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PhD Thesis

Mia Hamilton Jee

Skin and thymus connections - effects on T cell development

Principal supervisor: Professor Charlotte Menné Bonefeld

Co-supervisor: Professor Jeanne Duus Johansen

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Author: Mia Hamilton Jee, MSc.

PhD supervisors:

Principal supervisor
Charlotte Menné Bonefeld, Professor, PhD
LEO Foundation Skin Immunology Research Center
Department of Immunology and Microbiology
University of Copenhagen
Denmark

Co-supervisor
Jeanne Duus Johansen, Professor, MD, PhD
National Allergy Research Centre
Department of Dermatology and Allergy
University Hospital of Copenhagen
Denmark

Assessment committee:

Chair
Søren Buus, Professor, MD, PhD
Department of Immunology and Microbiology
University of Copenhagen
Denmark

Danish representative
Claus Johansen, Ass. Professor, PhD
Department of Clinical Medicine
Department of Dermatology and Venereology
Aarhus University Hospital
Denmark

International representative
Mark Anderson, Professor, MD, PhD
Diabetes Center
Department of Medicine
Department of Microbiology and Immunology
University of San Francisco
California, USA

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Preface

This PhD thesis was carried out from 1st of July 2016 to 12th of May 2020. The experimental work was performed at the LEO Foundation Skin Immunology Research Center, Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark in Carsten Geisler's and Charlotte Menné Bonefeld's group and at the MRC Centre for Immune Regulation, Institute for Immunology and Immunotherapy, University of Birmingham, United Kingdom in Graham Anderson's group. My principal supervisor was Professor Charlotte Menné Bonefeld, while Professor Jeanne Duus Johansen was my co-supervisor at the National Allergy Research Centre, Department of Dermato-Allergology, Copenhagen University Hospital Herlev-Gentofte, Denmark.

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This PhD thesis is based on the following three studies:

Mia H. Jee, Jeanne D. Johansen, Terkild B. Buus, Trine H. Petersen, Anne-Sofie Ø. Gadsbøll, Anders Woetmann, Niels Ødum, Jacob P. Thyssen, Andrea J. White, Graham Anderson, Carsten Geisler, Charlotte M. Bonefeld. ***Increased production of IL-17A-producing γδ T cells in the thymus of filaggrin-deficient mice*** (Frontiers of Immunology, 2018).

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Mia H. Jee, Gustav Ø. Jørgensen, Julie Friis Weber, Anders Woetmann, Niels Ødum, Jeanne D. Johansen, Carsten Geisler, Charlotte M. Bonefeld. ***Sensitization to contact allergens induces development and export of IL-17A-producing innate CD8$^+$ T cells from the thymus*** (Manuscript).

During my PhD I have also contributed to work in the following papers, not included in my thesis:

Trine H. Petersen, **Mia H. Jee**, Anne-Sofie Ø. Gadsbøll, Jonas D. Schmidt, Jens J. Sloth, Gregory F. Sonnenberg, Carsten Geisler, Jacob P. Thyssen, Charlotte M. Bonefeld. ***Mice with epidermal filaggrin deficiency show increased immune reactivity to nickel*** (Contact Dermatitis, 2018).

Terkild B. Buus, **Mia H. Jee**, Niels Ødum. ***OMIP-057: Mouse γδ T-Cell Development Characterized by a 14 Color Flow Cytometry Panel*** (Cytometry, 2019).

Anne-Sofie Ø. Gadsbøll, **Mia H. Jee**, Anders B. Funch, Maria Alhede, Veronika Mraz, Julie F. Weber, Lauren A. Callender, Elizabeth C. Carroll, Thomas Bjarnsholt, Anders Woetmann, Niels Ødum, Allan R. Thomsen, Jeanne D. Johansen, Sian M. Henson, Carsten Geisler, Charlotte M. Bonefeld. ***Pathogenic CD8$^+$ epidermis-resident memory T cells displace dendritic epidermal T cells in allergic dermatitis*** (Journal of Investigative Dermatology, 2019).

