice (**Figure 1a, b**). To determine whether the expansion of cells seen in $CD109^{-/-}$ mice skin was due to an increase in cell division in hematopoietic or non-hematopoietic cells, WT and $CD109^{-/-}$ skin were subjected to enzymatic digestion for cell counting and flow cytometry analysis. First, the dermis of $CD109^{-/-}$ mice had overall higher number of both live $CD45^+$ and $CD45^-$ cells compared to WT dermis, whereas no difference was observed in epidermal cell counts (**Figure 1c**). Despite histological evidence of epidermal thickening, a measure of epidermal cells that are no longer viable was not performed, potentially accounting for the discrepancy between these methods of measurement. Additionally, both $CD45^+$ and $CD45^-$ compartments in the $CD109^{-/-}$ epidermis and dermis showed higher frequency of $Ki67^+$ cells compared to WT controls (**Figure 1d**). This result translated to an increase in $Ki67^+CD45^-$ cell counts in epidermis of CD109 deficient animals, whereas the dermis had elevated numbers of proliferating $CD45^+$ and $CD45^-$ cells (**Figure 1e**). Thus, loss of CD109 results in spontaneous epidermal hyperplasia and increased proliferation of both hematopoietic and non-hematopoietic cells within the skin.

CD109 limits activation of the IL-23/IL-17 immune axis

To determine whether the observed skin abnormalities in $CD109^{-/-}$ mice correlated with dysregulation of the immune system, we performed transcriptional profiling of canonical T helper effector cytokines from the skin. In both the epidermis and dermis of $CD109^{-/-}$ mice, a substantial increase in *Il17a* mRNA was detected compared to WT mice by quantitative RT-PCR analysis (**Figure 2a**). However, no differences were observed in the expression of classical Th1 (*Ifng*) and Th2-associated cytokines (*Il4, Il5*and *Il13*) in the dermis, whereas the epidermis had a decrease in *Il13* transcription (**Figure 2a**). To validate these results on the protein level, IL-17 and IFNγ production was compared in WT and $CD109^{-/-}$ by intracellular staining following re-stimulation of epidermal and dermal cells with PMA and Ionomycin. Mice deficient in CD109 had more IL-17A

(IL-17) producing hematopoietic cells compared to WT mice, whereas no change in IFNγ production was detected (**Figure 2b**). An IL-17-dominated T cell response is often accompanied by production of IL-22 by the same cells, as IL-22 plays a key role in tissue repair and pathogen clearance[80]. However, little to no change in IL-22 transcripts was observed by RT-qPCR between steady state WT and CD109$^{-/-}$ mice (**Figure 2c**). IL-17 production by T cells is driven by IL-6, IL-23 and IL-1β signalling[80]. However, we did not observe differences in *Il6* and *Il1b* transcripts between skin from WT and CD109$^{-/-}$ mice. By contrast, *Il23p19* mRNA was significantly elevated in CD109$^{-/-}$ skin, as well as its associated specific receptor subunit, *IL23r* (**Figure 2c**). These results indicate an enhanced IL-23 signaling in CD109$^{-/-}$ skin, potentially sustaining elevated IL-17 production by T cells. These IL-17-producing T cells express the cell surface chemokine receptor CCR6, whose ligand CCL20 is used for homing to the skin[82]. Not surprisingly, CD109$^{-/-}$ skin was observed to express greater *Ccl20* transcripts than WT mice (**Figure 2c**). All together these data suggest CD109 acts as a negative regulator of the IL-23/IL-17 immune axis in the skin.

Cutaneous T cell infiltration and accumulation of γδ17 cells in the absence of CD109

So far, the data suggested the skin of CD109$^{-/-}$ mice has an IL-17-dominated immune response, compared to WT mice skin as evidenced by transcriptional profiling and intracellular cytokine staining. This enhanced signaling through the IL-23/IL-17 immune axis is also characteristic of pathological human skin conditions, the most common being psoriasis. As psoriatic disease is driven by aberrant dermal γδT cell-derived IL-17 production, we next determined the cellular source of IL-17 in CD109-deficient skin[28]. To this end, the skin of WT and CD109$^{-/-}$ mice was separated into epidermal and dermal sheets and processed for flow cytometric analysis. In the epidermis, the frequency of dendritic epidermal T cells (DETCs) remained the same between WT and CD109$^{-/-,}$ whereas the frequency and number of T cells expressing and αβTCR was increased in CD109$^{-/-}$ mice (**Figure 3a, b**). Similarly, the dermis of CD109$^{-/-}$ mice also had an increase in αβT cells frequency and number. However, a population of γδT cells expressing intermediate levels of their TCR were particularly elevated compare to controls (**Figure 3a, b**). This γδTCR-intermediate population has been described to be dermal residents, whereas the cells expressing high levels of their γδTCR are described to be contaminating DETCs[30]. Next,

epidermal and dermal cell suspensions were re-stimulated with PMA and ionomycin in the presence of monensin for detection of intracellular cytokine production. Consistent with previous studies, the majority of IL-17 producing T cells were dermal γδT cells and this population was significantly elevated in skin from CD109-/- mice compared to WT γδT cells (**Figure 3c, d**).

Skin γδT cells can be classified by their Vγ chain usage and expression of cell surface CD27, a member of tumor-necrosis factor 'superfamily' of membrane receptors[45]. A recently identified subset of IL-17-precommited dermal γδT cells, expressing Vγ4 receptors were described to migrate from the skin to lymph nodes and contribute to psoriatic-like inflammation[43]. Consistently, dermal γδT cells from CD109$^{-/-}$ mice were enriched for Vγ4^{+} cells compared to WT mice (**Figure 3e**). Further characterization of dermal γδT cells demonstrated that these cells do not express CD27 in either WT or CD109$^{-/-}$ mice, consistent with the notion that these cells become pre-committed during thymic development to become either CD27^{+} IFNγ-producing or CD27^{-} γδ17 cells[45, 78]. Additionally, all dermal γδT cells expressed high levels of the α_E integrin CD103, a TGF-β-dependent gene, but expression was not affected by the absence of CD109. Finally, to determine whether the absence of CD109 expression affects skin T cell proliferation, Ki67 expression was assessed in γδT cell subsets and αβT cells. In CD109$^{-/-}$ epidermis, greaterVγ4^{-} DETCs and the infiltrating αβT cells expressed Ki67, while in the dermis more Vγ4^{+} than Vγ4^{-} cells and αβT cells underwent the greatest increase in Ki67 expression, compared to WT mice (**Figure 3f, g**). Altogether, the absence of CD109 expression in mouse skin results in T cell infiltration and proliferation, as well as an increase in γδ17 cells.

CD109-deficient skin harbours more myeloid cells and anti-microbial peptide expression

Under steady state conditions, CD109$^{-/-}$ mice have a greater number of IL-17-competent γδT cells. Although these cells display cell surface phenotype characteristic of pre-committed γδT 17 cells and produce large quantities of IL-17 when re-stimulated *ex-vivo*, whether they are actively producing this IL-17 *in situ* is not known. IL-17 has been shown to directly induce the production of AMPs by keratinocytes, such as REG proteins, CRAMP and S100A peptides, while S100A8 and S100A9 both mediate cellular functions in myeloid cells such as neutrophils and macrophages[3, 83]. As a surrogate for functional IL-17 signaling, transcriptional profiling for

epithelial-derived anti-microbial factors was performed. In CD109$^{-/-}$ skin S100A7, 8 and 9 transcripts were greatly over-expressed compared to WT, as opposed to RegIIIγ or CRAMP, with emphasis on the epidermis where AMP-producing keratinocytes are located (**Figure 4a**). These data suggest that IL-17 signaling in skin epithelia is functionally enhanced in the absence of CD109. Additionally, IL-17 promotes the recruitment of neutrophils via induction of epithelial-derived chemokines such as CXCL1 and CXCL2. Consistent with this data, CD109$^{-/-}$ mice have higher frequencies and number of dermal Ly6G^{+}CD11b^{+} neutrophils compared to WT mice (**Figure 4b**). This steady state neutrophilia may also be an explanation for the substantial increase in S100A8 and S100A9 peptides. So far, these data suggest that absence of CD109 results in aberrant production of antimicrobial peptides and recruitment of neutrophils consistent with increased IL-17 production by dermal γδT cells, which may be the driving factor of this dysregulated skin environment.

Myeloid lineage cells, such as macrophages and dendritic cells (DCs) play important roles in skin health and disease and are the critical source of IL-23, a potent activator of γδ17 cells. To determine whether this population was also regulated by CD109, we assessed the number of dermal macrophages (CD11c-MHCII^{+}F4/80^{+}CD11b^{+}) and dendritic cells (MHCII^{+}CD11c^{+}) in the presence or absence of CD109$^{-/-}$ dermis compared to wild-type mice (**Figure 4c, d**). In the dermis of CD109$^{-/-}$ mice, the total population of macrophages was increased (**Figure 4c**), as well as dendritic cell populations, including both CD11b^{+}CD103^{-} and CD11b^{-}CD103^{+} subsets (**Figure 4d**), compared to wild-type controls. Together these results provide evidence that cutaneous IL-17 signalling is enhanced in CD109$^{-/-}$ mice as determined by higher expression of AMPs and elevated dermal myeloid cell accumulation.

Skin-draining lymph nodes of CD109$^{-/-}$ have more γδ17 cells

To determine whether the dysregulated IL-17 phenotype observed in the skin of CD109$^{-/-}$ mice translated to alterations in the skin-draining lymph node compartment, we investigated the cervical lymph nodes which drain the head and ear skin. Indeed, a higher frequency and number of lymphocytes from CD109$^{-/-}$ cervical lymph nodes (cerLNs) expressed the transcription factor retinoic-orphan receptor γt (RORγt), which regulates IL-17 production by T cells (**Figure 5a**). All of the RORγt^{+} cells found in the cerLNs expressed high levels of CD44, a cell-surface glycoprotein expressed by activated T cells and CD109$^{-/-}$ mice had more total CD44^{hi} cells than WT mice

(**Figure 5a**). Moreover, the majority of RORγt^{+}CD44^{+} cell population in CD109$^{-/-}$ expressed a γδTCR, essentially inversely mirroring the WT mice, where αβT cells were the main cell type (**Figure 5b, c, d**). Interestingly, a shift from CD27^{+}Vγ4^{-} γδT cells in wild-type mice to CD27^{-}Vγ4^{+} γδT cells in CD109$^{-/-}$ represents a large increase in IL-17-precommitted cells (**Figure 5e**). What is more, the increase was specific for γδT cells expressing Vγ4 receptors as opposed to the Vγ4^{-} population, consistent with reports demonstrating that Vγ4^{+} T cells represent a subset of γδT cells capable of migrating between the skin and skin-draining lymph nodes[43]. Consistent with the shift in CD27 and Vγ4 expressions, cerLNs of CD109$^{-/-}$ mice have more IL-17 producing T cells compared to WT mice (**Figure 5f**). Overall, skin-draining lymph nodes of CD109$^{-/-}$ mice have increased γδ17 cells, in a manner similar to the skin immune compartment.

Radio-resistant γδT cells in the skin require CD109 for control of IL-17 production

To determine whether the expression of CD109 on cells of hematopoietic origin was important to limit the observed changes in the immune cell repertoire, we generated bone marrow chimeric mice. We transferred CD109$^{-/-}$ bone marrow cells into lethally irradiated wild-type recipient mice (KO→WT) and wild-type bone marrow into CD109$^{-/-}$ mice (WT→KO). As a control we transferred wild-type bone marrow cells into wild-type recipients (WT→WT). We observed a specific and substantial increase in the frequency of IL-17-producing γδT cells in the dermis of CD109$^{-/-}$ mice transferred with wild-type bone marrow (**figure 6a**). However, the majority of these γδ17 cells are of host (CD109$^{-/-}$) origin (**figure 6b**), consistent with reports demonstrating that both DETCs and dermal γδT cells are radio-resistant[30]. Overall, IL-17 production by dermal γδT cells remains almost exclusive to host mice, regardless of chimeric transfer (**Figure c**). In spite of this, a greater frequency of donor wild-type bone marrow cells reconstituted the skin of CD109$^{-/-}$ mice compared to donor CD109$^{-/-}$ or wild-type cells transferred into wild-type host mice (**Figure d**). This might suggest, a specific dysregulation of cell recruitment into the skin of CD109$^{-/-}$ mice, in a non-hematopoietic intrinsic manner. From this set of studies, we can conclude that CD109 expression on radio-resistant cells is required to regulate IL-17 production by the γδT cell population and that CD109 expression on non-hematopoietic cell type is required to control leukocyte infiltration into the skin.

Contrary to the skin, reconstitution of cerLN was roughly 95% in the three chimeric groups. $CD109^{-/-}$ mice reconstituted with wild-type bone marrow had nearly a 6-fold increase in the frequency and 25-fold increase in the number of γδT cells in cerLNs, compared to the other 2 groups (**figure 6e, f**). These WT donor γδT cells also co-expressed high levels of CD44 in a $CD109^{-/-}$ host (**figure 6e**), suggesting a functional absence of CD109 on non-hematopoietic cells regulates γδT cell expansion. Roughly one quarter of the cerLN WT γδT cells that expanded in $CD109^{-/-}$ mice co-expressed RORγt and Vγ4, as opposed to $CD109^{-/-}$ γδT cells transferred into wild-type recipient mice, which did not undergo expansion of this γδT cell subset (**figure 6g**). What is more, this specific expansion of γδT cells in WT→KO mice was specific to lymphoid organs draining the skin (**figure** 6g). These data suggest that CD109 does not act in a γδT cell intrinsic manner and that CD109 expression by non-hematopoietic cells is required to regulate IL-17 producing γδT cell subsets present in the skin-draining lymphoid organs.