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The atmosphere at the 12th floor is exceptional. This is due to the members of the T-cell biology and skin inflammation group, the cancer immunology group and the eye immunology group. Not to forget Johan, who practically belongs to the 12th floor. Thank you to all of you! Especially to my office-mates Anna Knock Out Rode, Anne-Sofie Østergaard Gadsbøll and (often) Maria Gluud Grøndahl. Also, thank you Veronika Mraz and Terkild Brink Buus for help with illustrations.

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Summary

Our skin is a barrier that provides protection against assaults from the environment. To obtain this integrity, the outermost part of our skin, the epidermis, consists of differentiating keratinocytes in multiple layers. During differentiation, the keratinocytes begin expression of numerous proteins that are needed to form the outermost layer of the epidermis, the stratum corneum. These include filaggrin and mattrin, and not surprisingly, mutations in the genes encoding both proteins are associated with increased risk of developing the inflammatory skin disease atopic dermatitis. T cells roam the skin and play a large role in the development of inflammatory skin diseases.

The thymus is a primary lymphoid organ harbouring developing T cells. A distinct microenvironment, shaped from thymic stromal cells, is needed to support correct development of T cells. Thymic epithelial cells are an important part of the thymic stroma and have unique functions in the development and maturation of T cells. Interestingly, a subset of thymic epithelial cells express markers otherwise associated with differentiating keratinocytes. The role of these keratinocyte-like cells in the thymus is currently unknown.

Therefore, the overall aim of this thesis was to investigate if filaggrin and mattrin are expressed in mouse thymus. Furthermore, if these epidermis-associated markers were able to affect T cell development, and thereby inflammation status in the periphery. Finally, if local inflammation initiated in the epidermis could affect the thymus, and thereby T cell development.

In **manuscript I** we used the filaggrin-deficient flaky tail mice to investigate T cell composition in the thymus and periphery. We determined that filaggrin is expressed in wild-type mouse thymic medulla and that more Vγ2$^+$ IL-17A producing γδ T cells develop in flaky tail mice, and that this is correlated with more IL-17A producing γδ T cells in spleen and epidermis. The flaky tail mice, however, are double mutants harboring a mutation in the filaggrin gene and in the gene encoding mattrin. To ensure the conclusions from the first manuscript were correct, we backcrossed flaky tail mice onto a C57Bl/6 background and separated the two mutations creating *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice, with a mutation in filaggrin and mattrin, respectively, to use in **manuscript II**. In this study, we determined that the mutation in the mattrin gene, and not in the filaggrin gene, caused the IL-17A-skewed T cell profile, whereas the mutation in the filaggrin gene caused an IFNγ-skewed T cell profile. In **manuscript III** we wanted to investigate outside-in instead of inside-out. To this end, we determined that local inflammation in the skin was able to affect development of T cells in the thymus with an increased number of 'innate-like' IL-17A-producing CD8$^+$ αβ T cells. Taken together, this thesis describes connections between the thymus and the skin.

Dansk resumé

Vores hud udgør en vigtig barriere imod omverdenen. For at være i stand til det, er det yderste lag af huden, kaldet epidermis, bygget op af differentierende keratinocytter i talrige lag. Under differentieringen påbegynder keratinocytterne udtryk af adskillige proteiner, der er essentielle for den endelige, yderste struktur kaldet stratum corneum. Filaggrin og mattrin er vigtige proteiner under dannelsen af stratum corneum, og mutationer i gener der koder for begge proteiner er associeret med risiko for at udvikle den inflammatoriske hudsygdom atopisk dermatit. T-celler er vigtige celler i huden, men disse spiller også en stor rolle i sygdomsudviklingen af inflammatoriske hudsygdomme.

Thymus er et primært lymfoidt organ, der indeholder T-celler under udvikling. For at kunne understøtte korrekt udvikling, er thymus opbygget af stromale celler der giver et karakteristisk mikromiljø. Thymus epitelceller er en vigtig komponent af disse stromale celler og har unikke funktioner under udvikling og modning af T-celler. En undertype af thymus epitelceller udtrykker proteiner, som man normalt finder i differentierende keratinocytter, men den præcise funktion af denne undertype, er stadig ukendt. Derfor er det overordnede formål med denne afhandling at undersøge om filaggrin og mattrin er udtrykt i thymus fra mus, og om disse markører der normalt er associeret med epidermis er i stand til at påvirke T-celleudvikling i thymus og dermed inflammation i periferien. Derudover, er det også formålet at undersøge om inflammation i periferien er i stand til at påvirke thymus og dermed T-celleudvikling.