γδ17 cells of $CD109^{-/-}$ mice expand following imiquimod treatment

To induce psoriasis-like skin inflammation in wild-type mice, we topically administered 5mg of imiquimod (IMQ) to the ears of wild-type and $CD109^{-/-}$ mice for 7 consecutive days to determine whether $CD109^{-/-}$ skin would be pre-disposed to developing more severe psoriasis-like inflammation[41]. Ear thickness of wild-type and $CD109^{-/-}$ mice was measured daily and skin cross-sections were assessed by H&E staining. Between two independent experiments, ear thickness of $CD109^{-/-}$ mice treated with IMQ grew significantly thicker than wild-type treated mice in one experiment, whereas no difference was measured in a separate experiment (**Figure 7a**). No obvious changes were noticed by H&E staining either, however the histology sections remain to be quantitatively assessed (**figure 7b**). However, cerLNs of $CD109^{-/-}$ mice had an increase in frequency and number of $CD44^{+}$ γδTCR^{+} cells when treated with IMQ compared to wild-type treated mice (**figure 7c**). Interestingly, while there is an increase in γδT cells in treated $CD109^{-/-}$ mice cerLN, the frequency of these cells producing IL-17 is the same compared to $CD109^{-/-}$ non-treated mice, and albeit statistically higher compared to treated wild-type mice, it is by a mere 10% difference (**figure 7d**). These data suggest that IMQ treatment induces an expansion of an already pre-committed γδ17 cells in skin-draining lymph nodes and that CD109 may play an important role in regulating this particular cell type.

To test whether treatment of IMQ-induced psoriasis-like skin phenotype was dependent on CD109 expression by bone marrow-derived immune cells or non-hematopoietic cells, additional bone marrow chimeric mice were generated. A greater reconstitution time, allowed for more donor cells to populate the skin of recipient mice as suggested by Sumaria et al[30]. Ear thickness was measured daily for each mouse in the three chimeric groups (WT→WT, WT→KO, KO→WT) and while WT→WT and KO→WT ears began to swell by day 5, WT→KO mice ears were significantly thicker than the two other groups by day 3 and continued to increase throughout the regimen (**figure 7e**). The mice did indeed have greater degree of $CD3^+$ T cell reconstitution into the dermis following 22-24-week reconstitution (**figure 7f**), however there was no change in the percentage of donor-derived T cells across the three groups of chimeric mice (**figure 7g**). As a result of this low frequency of donor cells in the dermis, IL-17 production was assessed from total $CD3^+$ cells. Dermal tissue was digested in the presence of monensin, rather than re-stimulated *ex vivo* with PMA and ionomycin to get a more accurate representation of IL-17 producing cells *in situ* rather than cells with the potential to produce cytokine following ex vivo stimulation. While un-treated $CD109^{-/-}$ mice transferred with wild-type bone marrow cells had more γδ17 cells than WT→WT control mice, the frequency of these cells significantly increased following IMQ treatment (**figure 7h, i**). Moreover, the WT→KO mice had nearly a 10-fold increase in frequency and number of γδT cells producing IL-17 than KO→WT mice or WT→WT controls (**figure 7h, i**). Alas, the wild-type donor-derived γδT cells producing IL-17 in $CD109^{-/-}$ mice represent an average of 14% of $IL\text{-}17^+$γδT cells and is lower in the other two groups (**figure 7j**). Together these data indicate that CD109 acts in an extrinsic manner to limit the expansion of γδ17 cells during psoriatic-like inflammation.

$CD109^{-/-}$ exhibits changes in TGF-β target gene expression that may favour aberrant IL-17 production by skin γδT cells

To begin to address whether the immune dysregulation in the absence of CD109 requires adaptive immunity, $CD109^{-/-}$ mice were crossed to $Rag1^{-/-}$ mice to generate $CD109^{-/-}Rag1^{-/-}$ double knock out mice (henceforth referred to as Rag109DKO mice, $Rag1^{-/-}CD109^{+/+}$ (RagKO) mice were used as controls). The epidermis and dermis of RagKO and Rag109DKO mice were analyzed by RT-qPCR for transcription of key cytokines and chemokines important for IL-17-producing T

cells. In both the epidermis and dermis of Rag109DKO mice, transcription for *Il17* and *Ccl20* were lower than in Rag1 sufficient CD109$^{-/-}$ mice, suggesting a significant proportion the increased IL-17 and CCL20 observed in CD109$^{-/-}$ mice were a result of inflammatory lymphocytes in the skin (**figure 8a**). In contrast, *Ifng* and *Il1b* transcription was higher in Rag109DKO mice than in CD109$^{-/-}$ mice, potentially as a defense mechanism from innate cells compensating for the lack of cutaneous T cells (**figure 8a**). Interestingly *il23* mRNA remained unchanged between CD109$^{-/-}$ and Rag109DKO mice, regardless of presence or absence of adaptive immune cells (**figure 8a**), suggesting dysregulation upstream of IL-17 production by γδT cells. Finally, *Il6* and *Il23r* transcripts were unchanged in the epidermis of Rag109DKO mice compared to CD109$^{-/-}$ mice, although *Il23r* is just as elevated in Rag109DKO dermis as in CD109$^{-/-}$ dermis, suggesting the cells responsive to the increased IL-23 observed in CD109$^{-/-}$ are not exclusive (yet not excluding) to skin lymphocytes (**figure 8a**).

As CD109 has been described as a negative regulator of TGF-β signaling[10], transcriptional analysis of TGF-β target genes was performed on the skin of wild-type and CD109$^{-/-}$ mice. These results revealed that absence of CD109 in the epidermis significantly reduced *Smad7* transcription while both *Cola2* and *Smad7* transcripts were less abundant in CD109$^{-/-}$ mice compared to wild-type controls (**figure 8b**). In addition to TGF-β, CD109 has been suggested to regulate STAT-3 signaling in keratinocytes as well[11]. This prompted us to measure baseline p-Smad2/3 and p-STAT-3 in wild-type and CD109$^{-/-}$ T cells to determine whether CD109 was intrinsically controlling these signaling pathways. Our results identified higher mean fluorescence intensity (MFI) of p-Smad2/3 in CD109$^{-/-}$ in both γδ and αβ T cells compared to wild-type cells (**figure 8c**). Finally, no changes in p-STAT-3 levels in either T cell type between wild-type or CD109$^{-/-}$ mice were observed (**figure 8d**).

In summary, CD109 plays an indispensable role in maintenance of skin health by regulating the expansion of a dermal radio-resistant γδ17 cell subset. Based on our current dataset, we propose a potential mechanism in which keratinocyte-derived CD109 may act in an extrinsic manner to regulate both IL-23 producing myeloid cells and IL-17 producing γδT cells in order to limit cutaneous inflammation under steady-state conditions and following immune challenge.

Figures

Figure 1: CD109$^{-/-}$ mice develop spontaneous epidermal hyperplasia and leukocytosis