I **manuskript I** anvendte vi filaggrin-deficiente flaky tail-mus til at undersøge T-cellesammensætningen i thymus og periferi. Her fandt vi, at der udvikles flere Vγ2^{+} IL-17A-producerende γδ T-celler i flaky tail-mus og at dette var korreleret med flere IL-17A-producerende γδ T-celler i milt og epidermis. Flaky tail-mus er dog dobbeltmutanter med både en mutation i genet der koder for filaggrin og genet der koder for mattrin. For at sikre os at de fund, der fremkom af manuskript I var korrekte, tilbagekrydsede vi flaky tail-mus til en C57Bl/6 baggrund og separerede de to mutationer. Dette skabte de to nye musestammer *Flg*$^{ft/ft}$ og *Matt*$^{ma/ma}$, med en mutation i henholdsvis filaggrin og mattrin, som vi anvender i **manuskript II**. I dette studie viser vi at det er mutationen i mattrin-genet der giver den IL-17A-profil vi fandt i manuscript I. Derimod resulterer mutationen i filaggrin-genet i en IFNγ-drejet T-celleprofil. I **manuskript III** undersøgte vi udefra og ind i stedet for det omvendte. Vi viser at lokal hudinflammation er i stand til at påvirke T-celleudvikling i thymus med en øget udvikling af 'innate' IL-17A-producerende CD8^{+} αβ T-celler. Alt i alt beskriver denne afhandling sammenhænge mellem thymus og huden.

Abbreviations

ACD	Allergic contact dermatitis
AD	Atopic Dermatitis
Aire	Auto-immune regulator
AMP	Antimicrobial peptide
APC	Antigen-presenting cell
CD	Cluster of differentiation
CE	Cornified envelope
CHS	Contact hypersensitivity
cTEC	Cortical thymic epithelial cell
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DETC	Dendritic epidermal T cell
DL4	Delta-like 4
DN	Double negative ($CD4^-CD8^-$)
DNFB	2,4-dinitrofluorobenzene
DP	Double positive ($CD4^+CD8^+$)
EDC	Epidermal differentiation complex
Flg	Filaggrin gene (mice)
FLG	Filaggrin gene (humans)
FoxN1	Forkhead box N1
FoxP3	Forkhead box P3
ft/ft	Flaky tail
HC	Hassall's corpuscles
IFNγ	Interferon gamma
IL	Interleukin
ILC	Innate lymphoid cell
KC	Keratinocyte
LC	Langerhans cell
LN	Lymph node
LT	Lymphotoxin
LTβR	Lymphotoxin β receptor
ma	Matted gene
MHC	Major histocompatibility complex
mTEC	Medullary thymic epithelial cell
NMF	Natural moisturizing factor
NLR	NOD-like receptor
nTreg	natural regulatory T cell
OOA	Olive oil:Acetone
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptor
RANK	Receptor activator of nuclear factor κB
RANKL	RANK ligand
RORγt	Retinoic acid receptor-related orphan receptor γt
RTE	Recent thymic emigrant
SB	Stratum basale
SC	Stratum corneum
SG	Stratum granulosum
Skint-1	Selection and upkeep of intraepithelial T cells protein-1
SP	Single positive ($CD4^+$ or $CD8^+$)
SS	Stratum spinosum
Tbet	T-box transcription factor
Tc	Cytotoxic T cell
TCR	T cell receptor
TEC	Thymic epithelial cell
TEP	Thymic epithelial progenitor
Th	Helper T cell
TLR	Toll-like receptor
TRA	Tissue-restricted antigen
Treg	Regulatory T cell