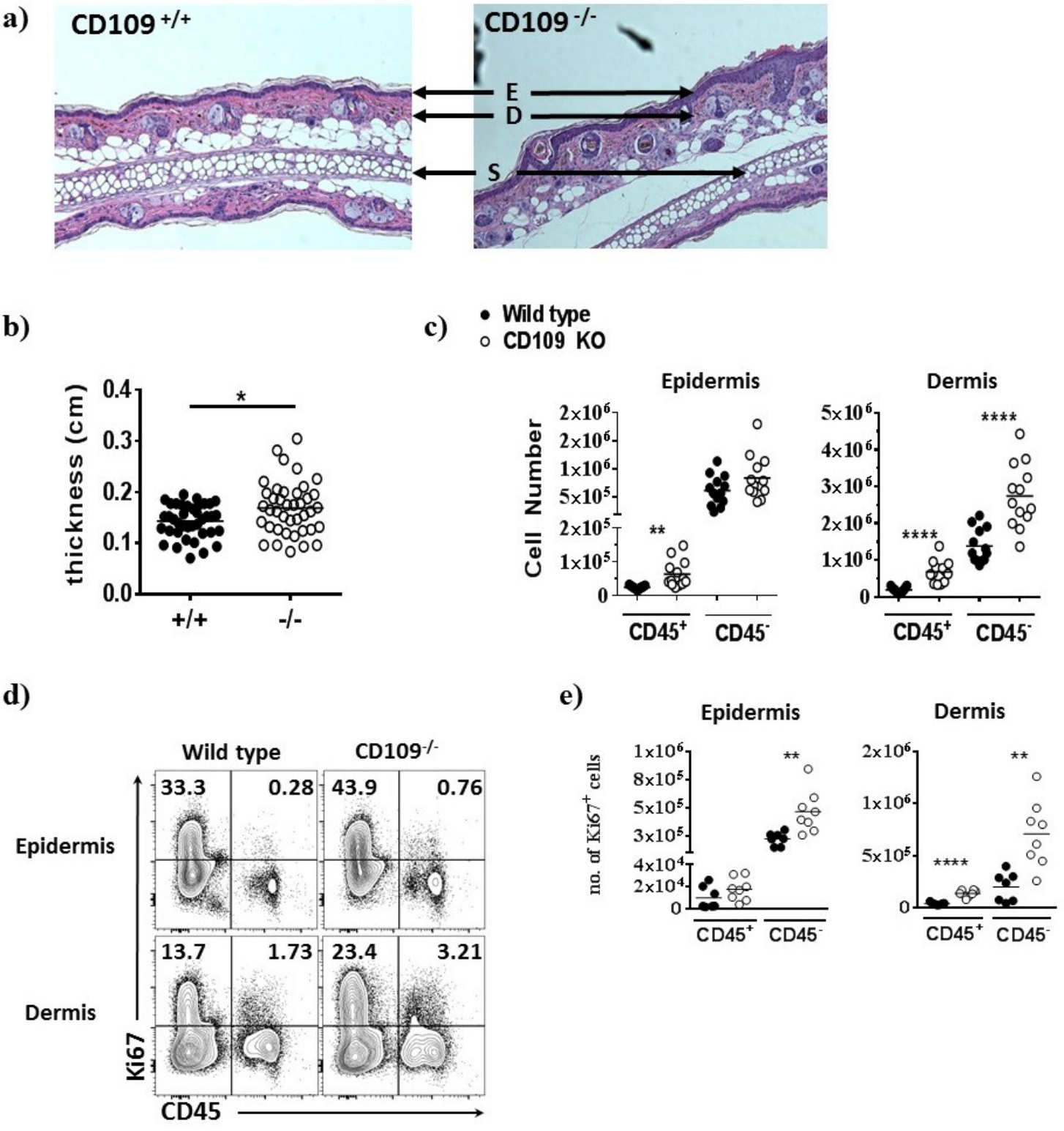


Figure 2: Skin of CD109$^{-/-}$ mice have increased IL-17 production

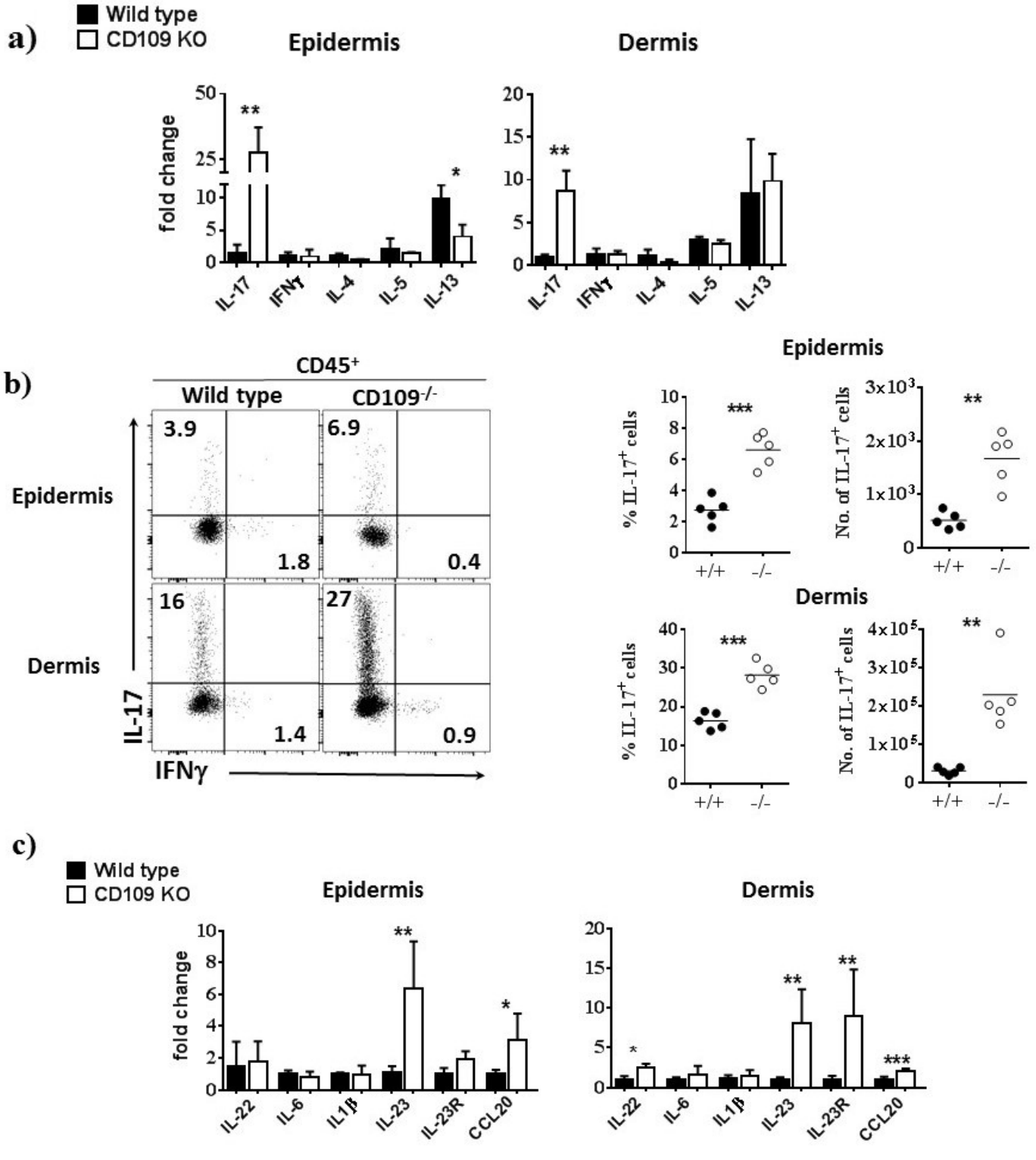
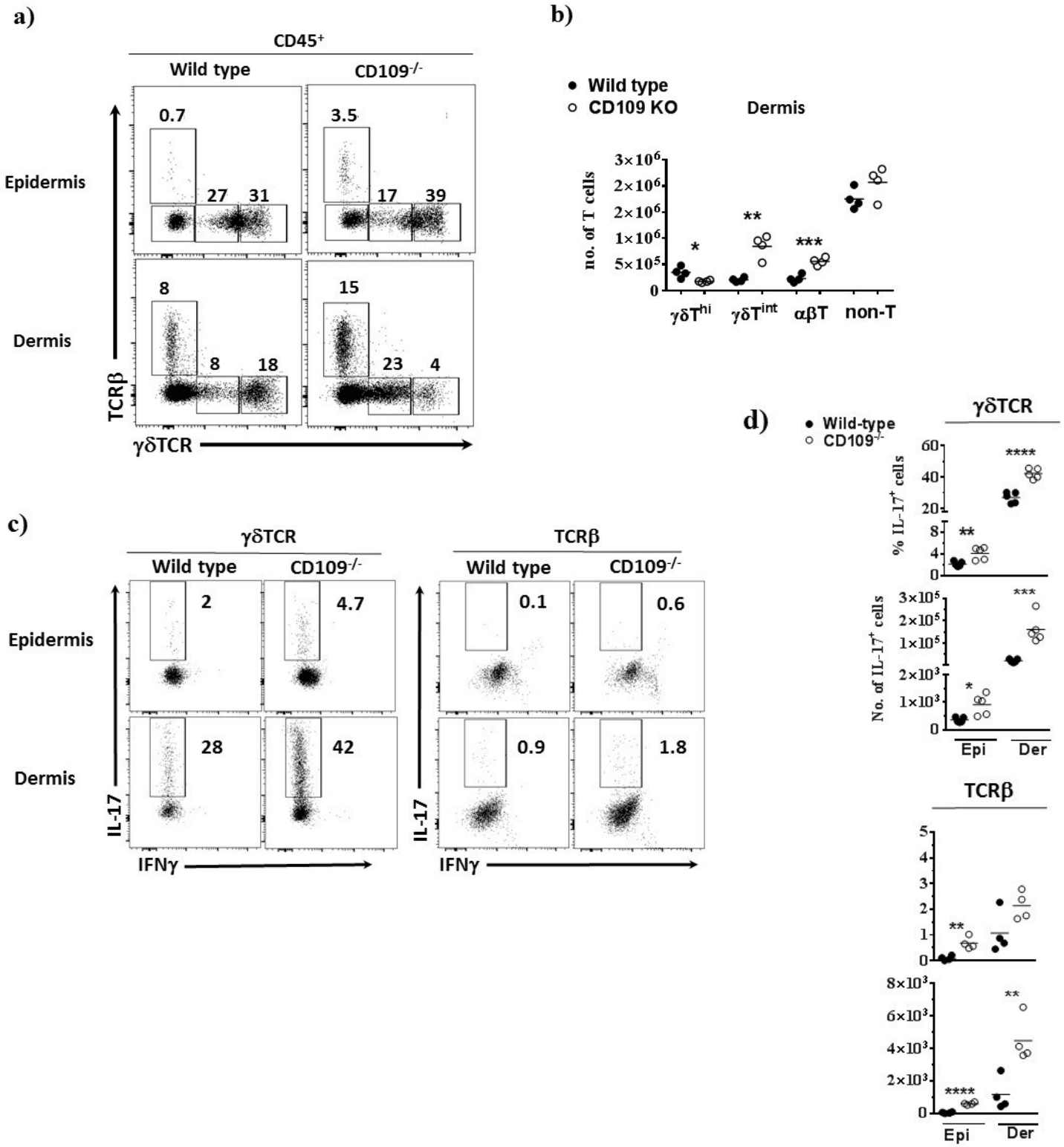


Figure 3: CD109$^{-/-}$ skin have specific increase in IL-17-producing γδT cells



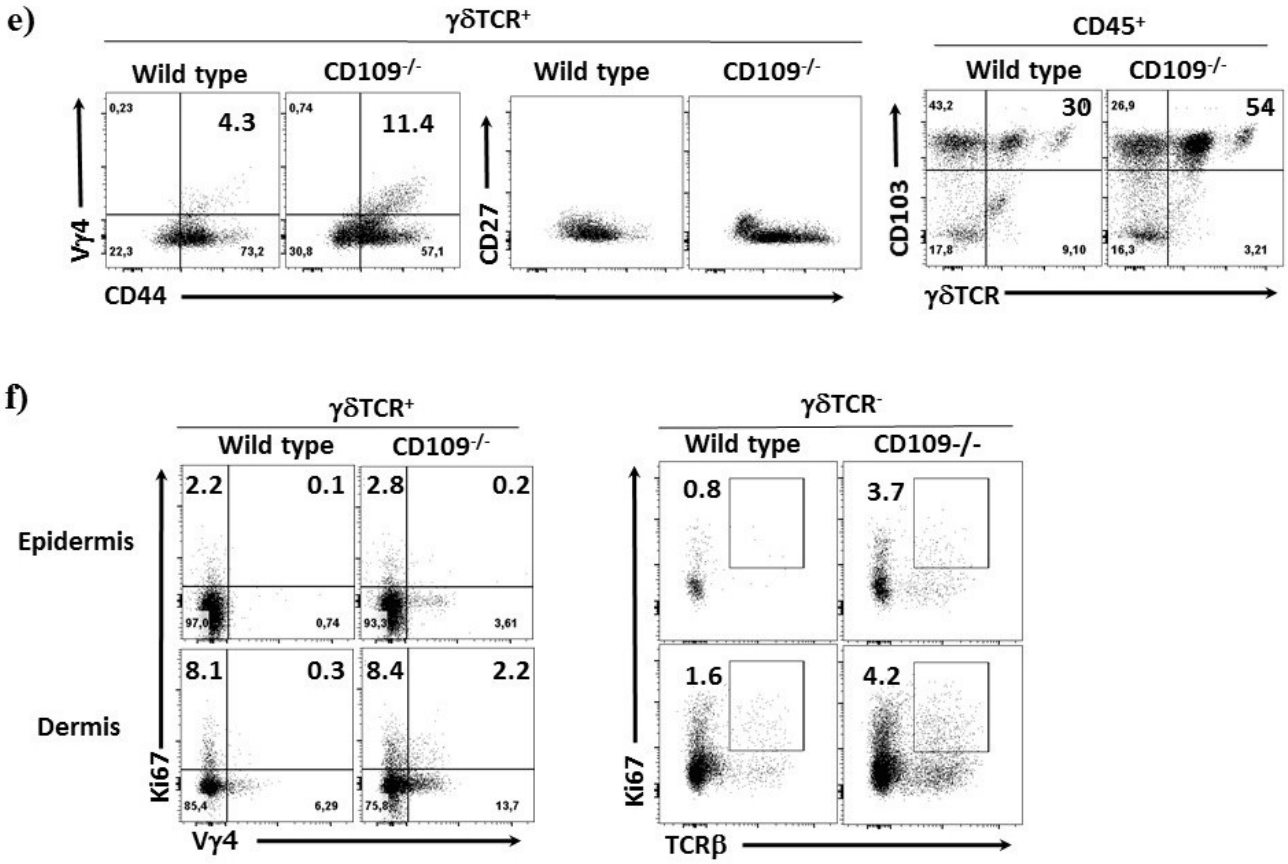

e)
γδTCR+
Wild type
CD109-/-
Wild type
CD109-/-
CD45+
Wild type
CD109-/-
0,23
4.3
22,3
73,2
0,74
11.4
30,8
57,1
43,2
30
17,8
9,10
26,9
54
16,3
3,21
Vγ4
CD44
CD27
CD103
γδTCR
f)
γδTCR+
Wild type
CD109-/-
γδTCR-
Wild type
CD109-/-
Epidermis
Dermis
2.2
0.1
97,0
0,74
2.8
0.2
93,3
3,61
8.1
0.3
85,4
6,29
8.4
2.2
75,8
13,7
0.8
3.7
1.6
4.2
Ki67
Vγ4
Ki67
TCRβ
g)
Wild type
CD109 KO
Epidermis
Dermis
% of Ki67+ cells
10
8
6
4
2
0
***
***
**
****
Vγ4-
Vγ4+
TCRβ+
Vγ4-
Vγ4+
TCRβ+
No. of Ki67+ cells
3×10⁴
2×10⁴
1×10⁴
3×10³
2×10³
1×10³
0
*
**
***
Vγ4-
Vγ4+
TCRβ+
Vγ4-
Vγ4+
TCRβ+

Figure 4: CD109$^{-/-}$ mice skin have increased IL-17-signature response as well as increased DCs

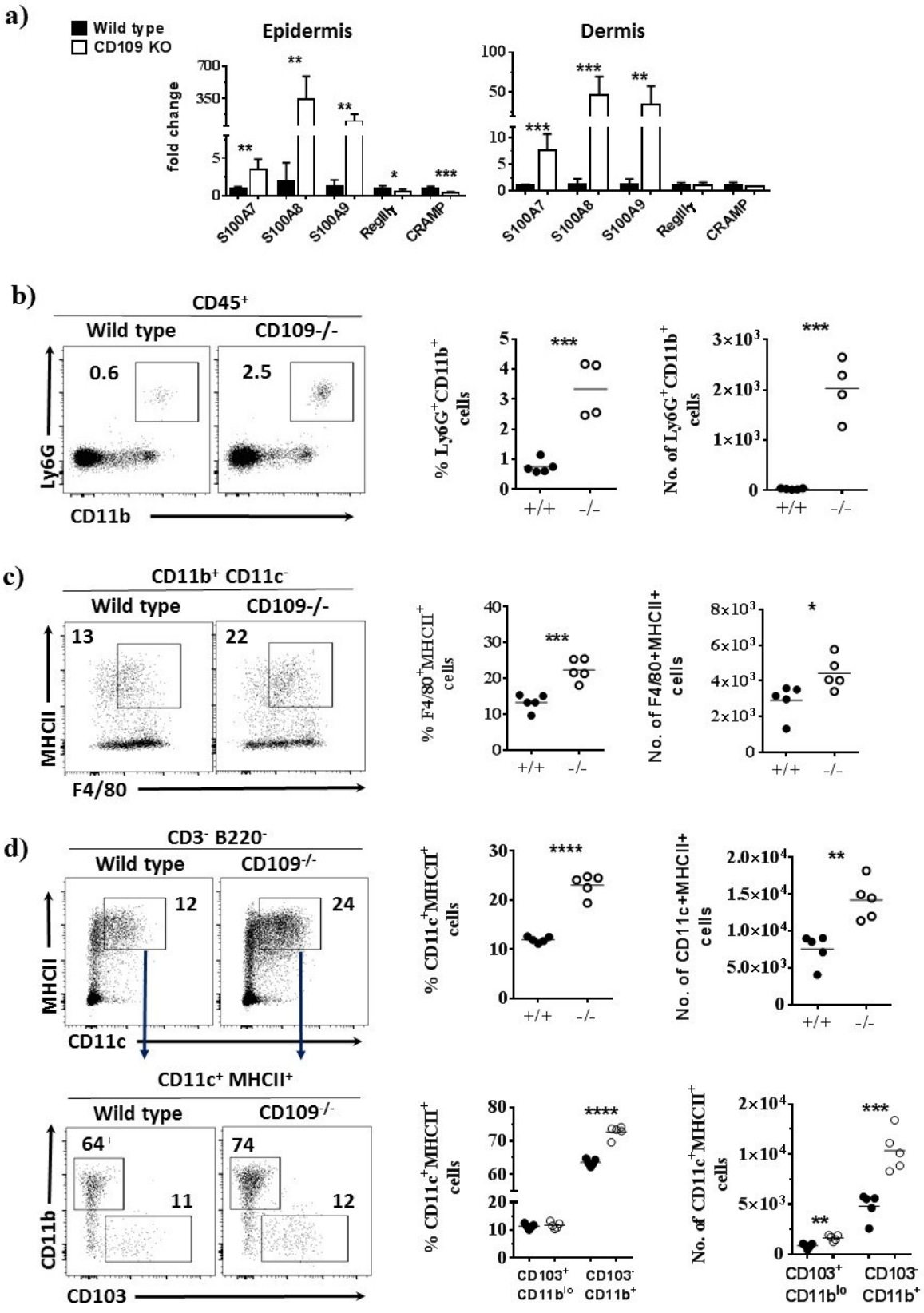
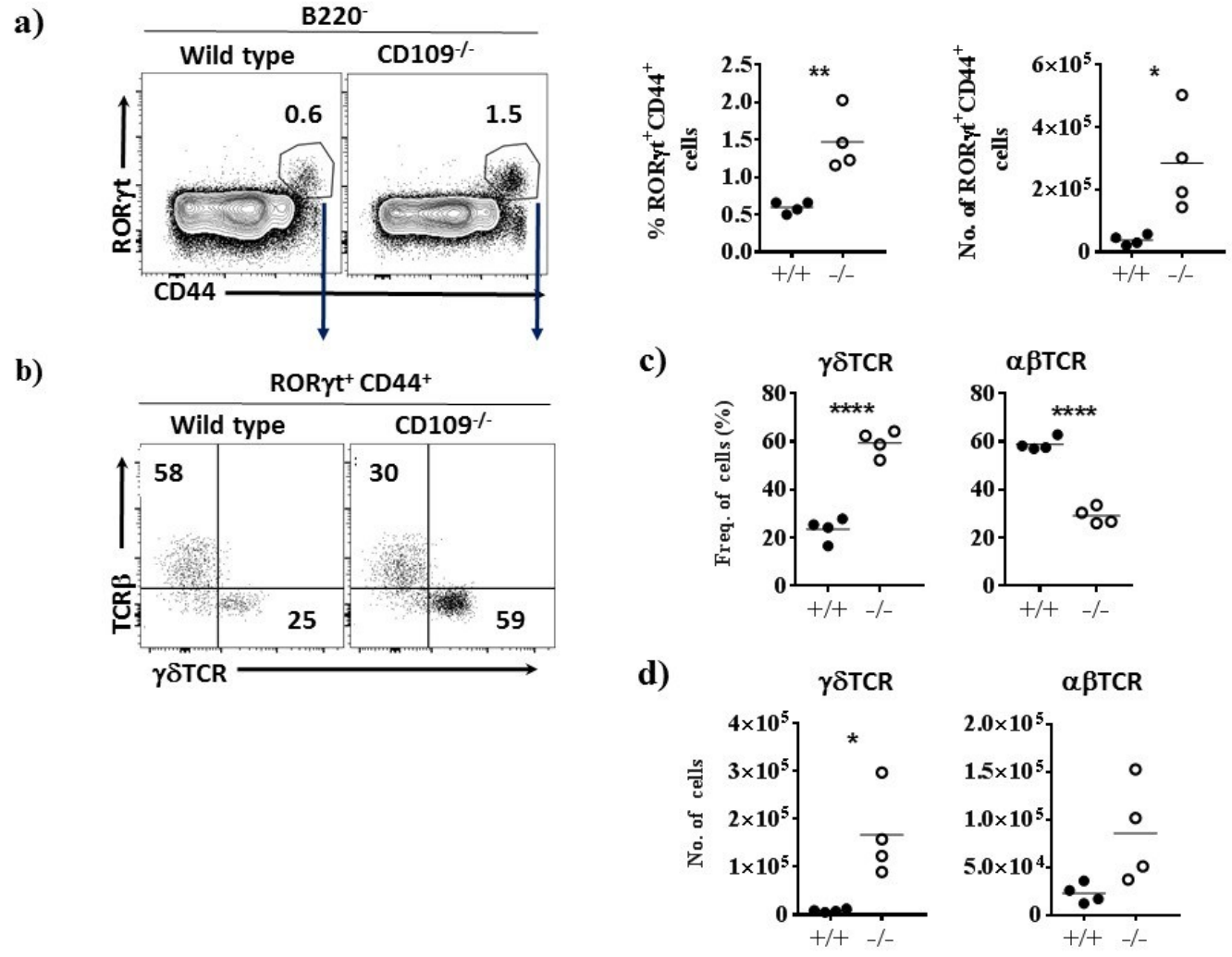