Introduction

1.1 The immune system

Our immune system is essential for the defense against the enormous number of pathogens we encounter during our lives. Furthermore, our immune cells play a key role in detecting and clearing out malignancies. A wide range of different immune cells exists, and each play a significant part in the complex structure that is our immune system. Although the immune system is not as simple, it can roughly be divided into two major subsystems. The innate system is our first line of defense and the cells of this system act fast and recognize features that are common to many different pathogens or danger signals, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Several immune cells act as innate effectors, and furthermore, the epithelial cells lining the interface between the host and the environment constitute an important barrier in the innate defense. The adaptive system is characterized by even greater specificity, and entails B and T lymphocytes that undergo gene rearrangement and clonal expansion to produce an impressive repertoire of antigen receptors, which upon encounter can discriminate between specific antigens. Additionally, a characteristic of the adaptive system is the ability to create memory, which permits a faster clearing of a specific pathogen upon secondary encounter[1]. Each subset of immune cells contributes with important functions, but in this thesis, the focus is on T cells.

1.1.1 The thymus and T cell biology

The thymus is a crucial organ for the adaptive immune system, supporting the development of both conventional and non-conventional T cells. Lymphoid progenitor cells arising from the bone marrow colonise the thymus and undergo proliferation, differentiation, lineage choices and selections[2–4].

T cells are characterized by expression of the T cell receptor (TCR). During their development, each T cell undergo gene rearrangement to form a unique TCR. Following the formation of the TCR, the conventional thymocytes are positively selected for their ability to recognize self in the form of major histocompatibility complex (MHC) molecules. Subsequently, thymocytes reacting

too strongly to self-peptides on MHC molecules are deleted or rendered regulatory in a process termed negative selection.

T cells occur in two lineages defined by the usage of either the TCRα and TCRβ subunits or the TCRγ and TCRδ subunits, constituting the αβTCR and the γδTCR, respectively. Besides these subunits, the entire TCR complex also contains subunits required for signaling that are shared between the two lineages. These include a TCRζ and two CD3 heterodimers consisting of CD3ε paired with either CD3γ or CD3δ. Conventional αβ T cells are divided into two large groups based on their expression of either the CD4 or CD8 co-receptor that recognizes constant parts of the MHC II or MHC I molecules, respectively[1].

Activation of a conventional T cell occurs in the periphery and requires stimulation through the TCR by its cognate ligand (peptide:MHC), co-stimulation and cytokines to differentiate the response. Upon stimulation, intracellular signaling is relayed by phosphorylation of the TCR complex by associated kinases, leading to recruitment and activation of more kinases that phosphorylate other targets leading to activation, differentiation and expansion of the T cell. Following priming, $CD4^+$ T cells differentiate into different T helper (Th) subsets, and their helper function depends on the nature of their cytokine secretions. The same is true for $CD8^+$ T cells, but they are also able to kill target cells directly and are therefore termed cytotoxic T (Tc) cells[1]. Most of these effector T cells die by apoptosis once the pathogen is cleared, but a few become memory T cells with the ability to produce a stronger and faster response upon reencounter with the pathogen[5].

A large fraction of T cells present in non-lymphoid tissues are non-conventional and can be of both the αβ and γδ T cell lineage. Notably, these T cells are mostly effector primed in the thymus and do not just recognize peptide:MHC complexes, but can be activated by other molecules[6]. For example γδ T cells respond to a broad range of antigens, such as non-classical MHC molecules, heat shock proteins and lipids[7]. Non-conventional lymphocytes are able to act rapidly and are sometimes referred to as innate-like lymphocytes. Interestingly, non-conventional cells recognize self-antigens that are normally upregulated upon cell stress, activation or transformation[8]. Looking at the multiple populations of non-conventional T cells, they are able to recognize a wide array of molecules, however, the individual populations mostly have oligoclonal or invariant TCRs. The non-conventional T cells include γδ T cells, natural regulatory T cells (nTregs), natural IL-17-producing Th (nTh17) cells, Natural Killer T (NKT) cells (both αβ and γδ subsets), mucosal-

associated invariant T (MAIT) cells and various intraepithelial lymphocytes (IELs)[9–12]. Conventional αβ T cells and non-conventional γδ T cells are described in more detail in section 1.3.