Figure 5: Cervical lymph nodes of CD109$^{-/-}$ mice contain more IL-17-producing γδT cells



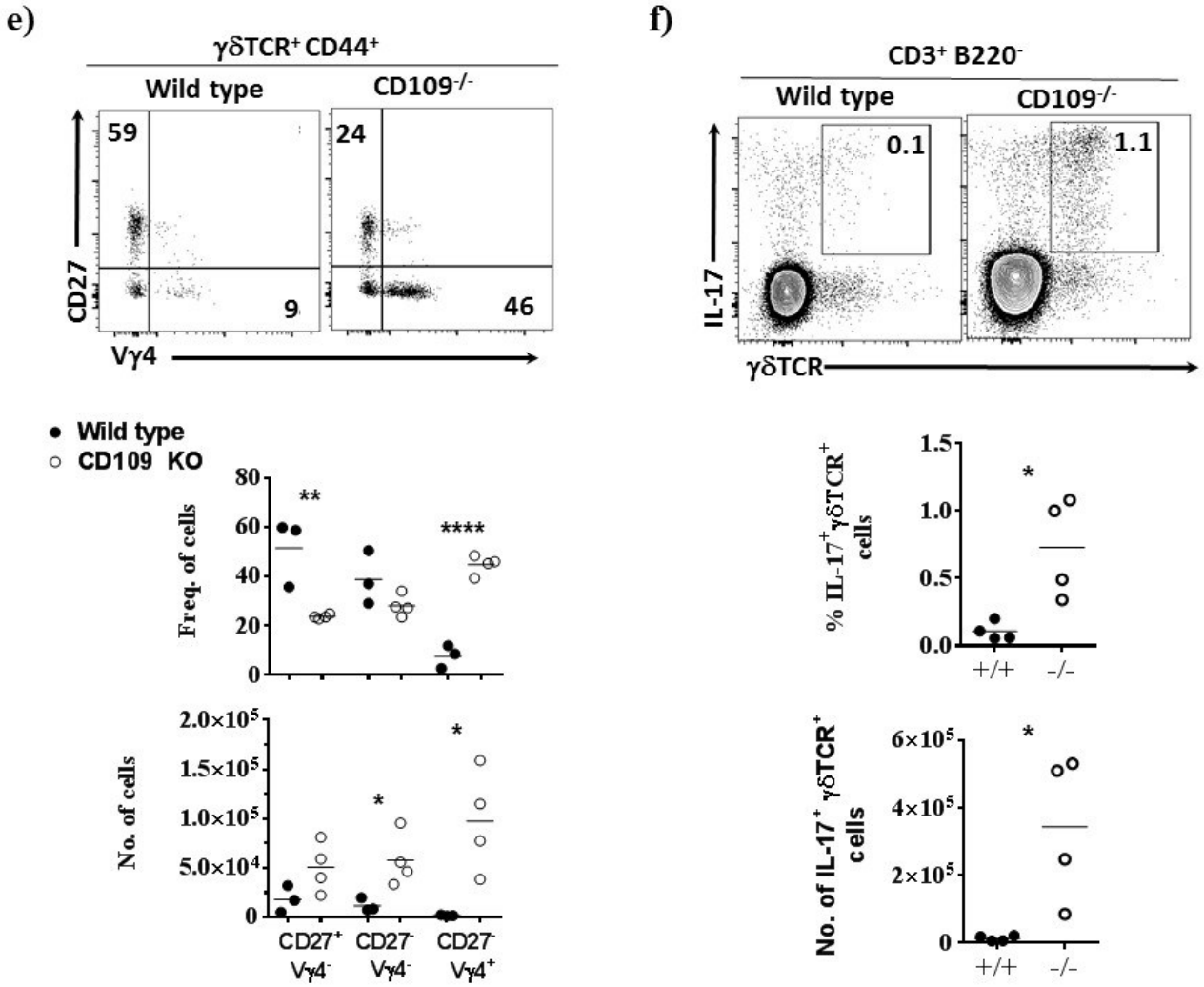
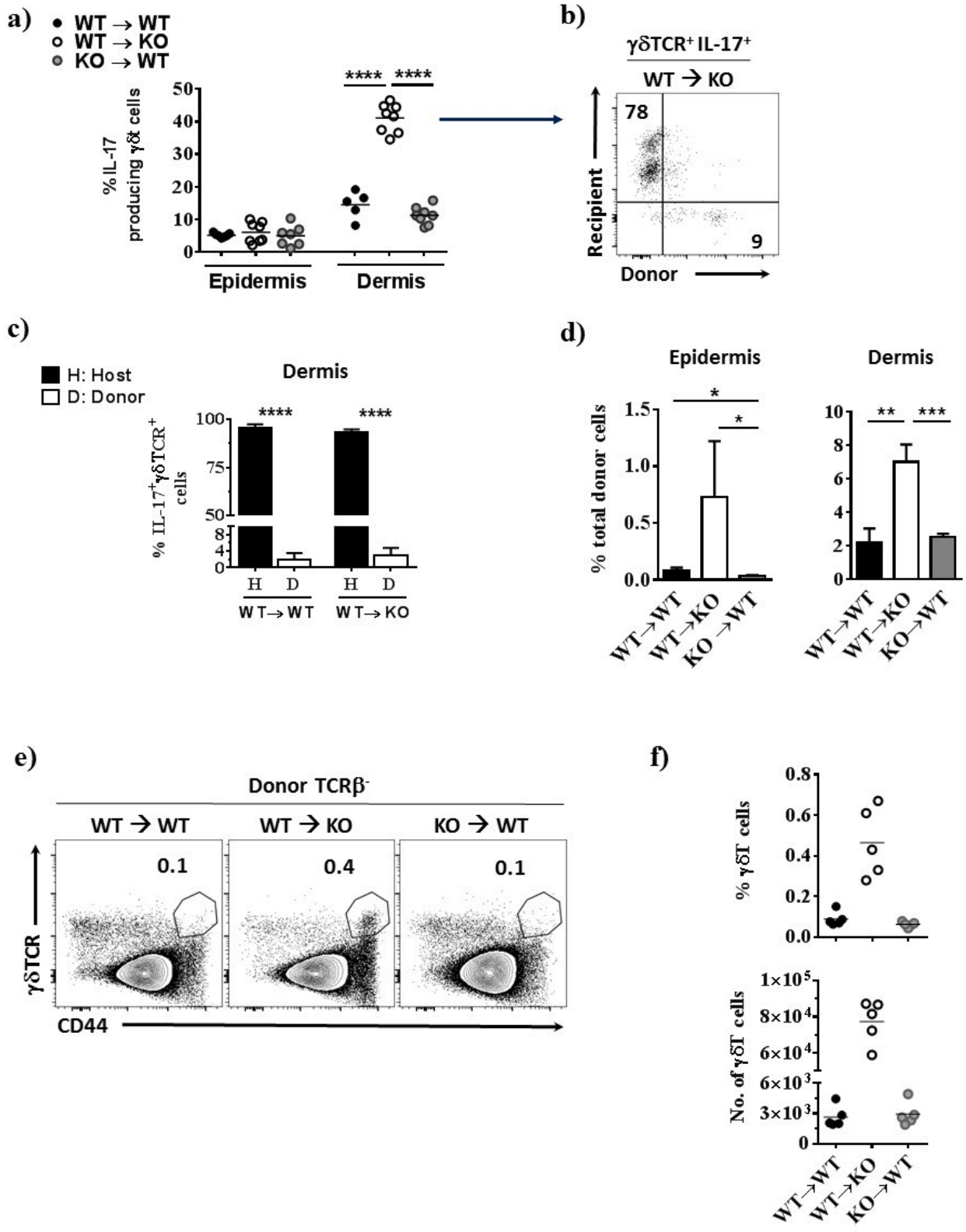

e)
γδTCR+ CD44+
Wild type
CD109-/-
59
24
9
46
CD27
Vγ4
Wild type
CD109 KO
Freq. of cells
No. of cells
CD27+ Vγ4-
CD27- Vγ4-
CD27- Vγ4+
f)
CD3+ B220-
Wild type
CD109-/-
0.1
1.1
IL-17
γδTCR
% IL-17+ γδTCR+ cells
No. of IL-17+ γδTCR+ cells
+/+
-/-

Figure 6: CD109 regulates a radio-resistant γδT cell population in the skin



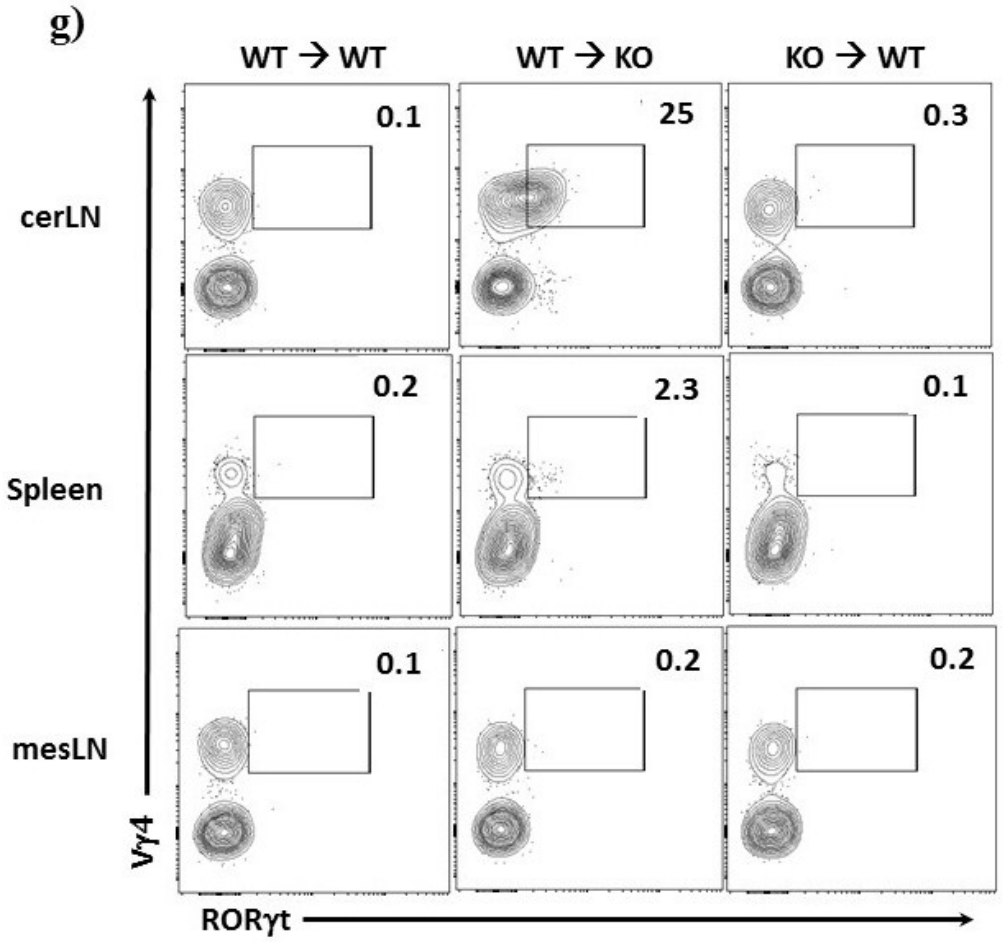
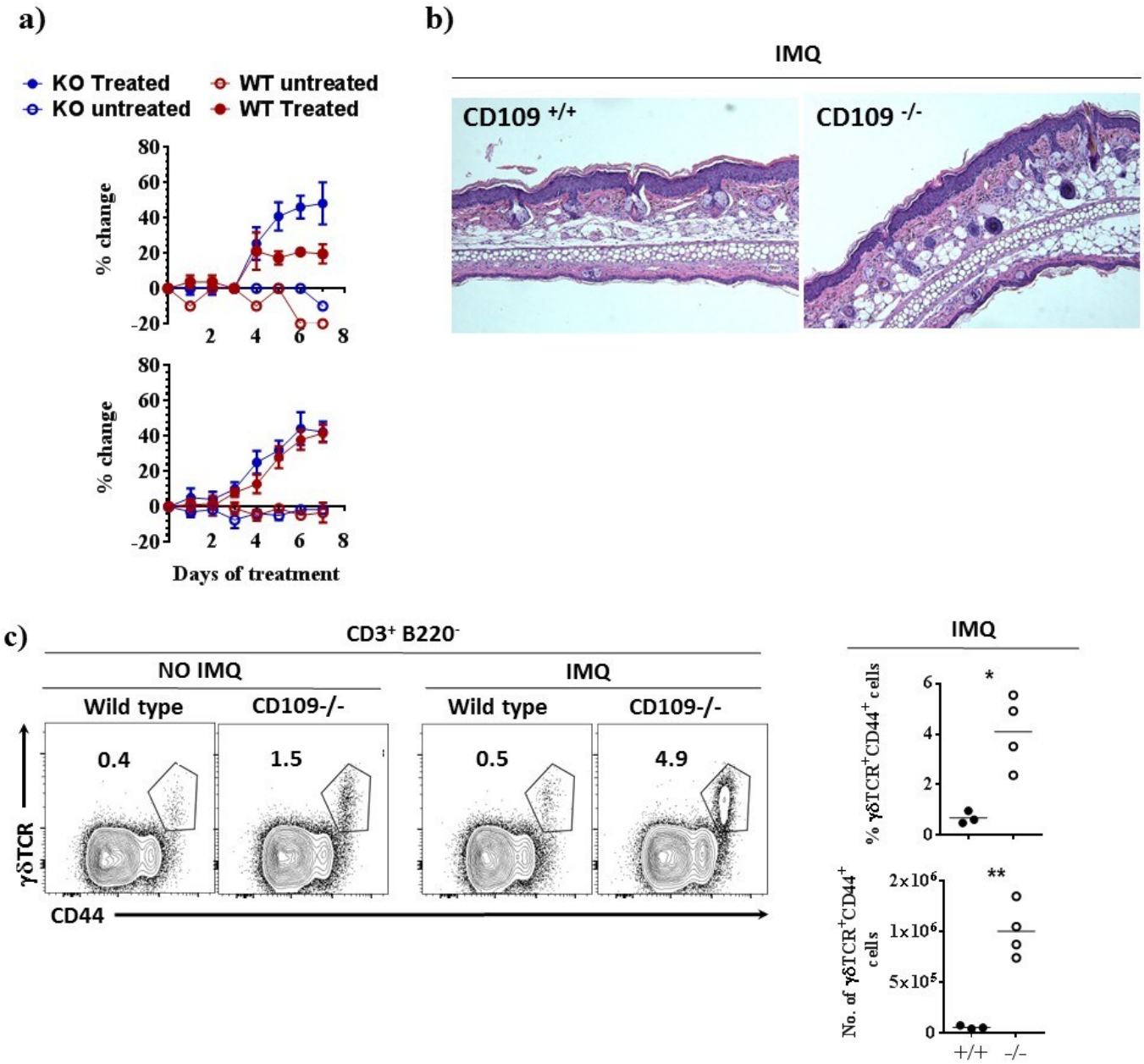
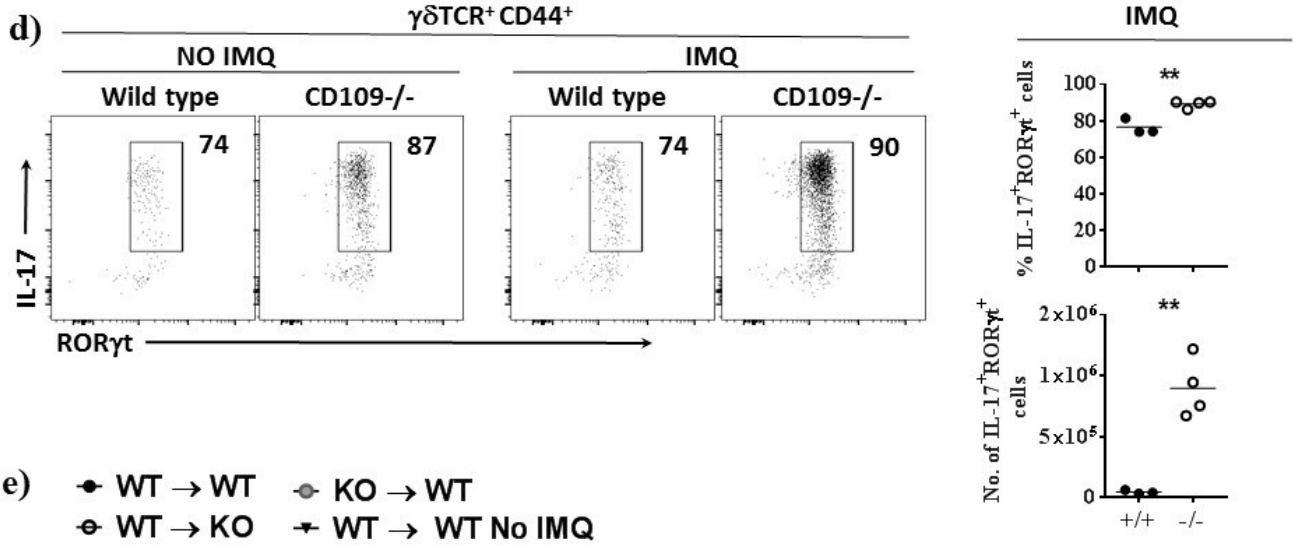
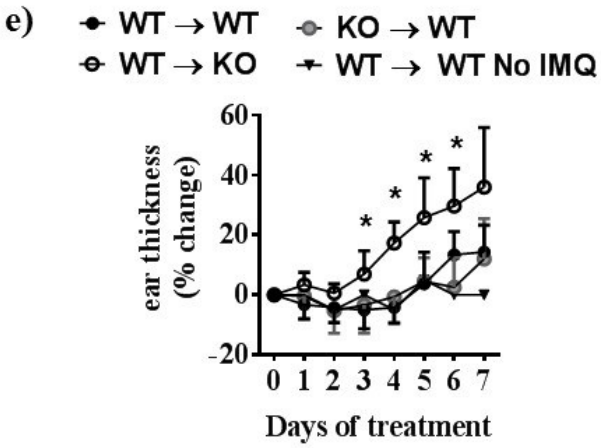
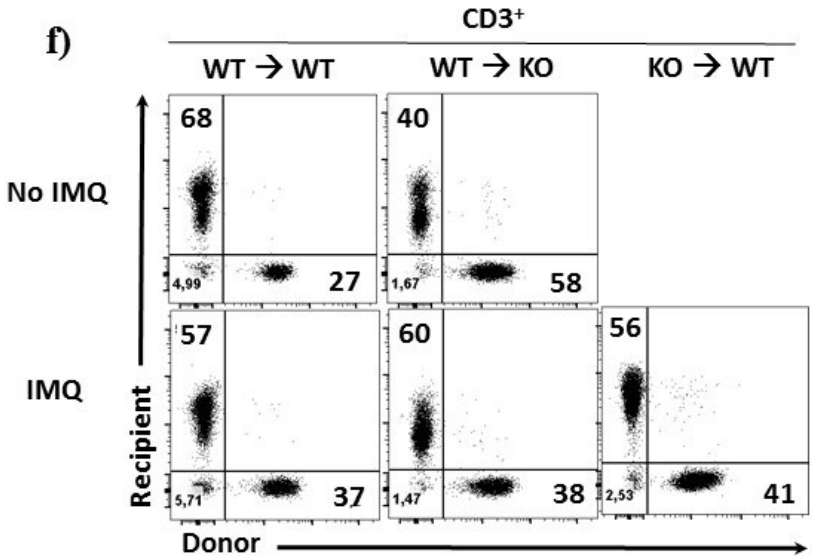

g)
WT → WT
WT → KO
KO → WT
cerLN
Spleen
mesLN
0.1
25
0.3
0.2
2.3
0.1
0.1
0.2
0.2
Vγ4
RORγt

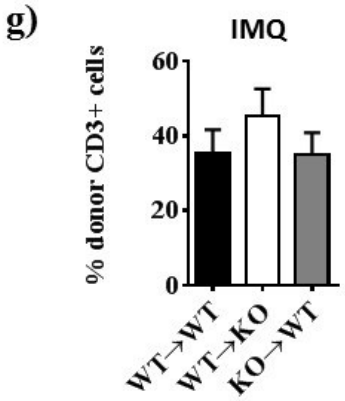
Figure 7: CD109 expression is required to limit pathogenic IL-17 response between skin and skin-draining lymph nodes




d)
γδTCR+ CD44+
NO IMQ
IMQ
Wild type
CD109-/-
Wild type
CD109-/-
74
87
74
90
IL-17
RORγt
IMQ
% IL-17+RORγt+ cells
100
80
60
40
20
0
**
No. of IL-17+RORγt+ cells
2x10^6
1x10^6
5x10^5
0
**
+/+
-/-


e)
WT → WT
KO → WT
WT → KO
WT → WT No IMQ
ear thickness (% change)
60
40
20
0
-20
0 1 2 3 4 5 6 7
Days of treatment
*
*
*
*


f)
CD3+
WT → WT
WT → KO
KO → WT
No IMQ
IMQ
Recipient
Donor
68
4,99
27
40
1,67
58
57
5,71
37
60
1,47
38
56
2,53
41


g)
IMQ
% donor CD3+ cells
60
40
20
0
WT→WT
WT→KO
KO→WT

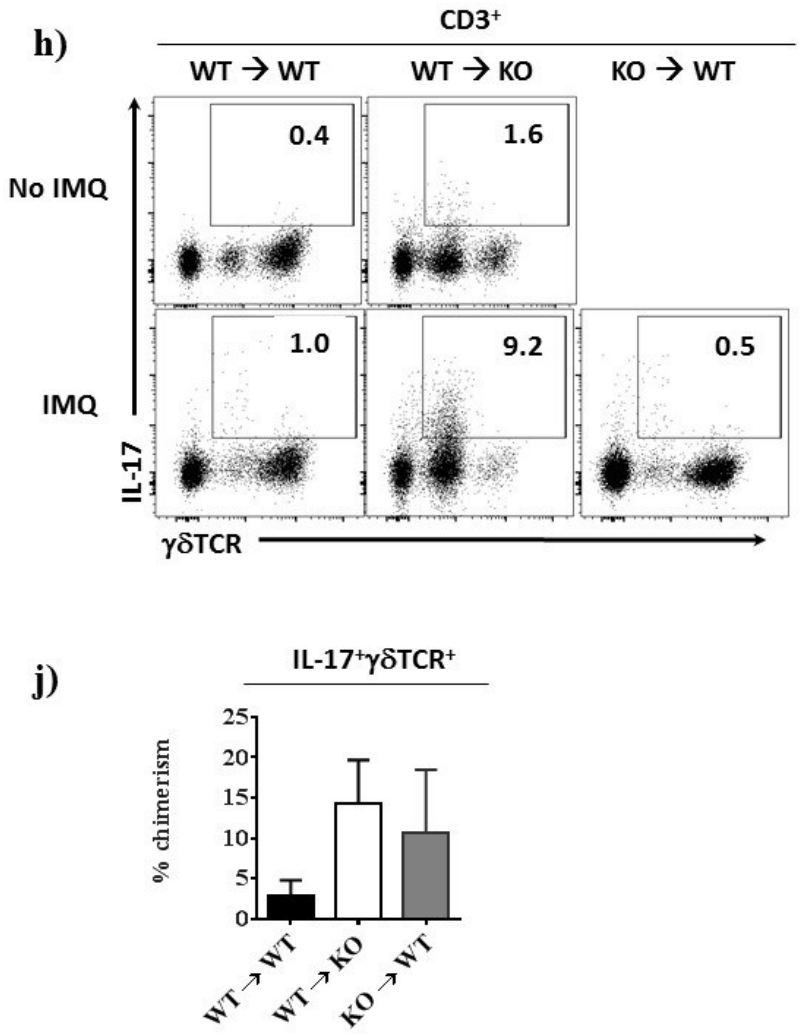
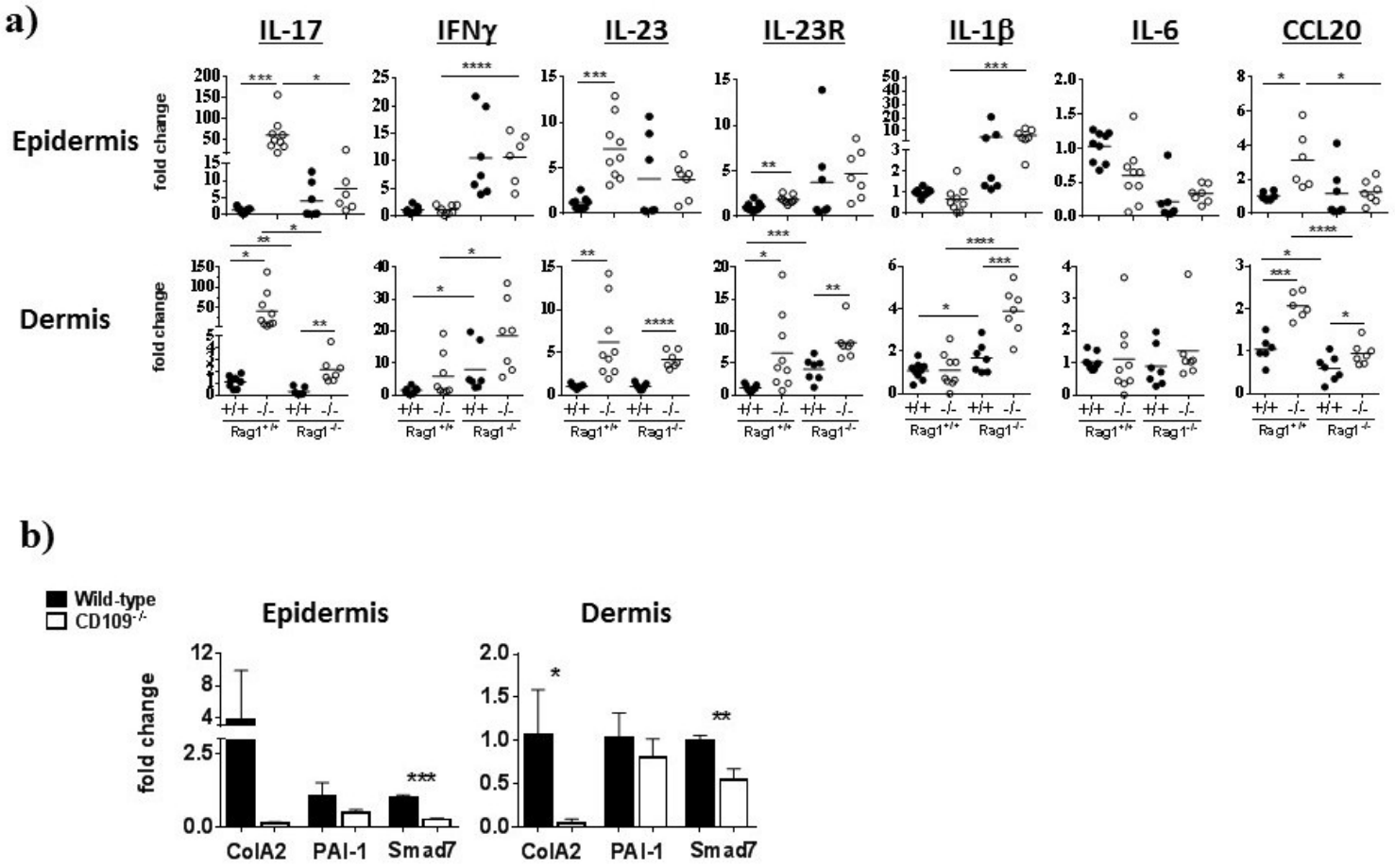