1.2 The skin

The skin constitutes the largest organ of the body and acts as our first line of defense against assaults from pathogens, chemicals and allergens. Our skin is not just a passive barrier it is a complex network containing many different cell types. Many of these cells maintain skin homeostasis by providing a microbial, a chemical, a physical and an immunological barrier[13]. Anatomically, the skin is divided into the epidermis and the dermis that are separated by a basement membrane.

1.2.1 Keratinocytes

The epidermis consists mainly of keratinocytes (KCs)[14,15]. Based on the histological characteristics of the KCs, the epidermis is further divided into four layers that are termed the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG) and stratum corneum (SC) moving from the basement membrane and outwards[14–17] (Figure 1).

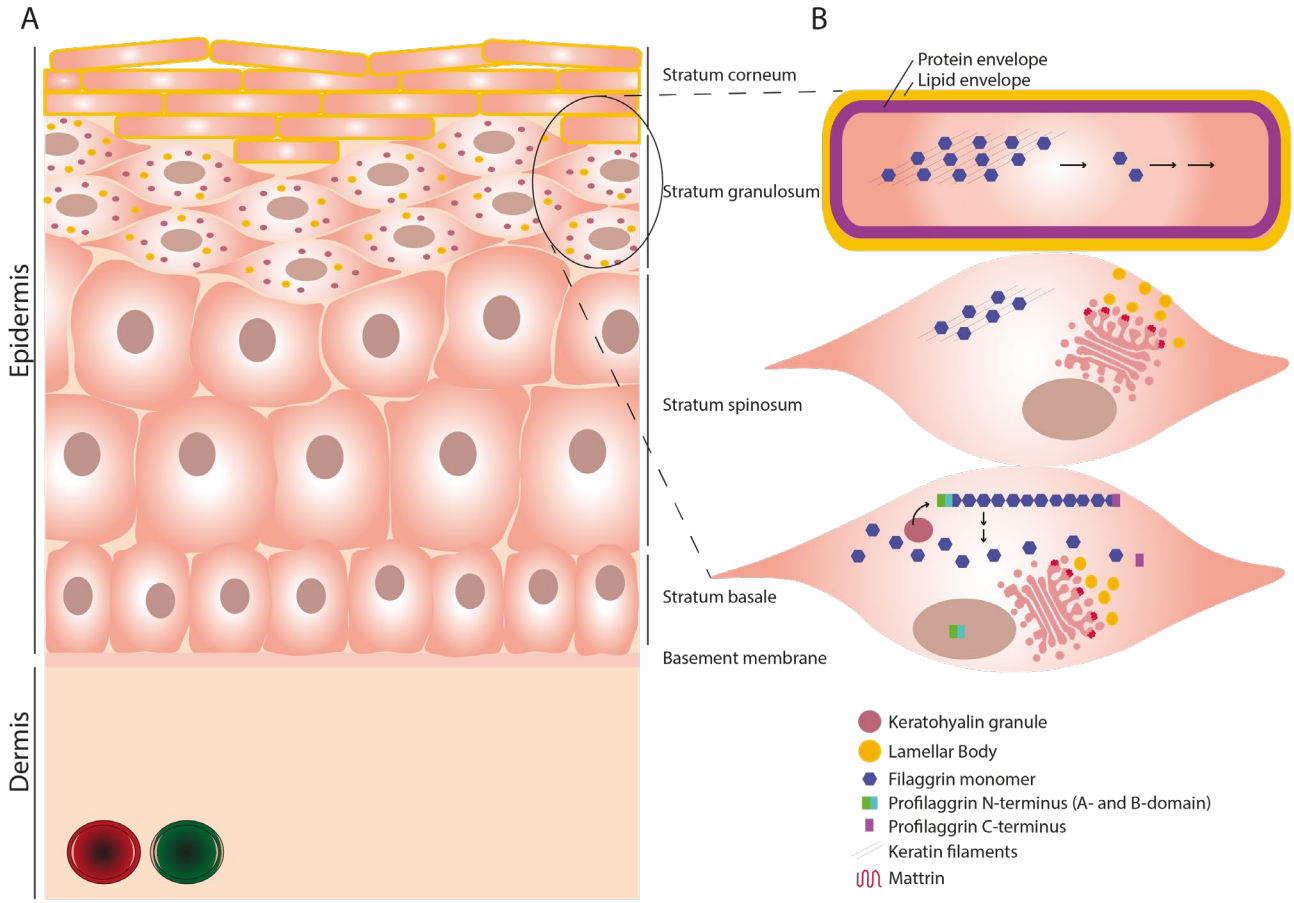

Figure 1. The strata of the skin. A. *Overall, the skin is divided into the epidermis overlaying the dermis and separated by the basement membrane. The epidermis is further divided into the stratum basale, stratum spinosum, stratum granulosum and stratum corneum (see text for more details). During differentiation, keratinocytes from stratum basale move apically. Reaching the stratum granulosum, expression of profilaggrin and mattrin appear (**B.**) Here, filaggrin is degraded into filaggrin monomers that aggregate keratin and are later degraded into constituents of natural moisturizing factor. Mattrin expression co-localizes with the trans-Golgi network membrane and possibly plays a role in the formation of lamellar bodies. Figure inspired by* [18–22].

The SB consists of a single layer of epidermal stem cells that constantly renew by proliferation and attach to the basement membrane. When an SB KC withdraw from the cell cycle, it detaches from the basement membrane and becomes part of the SS. In the SS, the KCs become more spinous and begin a strengthening of the keratin filament cytoskeleton.