h)
CD3+
WT → WT
WT → KO
KO → WT
No IMQ
IMQ
0.4
1.6
1.0
9.2
0.5
IL-17
γδTCR
i)
IMQ
% IL-17+ γδ T cells
15
10
5
0
****
****
no. of IL-17γδ T cells
2×10^4
2×10^4
1×10^4
5×10^3
8.0×10^2
4.0×10^2
0
***
***
WT→WT
WT→KO
KO→WT
j)
IL-17+γδTCR+
% chimerism
25
20
15
10
5
0
WT→WT
WT→KO
KO→WT

Figure 8: CD109 plays a key role in regulating T-cell responses in the skin through a potential dysregulated TGF-β signaling pathway



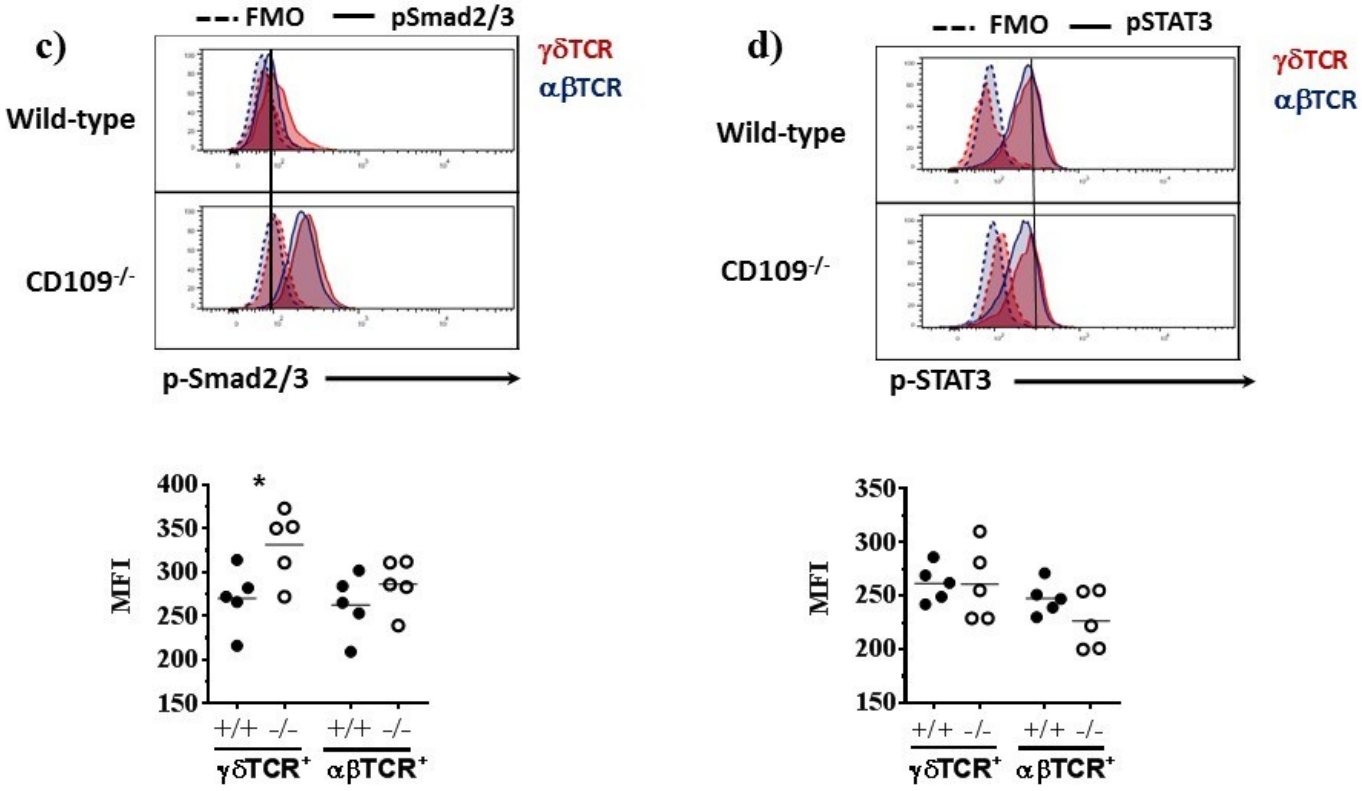

c)
FMO
pSmad2/3
γδTCR
αβTCR
Wild-type
CD109-/-
p-Smad2/3
MFI
400
350
300
250
200
150
*
+/+ -/-
+/+ -/-
γδTCR+
αβTCR+
d)
FMO
pSTAT3
γδTCR
αβTCR
Wild-type
CD109-/-
p-STAT3
MFI
350
300
250
200
150
+/+ -/-
+/+ -/-
γδTCR+
αβTCR+

Figure Legends

Figure 1. (a) Cross-sections of hematoxylin and eosin (H&E) stained wild-type and CD109$^{-/-}$ ears. E=Epidermis, D=Dermis, S=Subcutaneous fatty layer. **(b)** Quantification of epidermal thickness measurements of H&E ear cross-sections. **(c)** Epidermis and dermis from wild-type and CD109$^{-/-}$ ears were separated and digested prior to staining with anti-CD45 and analysis by flow cytometry. Graphs represent combined cell numbers from three independent experiments. **(d)** Representative flow cytometry plots of CD45 and intracellular Ki67 expression from wild-type and CD109$^{-/-}$ ear cells. **(e)** Enumeration of Ki67-positive cells based on expression of CD45, from flow cytometry analysis shown in d). At least 2 independent experiments were performed with 4-5 mice per genotype, aged 6-12 weeks of age. *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001

Figure 2. (a) RT-qPCR analysis for mRNA expression of T helper cytokines in wild-type and CD109$^{-/-}$.epidermis (left) and dermis (right) **(b)** Wild type and CD109$^{-/-}$ epidermis and dermis analyzed by flow cytometry for intracellular IL-17 and IFNγ production following *ex vivo* re-stimulation with PMA and ionomycin for 4h with Golgistop. Representative flow cytometry plots (left) and graphical representation of frequency and cell numbers of IL-17^{+} cells (right). **(c)** RT-qPCR analysis as in a) for IL-17-T cell related cytokines and CCL20. All RT-qPCR graphs show fold change expression relative to averaged wild-type ddCt values, normalized to HPRT expression, values from 3 independent experiments were combined. At least 3 independent replicate experiments were performed with 3-5 mice per genotype, aged 6-12 weeks of age. Error bars show standard deviation, *p <0.05, **p<0.01, ***p<0.001.

Figure 3. (a) Flow cytometry analysis of wild-type and CD109$^{-/-}$ epidermis and dermis showing TCRβ and TCRδ expression of total live leukocytes. **(b)** Enumeration of dermal T cell populations shown in (a). **(c)** Flow cytometry analysis of wild-type and CD109$^{-/-}$ epidermal and dermal γδ or αβT cell production of IL-17, following *ex vivo* re-stimulation of PMA and ionomycin for 4h with Golgistop. **(d)** Graphical representation of frequency (top) and cell number (bottom) of IL-17-producing T cells. **(e)** Representative flow cytometry plots for γδT cell expression of Vγ4 receptors, CD27, CD44 (left) and CD103 (right). **(f)** Flow cytometry analysis of wild-type and CD109$^{-/-}$ epidermis and dermis for intracellular Ki67 expression in T cell subsets. **(g)** Graphical representation of (f) for frequency (top) and cell number (bottom) of Ki67-expression and T cell subsets. At least 3 independent replicate experiments were performed with 3-5 mice per genotype, aged 6-12 weeks of age. Error bars show standard deviation, *p <0.05, **p<0.01, ***p<0.001.

Figure 4. (a) RT-qPCR analysis of wild-type and CD109$^{-/-}$ epidermis and dermis for expression of anti-microbial peptide expression. All RT-qPCR graphs show fold change expression relative to averaged wild-type ddCt values, normalized to HPRT expression, values from 3 independent experiments were combined. **(b)** Flow cytometry analysis of neutrophils in wild-type and CD109$^{-/-}$ dermis, with graphical representation of frequency and cell number of Ly6G^{+}CD11b^{+} cells below. **(c)** Flow cytometry analysis of dermal macrophages, gated on CD45^{+}CD3^{+}CD11b^{+}CD11c^{-} in wild-type and CD109$^{-/-}$ dermis (left), with graphical representation of frequency and cell number of MHCII^{+}F4/80^{+} cells to the right. **(d)** Flow cytometry analysis of dermal dendritic cells (DC) in wild-type and CD109$^{-/-}$ dermis (left), with graphical representation of frequency and cell number of MHCII^{+}CD11c^{+} cells below, with subsequent analysis of DC subsets (right). At least 2 independent replicate experiments were performed with 3-5 mice per genotype, aged 6-12 weeks of age. +/+ denote wild-type mice, $^{-/-}$ for CD109$^{-/-}$ mice. Error bars show standard deviation, *p <0.05, **p<0.01, ***p<0.001.

Figure 5. (a) Flow cytometry analysis of wild-type and CD109$^{-/-}$ cervical lymph nodes (cerLNs). Representative plots following intracellular staining for RORγt^{+} cells with graphical representation of frequency and cell number of cells to the right. **(b)** Flow cytometry analysis as in (a). **(c)** Graphical representation of cell frequencies for γδ and αβT cells from analysis in (b). **(d)** Graphical representation of cell numbers for γδ and αβT cells from analysis in (b). **(e)** Flow cytometry analysis as in (a), gating on viable B220^{-} cells, with graphical representation of frequency and cell number for γδT cell subsets below. **(f)** Flow cytometry analysis of wild-type and CD109$^{-/-}$ cerLNs following *ex vivo* re-stimulation with PMA and ionomycin for 4h, with Golgistop. Cells were intracellularly staining with anti-IL-17 to measure cytokine production. At least 3 independent replicate experiments were performed with 3-5 mice per genotype, aged 6-12 weeks of age. +/+ denote wild-type mice, $^{-/-}$ for CD109$^{-/-}$ mice. *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6. (a) Wild-type or CD109$^{-/-}$ mice transferred with 5x10^{6} of either CD45 congenic wild-type or CD109$^{-/-}$ bone marrow cells. Graph represents frequency of total γδ17 cells in epidermis and dermis of chimeric mice following *ex vivo* PMA and ionomycin re-stimulation with Golgistop for 4h. **(b)** Flow cytometry analysis of congenic source of dermal γδ17 cells, in (a), from WT → KO chimeric mouse. **(c)** Graphical representation of congenic source of γδ17 cells in dermis from WT → KO and WT → WT control mice. **(d)** Degree of chimerism in the epidermis and dermis, gating on CD45.1^{+} or CD45.2^{+} viable cells. **(e)** Flow cytometry analysis of cervical lymph nodes for analysis of donor γδT cells. **(f)** Graphical representation of (e) showing frequency and cell numbers of donor γδT cells in cervical lymph nodes. **(g)** Flow cytometry analysis of wild-type and CD109$^{-/-}$ cervical and mesenteric lymph nodes and spleen of chimeric mice. Gating on donor population of γδTCR^{+} TCRβ^{-} cells. At least 2 independent replicate experiments were performed with 3-5 mice per genotype, aged 6-12 weeks of age. +/+ denote wild-type mice, $^{-/-}$ for CD109$^{-/-}$ mice *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 7. (a) Ears of wild-type and CD109$^{-/-}$ mice were measured daily with a caliper, and an average measurement for both ears were used per mouse per day. Values for 2 independent experiments shown. **(b)** H&E staining of IMQ-treated wild-type of CD109$^{-/-}$ ear cross-section after 7-day treatment. **(c)** Flow cytometry analysis of IMQ treated or non-treated wild-type or CD109$^{-/-}$ cerLNs, with graphical representation of frequency and cell number. **(d)** Intracellular cytokine and transcription factor staining in IMQ treated or non-treated wild-type or CD109$^{-/-}$ cerLNs, numerical graphs as in d). **(e)** Ears of IMQ or untreated WT→WT, WT→KO and KO→WT chimeric mice measured for epidermal thickness as in a). **(f)** Flow cytometry plots of congenic source of CD3^{+} cells of dermis in 3 chimeric mice treated with IMQ or WT→WT and WT→KO non-treated controls. **(g)** Graphical representation of frequency of CD3^{+} donor cells in IMQ treated groups. **(h)** Flow cytometry plots of total γδT cell IL-17 staining for mice as in f). **(i)** graphical representation of IMQ treated chimeric mice for frequency and cell number of IL-17^{+}γδTCR^{+} cells. At least 2 independent replicate experiments were performed with 3-5 mice per genotype, aged 6-12 weeks of age. +/+ denote wild-type mice, $^{-/-}$ for CD109$^{-/-}$ mice *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 8. (a) RT-qPCR analysis of dermis from Rag1$^{-/-}$CD109$^{+/+}$, Rag1$^{-/-}$CD109$^{+/-}$, and Rag1$^{-/-}$CD109$^{-/-}$ mice and compared to wild-type and Rag1$^{+/+}$CD109$^{-/-}$ controls. **(b)** RT-qPCR analysis of epidermis and dermis wild-type and CD109$^{-/-}$ mice for mRNA expression of TGF-β signaling target genes. All RT-qPCR graphs show fold change expression relative to averaged wild-type ddCt values, normalized to HPRT expression, values from 3 independent experiments were combined. **(c)** Baseline expression of pSmad2/3 in lymphocytes from pooled skin-draining lymph nodes, comparing γδT (red) and αβT cells (blue) between wild-type and CD109$^{-/-}$ mice. **(d)** Baseline expression of pSmad2/3 in lymphocytes from pooled skin-draining lymph nodes, comparing γδT (red) and αβT cells (blue) between wild-type and CD109$^{-/-}$ mice. At least 2 independent replicate experiments were performed with 3-5 mice per genotype, aged 6-12 weeks of age, with the exceptions of 8c and d, which were performed only once at the time of thesis submission. +/+ denote wild-type mice, $^{-/-}$ for CD109$^{-/-}$ mice Error bars show standard deviation, *p <0.05, **p<0.01, ***p<0.001.

Discussion

The studies described in this thesis provide evidence that CD109 plays a critical role in regulating the cutaneous IL-23/IL-17 immune axis during homeostasis and inflammation. In addition, the absence of CD109 results in distinct changes to the skin stromal cell compartment most strikingly indicated by epidermal thickening and increased stromal cell proliferation. However, increased cell division did not translate into an increased number of cells as determined by tissue digestion. This result could in part be due to inefficient digestion of the epidermis when isolating total cells, since the tissue essentially consists of densely packed epithelial cells connected by tight junctions. This is opposed to the more fibrous and collagen rich dermis, which digestive enzymes can effectively break down. To substantiate this point, future studies should focus on in situ measurements of cell proliferation and apoptosis using various in vivo imaging strategies. Nevertheless, our results indicate that CD109 plays an important role in keeping the proliferative nature of keratinocytes and skin T cells under strict control.

Analysis of the T cell compartment in CD109$^{-/-}$ mouse skin, showed a dramatic expansion of IL-17 producing cells of the γδ T cell lineage. Transcriptional profiling of the skin also confirmed CD109$^{-/-}$ mice exhibit specific increases in transcripts encoding for the *Il23* gene, which promotes activation and maintenance of IL-17 production by T cells. As a result, *Il23r* gene transcripts were also higher in CD109$^{-/-}$ mice, suggesting that an active IL-23 signaling pathway is dysregulated in these mice. IL-17-producing T cells express the skin-homing chemokine receptor CCR6, which binds its ligand CCL20. Interestingly CCL20 is up-regulated by epithelial cells upon IL-17 signaling[38] and more of its transcripts were detected in CD109$^{-/-}$ mice, providing a plausible explanation for the enhanced recruitment of more IL-17^{+} T cells to the skin of these mice. The major producers of IL-17 in the skin of CD109$^{-/-}$ mice were γδT cells. Consistent with previous studies, we determined that most of the IL-17 was being produced in the dermis, although it is worth mentioning the small but significant increase in αβ and dermal γδT cells which infiltrate into the CD109$^{-/-}$ epidermis. It is unclear at this point whether the increase in frequency and number of dermal γδ17 cells in CD109$^{-/-}$ mice is due to local expansion in the tissue of a small IL-17-producing population or recruitment of thymic-imprinted γδ17 cells and upon exposure to dermal IL-23 signals results in enhanced IL-17 secretion. It important to mention that intracellular

cytokine staining was performed following *ex vivo* stimulation of isolated epidermal and dermal cells with PMA and ionomycin in the presence of Golgistop for four hours. This method may mask the true difference in γδ17 cells between wild-type and CD109$^{-/-}$ mice as this "non-physiological" stimulus may induce all IL-17-competent γδT cells to start secreting cytokine. An alternative approach, which had been used for experiments involving bone marrow chimeric mice but not for steady state experiments, is to simply digest ear samples for two hours in the presence of Golgistop. These methods more accurately represent the cytokine-producing cells *in situ*.

Another striking difference between wild-type and CD109$^{-/-}$ mice is the distinct outgrowth of Vγ4^{+} subset of cells which are relatively rare in wild-type mice. The finding of Vγ4^{+} cell expansion in the skin-draining cerLNs of CD109$^{-/-}$ mice may also suggest enhanced trafficking of these cells to and from the inflamed skin and associated lymph nodes (LNs). As reported, these cells have been shown to expand in the cerLNs prior to returning to the skin, possibly suggesting our dermal staining for Vγ4 may actually be under-representing the actual amount of cells that may be dysregulated by the loss of CD109. In light of this, DC subsets and macrophages in cerLNs will have to be characterized as well, to establish whether these antigen-presenting cells (APCs) also migrate between the skin and lymph nodes. If so, aberrant production of IL-23 within the skin-draining lymph nodes may further contribute to the expansion of mobile γδ17 cells. Alternatively, enhanced DC migration from the skin may also enhance presentation of skin-derived antigens to circulating αβT cells to facilitate adaptive immunity. If this were the case, it could explain the influx of αβT cells into the dermis and epidermis of CD109$^{-/-}$ mice. αβT cell priming could also be facilitated by the increased numbers of activated γδT cells in CD109$^{-/-}$ skin. Indeed, both human and mouse γδT cells have been shown to enhance dendritic cell cross-presentation and the development of an adaptive immune response[84, 85]. Of further interest, both human and mice γδT cells has been described to express MHCII and possess antigen processing and presentation capabilities to naïve αβT cells, in the context of *in vitro* microbial antigen stimulation[86] or with monoclonal TCR agonists[87]. The human γδT cells were the same Vγ9^{+}Vδ2^{+} γδT (Vγ9^{+}) cells identified to be recruited to inflamed skin of psoriasis patients[44]. Thus it is possible that in the mouse, Vγ4^{+} T cells are the equivalent to human Vγ9^{+} cells and may behave in similar fashion in CD109$^{-/-}$ mice.

Consistent with human psoriatic lesions and mouse models of psoriasis (Cai, et al), CD109$^{-/-}$ mice also had an expansion of DCs and macrophages in the skin. These cells may be the source of IL-23 which might be driving γδT17 cell expansion, however this needs to be further investigated in the context of CD109$^{-/-}$ mice at steady state.

In an attempt to determine whether CD109 is acting in an intrinsic manner to the T cells, we generated bone marrow chimeric mice with CD45 disparate donor and host cells. This approach was not optimal due to the radio-resistant nature of both epidermal and dermal γδT cells, but also of Langerhans cells. Nevertheless, key information was acquired from secondary lymphoid organs analysis which indicated that only the skin-draining cerLNs of CD109$^{-/-}$ mice that received WT cells had an increase in donor wild-type Vγ4^{+} cells and γδ17 cells. As this result was not observed in CD109$^{-/-}$ Vγ4^{+} cells transferred into wild-type mice, this clearly indicates that expression of CD109 by a source extrinsic to γδT cells is sufficient to control IL-17 production by this innate subset.

To further address whether γδ17 cells are a critical source of immune dysregulation in the absence of CD109, we analyzed the skin from CD109$^{-/-}$Rag$^{-/-}$ (Rag109DKO) mice by qRT-PCR. Our preliminary results have confirmed some observations in Rag-sufficient CD109$^{-/-}$ mice but also provided some unexpected results. For example, the increase in IL-17 expression in CD109$^{-/-}$ mice was significantly decreased in Rag109DKO mice, strongly suggesting γδ17 cells as a key source of this cytokine in vivo. By contrast, IL-23 levels were relatively unaffected regardless of the presence or absence of mature T cells. These data suggest that the stimulus driving IL-23 production in the absence of CD109 is upstream of γδT cells. However, these studies still fail to examine a potential role for CD109 expression by dermal γδT cells themselves. To directly address this question, reconstitution of the dermal γδ17 cell population with CD109 sufficient or deficient γδT cells would need to be performed. To this end, transfer of neonatal thymocytes into adult Rag-deficient mice has proven a useful method to specifically manipulate dermal γδT cells[43]. Using this approach, future studies should include transferring wild-type or CD109$^{-/-}$ thymocytes into Rag1$^{-/-}$ or Rag109DKO and assessing the resulting immune phenotype of recipient mice during steady-state and inflammatory conditions.

We showed that while application of IMQ to the ears of wild-type and CD109$^{-/-}$ mice enhanced expansion of IL-17 pre-committed γδT cells in CD109$^{-/-}$ cerLNs compared to controls,

a discrepancy in ear thickness was observed between the two experiments. Although additional experiments need to be performed to determine whether either phenotype is reproducible, quantitating epidermal thickness by analyzing histological tissue sections should also be performed as a more objective readout of skin inflammation. In addition, assessing "physiological" IL-17 production by γδT cells using methods described in Figure 7 in wildtype and germline CD109$^{-/-}$ mice should be performed following IMQ treatment to confirm the in situ dysregulation of γδ17 cells during psoriatic-like inflammation.

It will be important for future studies to focus on the mechanism by which CD109 regulates γδ17 cells. To gain initial insight into this question and address whether regulation of TGF-β signalling, a known function of CD109, is indeed altered in the absence of CD109, we examined mRNA levels of TGF-β target genes in the skin of wildtype and CD109$^{-/-}$ mice. These include collagen type 1 α2 (*Col1a*), a fibril-forming collagen found in the extracellular matrix[88] and Smad7, a well-established negative regulator of TGF-β signaling[89]. Contrary to our expectations, we observed a decrease in transcript expression for these genes in CD109$^{-/-}$ compared to wild-type controls, whereas no change in plasminogen activator inhibitor (PAI-1) expression, another well-defined TGF-β target gene, was observed. Although we expected TGF-β target genes to be up-regulated in CD109$^{-/-}$ mice, Mii et al have also failed to observe increases in canonical TGF-β signalling[12]. While it remains difficult to conclude from our transcriptional analyses how TGF-β signaling is affected *in vivo* by the absence of CD109, we must take into consideration the possibility that excessive TGF-β signaling may be resulting in a decrease in target gene expression, due to downregulation of TGF-β receptor[76]. In addition, compensatory mechanisms might have taken over in a physiological context and may be masking any *in vivo* defects[12].

We also looked at baseline levels of p-Smad2/3 and p-STAT3 protein in T cells of wild-type and CD109$^{-/-}$ mice. Although these preliminary results are promising, further *in vitro* analyses are necessary to decipher the cellular and molecular mechanisms regulating CD109 function. For example, culturing WT or CD109$^{-/-}$ keratinocytes with either WT or CD109$^{-/-}$ γδT cells in the presence IL-17 polarizing cytokines and assessing their impact on IL-17 production is warranted. A secondary experiment to this would be treatment of keratinocyte culture with PI-PLC to ensure release of GPI-linked proteins, and assess whether the soluble CD109 interacts and alters WT or CD109$^{-/-}$ physiology. These experiments could help identify potential cross-talk between

keratinocytes and γδT cells and determine the extent to which soluble or surface-bound CD109 mediates changes in IL-17 producing γδT cells. These culture experiments could also be expanded to analyze the potential involvement of DCs in the development of psoriasis-like inflammation of $CD109^{-/-}$ mice. Ideally crossing CD11c-DTR transgenic mice to $CD109^{-/-}$ mice would be the best scenario to test the role DCs play in the $CD109^{-/-}$ skin phenotype, since CD11c-DTR mice have been genetically engineered so that diphtheria toxin receptor expression is under the control of the CD11c promoter[90]. Hence, this results in specific deletion of DCs upon injection of diphtheria toxin and this deletion can be temporally controlled. As an alternative approach, imaging of the skin to identify location of DCs and potential interactions with γδT cells or stromal cells, could also be performed.

Other possible factors that could be contributing to the skin phenotype of $CD109^{-/-}$ mice is whether skin barrier integrity is altered. One readout of skin integrity and proper functioning is trans-epidermal water loss; this is a measure of the amount of water that is evaporated from the body via the skin[91]. Additionally, skin integrity is maintained by tight-junction expression in keratinocytes, which prevents passage of small molecules at barrier surfaces. A common test for whether defective tight-junctions compromise barrier integrity, is to use sulfo-NHS-biotin, a small water-soluble molecule which under normal conditions cannot passage via the inter-cellular space[91]. Sulfo-NHS-biotin can be applied to the surface of wild-type and $CD109^{-/-}$ skin sections and subsequently stained with fluorescently labelled streptavidin antibody and assessed by confocal microscopy. If in fact the skin barrier is compromised in $CD109^{-/-}$ mice, this could result in increased penetration of environmental antigens or microbes that activate the immune system or damage keratinocytes which would in turn cascade into a state of chronic inflammation. One way to test the contribution of commensal microbes on the skin of $CD109^{-/-}$ mice, would be to re-derive the mice into a germ free facility. It is possible that an altered skin microflora may be impacting cutaneous immune responses regardless of impaired barrier integrity as well. Therefore, future experiments could include sequencing the skin microbiome and generating a population study to compare with wild-type mice. Finally, IL-17 producing γδT cells have been shown to be important for protection against *S. aureus* infections in mice, and this could also be of important relevance to humans as well[92]. Future experiments could also include infecting $CD109^{-/-}$ mice with *S. aureus* to see the role of CD109 in regulating cutaneous immune responses by microbes.