The SG contains lamellar bodies[23] derived from the Golgi apparatus and keratohyalin granules. The KCs start a vigorous lipid metabolism and synthesis of keratinization-specific lipids in the SG that are stored in these lamellar bodies[24]. Reaching the upper SG the lamellar bodies are extruded to the intercellular space and the membrane of the lamellar body fuses with the plasma membrane[25,26]. Besides lipids, the lamellar bodies contain enzymes needed for lipid processing, enzymes for orderly desquamation and antimicrobial peptides[27]. During the transition through SG,

the protein profilaggrin is a key component of the keratohyalin granules, and reaching the junction between the granular and cornified layer, profilaggrin is dephosphorylated and subsequently cleaved to yield functional filaggrin monomers[20]. Once free, filaggrin monomers bind and aggregate keratin filaments forming tight bundles within the KC cytoskeleton. Bundle formation facilitates the collapse of KCs in the outermost SC, flattening the cells and creating the integrity of the SC[28]. Reaching the SC, the cells no longer contain nuclei and organelles. The cell membrane is replaced by an envelope structure, termed the cornified envelope (CE). The CE is built from a lipid envelope, from the lipids extruded from the lamellar bodies, overlaying a protein envelope that contains structural proteins, e.g. periplakin, envoplakin, involucrin and loricrin[18,29]. The CE is formed by successive deposition of proteins on the intracellular surface of the plasma membrane that begins already at the upper SG[30,31]. Thus, the protein-rich, hard, flattened dead corneocytes of the SC are described as bricks, while the intercellular lipids are described as mortar[29,32]. The brick and mortar structure provides both mechanical strength and reduces water loss from the skin. As filaggrin reaches the upper layer of the SC, it is degraded, and the major metabolites are trans-urocanic acid (trans-UCA) and pyrrolidone-5-carboxylic acid (PCA). These degradation products together with sodium and chloride ions, lactate and urea, form natural moisturizing factor (NMF) that is crucial for the hydration of the skin, and the organic acid degradation products maintain a low pH of the skin[20]. The thickness of the SC is maintained by desquamation, where the outermost corneocytes detach and are constantly replaced by new ones from underneath[33].