In conclusion, the results included in this thesis provide a sound and in-depth characterization of the mouse skin T cell populations and the role of CD109 on the cutaneous immune system in general. While we have demonstrated that CD109$^{-/-}$ mice represent a novel mouse model to study IL-17-driven skin diseases, such as psoriasis, there remains an extensive body of work to be fulfilled to fully understand the role CD109 has in promoting skin health. I believe research on CD109 and its role in immunity and homeostasis has tremendous potential for future graduate students and scientists. The initial work presented in this thesis, is the start of many fruitful years ahead filled with exciting and revolutionary discoveries. I recommend that experiments in the near future should strive to delineate clear molecular and cellular mechanisms of CD109 action on immune cells in the skin. Ultimately, knowledge obtained from rodent research should transition towards basic human research, in the scope to one day apply the extent of the knowledge acquired about the role of CD109 in skin disease pathogenesis and address its therapeutics potential.

Materials and Methods

Mice

CD109$^{-/-}$ mice were generated from CD109$^{+/-}$ parents obtained from Dr. Masahide Takahashi at Chubu University in Japan[12], while C57BL/6 mice were purchased from Jackson Laboratories and bred with CD109$^{+/-}$ mice to generate littermate controls. Rag1$^{-/-}$ mice were purchased from Jackson Laboratories and crossed to CD109$^{-/-}$ to generate double knock-out mice. All experimental mice used were aged between 6-12 weeks.

Cell Isolations

Skin: Mouse ears were cut above the cartilaginous region, manually separated into dorsal and ventral sheets and placed dermal-side down in 35mm petri dish containing 1mL RPMI 1640 buffer containing 1mg/mL Collagenase/Dispase (Roche, Cat. 10269638001) for 1 hour at 37°C. Following this, epidermis and dermis were separated manually, using forceps, and briefly soaked in PBS before being transferred into a 24 well plate, with 1mL RPMI 1640 per well, containing 10% fetal bovine serum (FBS), 154 U/mL of Collagenase from Clostridium Histolyticum-Type IV (Sigma Aldrich, Cat. C5138) and 200 U/mL of DNaseI (Roche, Cat. 11284932001). Ear sections were and biological replicates were kept in individual wells and cut using fine scissors into 2-3 mm^3 pieces and digested at 37°C for 2h. Samples were then homogenized by aspirating up and down with a p1000 pipette, filtered through a 70µm cell strainer into a 50mL conical tube, where remaining pieces of tissue were crushed using plunger of 5mL plastic syringe. Subsequently, 8mL of R10 buffer (RPMI 1640 containing 10% FBS, 15mM HEPES and 1% penicillin streptomycin) were passed through each filter to dislodge any adherent cells. Samples were then centrifuged for 10 minutes at 1800 rpm (Sorvall Legend X1R, Thermo Scientific) at 4°C. Pellets were re-suspended in RPMI solution containing 2% FBS and 10mM HEPES for cell counting on a hemocytometer and ready for subsequent use.

Lymph Nodes: Skin-draining lymph nodes were harvested from wild-type and CD109-/- mice, placed in 70µm filter sitting in HBSS containing 2% FBS and 10 mM HEPES (HBSS buffer). Lymph nodes were then crushed plunger of a 5mL plastic syringe.

Spleens: Spleens were harvested and treated as described for lymph nodes. Prior to crushing, spleens were injecting with 1mL HBSS buffer using a 25G needle and syringe. This process swells the spleen and dissociates the spleenocytes for better isolation. Next, spleens were crushed as described above. Following crushing of the tissue, single cell suspensions were generated for both lymph nodes and spleen by repeated up and down pipetting and total volume was transferred into 15 mL conical tubes, centrifuged at 1500 rpm, at 4°C for 5 minutes, and re-suspended in HBSS buffer. Thereafter, cells were manually counted on a hemocytometer and ready for subsequent use.

Flow Cytometry and Intracellular Cytokine Staining

Extracellular staining: In 5mL polystyrene round-bottom tubes, 1-2 x 10^6 cells were washed once in 2mL and subsequently with 1mL PBS solution, centrifuging samples for 5 minutes at 1800 rpm in 4°C after each wash, before staining for 22 minutes with efluor 506-conjugated fixable viability dye (ebioscience, cat no: 66-0866-18), in 100 µl PBS on ice, in the dark. Following viability staining, cells were washed in 1mL PBS containing 2% FBS and 10 mM HEPES (FACS buffer), centrifuged as previously described and re-suspended in FACS containing <1µg anti-Fc receptor (clone: 2.4G2, BD biosciences, Cat. BE0008) for 10 minutes on ice, in the dark. Antibody cocktail in 100µl FACS buffer were then added directly to all cells. Following 30 minutes of on ice incubation in the dark, all samples were washed with 1mL FACS buffer, and re-suspended in 200-400 µl FACS buffer for flow cytometry analysis.

Intracellular staining: Samples needing intracellular staining were centrifuged following 30 minutes of extracellular staining (see above) and supernatant was decanted. Each sample was subsequently vortexed to dislodge pellet and resuspended in 1mL of BD Fix/Perm Working Solution (From ebioscience FoxP3/Transcription Factor Buffer Staining Set, Cat. 00-5523) and vortexed again before letting fix for 45-60 minutes on ice, in the dark. After which, cells were washed once with 2mL 1X Perm Buffer (Cat. 00-5523) and once with 1mL 1X Perm buffer, centrifuging samples for 5 minutes at 1800 rpm in 4°C after each wash. Subsequently samples were staining with intracellular antibody(ies) diluted in 1X Perm buffer, for 1 hour on ice, in the dark. All the samples were washed with 1X Perm Buffer as previously described, supernatant was aspirated and pellet was resuspended in 200-400µl PBS for flow cytometric analysis.

Antibodies used for Flow Cytometry:

From ebioscience: CD3e-APC, (145-2C11), B220-Alexafluor 700 (RA3-6B2), CD45.2-Pacific blue, PE-efluor 710 (104), TCRβ-APC-efluor 780 (H57-597), Vγ2, a.k.a Vγ4-PE-Cy7 (UC3-10A6), CD4-efluor 450 (RM4-5), CD8β-PE (eBioH35-17.2), CD27-PE (LG.7F9), CD103-PE-Cy7 (2E7), CD11c-APC (N418), CD11b-APC efluor 780 (M1/70), MHCII-Pacific Blue (M5/114.15.2), F4/80-PerCP-Cy5.5 (BM8), RORγt-APC (B2D), IL-17-PE (ebio17B7), IFNγ-FITC (XMG1.2), Ki67-APC (solA15), pSmad2(pS465/pS467),pSmad3(pS423/pS425)-PE (072-670).

Biolegend: TCRδ-PerCP-Cy5.5 (GL3), Ly6G-FITC, AF700 (1A8)

BD biosciences: CD44-BV421, BV786 (1M7), CD3e-BV650 (145-2C11), p-STAT-3(pY705)-PE (4/p-STAT3)

Phospho-protein assays

Lymphocytes were isolated and washed as previously described, counted and equal number of cells were transferred to 5mL round bottom tubes in a pre-heated water bath to 37°C. There the cells were immediately fixed with 100 µl of Phosflow fix buffer (BD bioscience, Cat no. 557870) for 16 minutes, with viability dye being added during the remaining 8 minutes. Cells were then washed with PBS and resuspended in 1mL pre-chilled Phosflow Perm Buffer III (BD biosciences, Cat no. 558050) for 30 minutes on ice. Samples are then washed in FACS buffer and stained with extracellular and phospho-antibody together (see above for antibodies) in 100µl FACs buffer for 30 minutes at room temperature. Following 30 minutes of on ice incubation in the dark, all samples were washed with 1mL FACS buffer, and re-suspended in 200-400 µl FACS buffer for flow cytometry analysis.

RNA extraction & Real-Time qPCR

Tissue samples of approximately 30 mg were lysed in RLT buffer using Fisher Sonic Dismembrator model 300, for 30 seconds then further homogenized by pipetting. The RNA extraction was continued following QIAGEN RNAeasy Mini Kit protocol (including optional DNase treatment). RNA samples were quantified using a NanoDrop 2000 and between 100 to 500 µg of RNA was used to generate cDNA. Synthesis of cDNA was accomplished by pre-heating

RNA at 65°C for 5 minutes in 1.5mL eppendorf RNase/DNase-free tube, in a water solution containing oligo(dT) 12-18 primer (Invitrogen, Cat. 18418-012) and dNTPs. Following this, tubes were chilled on ice for 2 minutes before adding First Strand RT buffer (1x, Invitrogen), DTT (Invitrogen) and RNAse inhibitor (20U, Promega/Fisher, Cat. PR-N2615). RNase/DNase-free water and M-MLV Reverse Transcriptase (200U, Invitrogen, Cat. 28025-013) was added to each tube and incubated for 60 minutes at 37°C. The M-MLV was heat-denatured for 5 minutes at 70°C to terminate the cDNA synthesis step.

For RT-qPCR, each cDNA sample was diluted two-fold in RNase/DNase-free water and loaded into a 96 well plate in duplicate analytical samples. The cDNA was diluted ten-fold in each well in SSO ADV SYBR Green Supermix (Biorad, Cat. 1725260). The qPCR reaction was analyzed by BioRad CFX96 C1000 Touch Thermocycler using the following cycle: 1) 95°C: 30 secs, 2) 95°C: 5 secs, 3) 58°C: 30 secs, plate read, repeat from step 2) 39 times. Melt curve: 4) 65°C: 31 secs, 5) 65°C: 5 sec, +0.5°C/cycle Ramp 0.5°C/s, plate read, repeat from step5) 60 times.

List of primers used for RT-qPCR analysis, including reference gene (*Hprt*) and sample genes.

Gene	***Forward***	***Reverse***
Hprt	AGGACCTCTCGAAGTGTTGG	AACTTGCGCTCATCTTAGGC
Il17a	ACTCTCCACCGCAATGAAGA	CTCTCAGGCTCCCTCTTCAG
Ifnγ	TTCTTCAGCAACAGCAAGGC	ACTCCTTTTCCGCTTCCTGA
Il22	ATACATCGTCAACCGCACCTTT	AGCCGGACATCTGTGTTGTTAT
Il4	TCACAGCAACGAAGAACACC	ATTCATGGTGCAGCTTATCG
Il5	TCCAATGCATAGCTGGTGATTT	AGCACAGTGGTGAAAGAGACCTT
Il13	ATTGCATGGCCTCTGTAACC	TGAGTCCACAGCTGAGATGC
Il23p19	GACTCAGCCAACTCCTCCAG	GGCACTAAGGGCTCAGTCAG
Il23r	AGCAAAATCATCCCACGAAC	GCCACTTTGGGATCATCAGT
Il1β	GGGCCTCAAAGGAAAGAATC	TACCAGTTGGGGAACTCTGC
Il6	ACTTCACAAGTCCGGAGAGG	TTCTGCAAGTGCATCATCG
Ccl20	AATCTGTGTGCGCTGATCC	AGGTCTGTGCAGTGATGTGC
S100a7	CAGGCAGTCTCTCATCACCA	CCCAGAACCATGACCTGAGT
S100a8	GGAAATCACCATGCCCTCTA	TCCTTGTGGCTGTCTTTGTG
S100a9	CAGCATAACCACCATCATCG	GTCCTGGTTTGTGTCCAGGT
Cramp	CTCCGCAAAGGTGGGGAGAAG	GGGAATCCAGAAACAGGCCAAGG

RegIIIγ	AACAGAGGTGGATGGGAGTG	ATTTGGGATCTTGCTTGTGG
Col1a2	ATTGCACCTCTGGACATTGG	CACACTGCTCTGACCAATCC
Pai-1	GGTGCTGCGATATAGCTTCC	CATGTAATCCAGGCTGTTGC
Smad7	GAAGTCAAGAGGCTGTGTTGC	CAGGCTCCAGAAGAAGTTGG

Generation of Bone Marrow Chimera

In order to generate bone marrow chimera, C57BL6 wild type (WT) CD45.2^{+} or CD109$^{-/-}$ CD45.2^{+} recipient mice were lethally irradiated with 9 Gy split-dose irradiation (2 doses of 4.5 Gy of radiation, separated by a 3-hour interval). Irradiated recipients received by tail vein injection, 5x10^{6} bone marrow CD45.1 or CD45.2 cells from WT or CD109$^{-/-}$ donors. Chimeric mice were given normal drinking water (no antibiotics added) and allowed 12-24 weeks for bone marrow reconstitution before conducting any experiments.

Imiquimod-induced psoriasis-like model

To induce psoriasiform plaques WT or CD109$^{-/-}$ mice we treated with 5mg/ear with Aldara cream (5% Imiquimod, Valeant Pharmaceuticals) daily for 7 days, using the convex side of 5mm lab spoon. To allow proper and even absorption of applied cream, mice were kept for 10 minutes under low anesthetics (1.5% Isoflurane) prior to being returned to their respective cages. Mice were anesthetised by vapour isoflurane for the duration of the procedure and caged with Isopad bedding.

Histology

Tissue were fixed in 10% formalin for a minimum of 5 days, prior to be transferred into tissue processing cassettes and stored in 70% ethanol. Tissues were transferred to McGill University Histology Core facility for processing, embedding, sectioning and H&E staining. Epidermal thickness was quantified from H&E stained sections using FIJI is just ImageJ software and measuring various regions of the epidermis of wild-type and CD109$^{-/-}$ mice from random un-identified images. Measurement was then converted from pixels into length in centimeters (cm).

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