1.2.2 Filaggrin and filaggrin deficiency

As mentioned above, filaggrin is a structural protein expressed in the terminal stages of KC differentiation. It is synthesized as profilaggrin, which is a large (>400 kDa), highly phosphorylated polyprotein. Besides the filaggrin monomer repeats, profilaggrin contains an N-terminal domain and a C-terminal tail that flank the repeats. The N-terminal domain is a S100-like calcium-binding domain[34] and profilaggrin belongs to a family of high molecular weight proteins with N-terminal S100 domains fused to repetitive regions[35,36]. The S100 terminus comprises an A and B domain, where the A domain contains calcium binding sites and the B domain contains a nuclear localization sequence, which has been proved functional in both mice and humans[35]. The N-terminal undergoes processing in several steps, where proteolytic cleaving first releases it from the rest of the protein with exception of a small peptide, and subsequently freeing the N-terminal completely, enabling it to translocate to the nucleus. The last cleaving is therefore suggested to be

a form of checkpoint or regulation for the localization of the N-terminal. The function of the N-terminal is not fully uncovered, but it is suggested to provide a feedback mechanism that controls epidermal homeostasis[37]. Likewise, the exact function of the C-terminal is not yet understood, but the C-terminal is essential for correct processing of profilaggrin. This is underpinned by the fact that truncated profilaggrin, without the C-terminal, results in almost complete absence of epidermal filaggrin[38].

In humans, the filaggrin gene (*FLG*) is located on chromosome 1q21 within a cluster of epithelium-related genes, termed the epidermal differentiation complex (EDC). *FLG* consists of three exons, although almost the entire profilaggrin protein is encoded in exon three[39]. The skin disease Ichthyosis Vulgaris is characterized by dry, thickened and scaly skin, and in 2006 two loss-of-function mutations within the *FLG*, R501X and 2282del4, were identified in families of Northern European ancestry with Ichthyosis Vulgaris. Both mutations cause loss of processed filaggrin in the epidermis of homozygous and compound heterozygous carriers[40]. Following the discovery of the mutations in *FLG* causing Ichthyosis Vulgaris, the same mutations were also linked to an increased risk of developing Atopic Dermatitis (AD)[41], especially in AD with early onset and a more severe, persistent disease course[42]. AD is a chronic, relapsing disease with skin inflammation characterized by eczematous lesions and itching (Fig. 2).

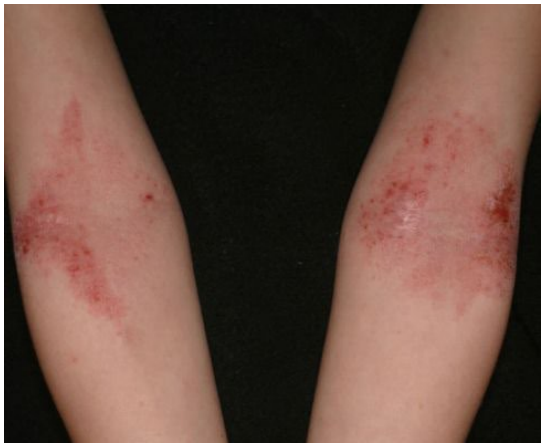

Figure 2. Example of atopic dermatitis on the flexural surface of the elbow. *Image provided by the National Allergy Research Centre.*

Moreover, AD is commonly associated with other atopic disorders like allergic rhinitis and asthma. In industrialized countries, AD prevalence is high in especially Northern Europe, affecting 15-30 % of children and 2-10 % of adults[43,44]. Genome-wide association studies have revealed several possible AD-related loci, and the EDC region had the highest linkage. Remarkably, the disease

occurs in the context of two distinct groups of genes; genes encoding epidermal proteins or genes encoding important immune elements[44].

Notably, only 30 % of European patients with AD harbor an *FLG* mutation and up to 60 % of *FLG* mutation carriers never develop AD, therefore a *FLG* mutation in itself is not enough to develop AD[45]. It is worth mentioning, however, that filaggrin deficiency is a problem in patients with AD, regardless of mutation status, which suggests that the absence of filaggrin is an important factor in the pathogenesis of AD in all patients[46]. In fact, a wide range of inflammatory cytokines have been shown to downregulate the expression of epidermal filaggrin, including IL-1β[47], IL-4, IL-13[48], IL-17A[49], IL-22[50], IL-25[51,52], IL-31[53], TNFα[54] and thymic stromal lymphopoietin (TSLP)[55], which suggests that skin inflammation contribute to barrier defects of the skin.

Around 10 % of the Northern European population are heterozygous carriers of an *FLG* mutation and 0.1 % are homozygous carriers, resulting in complete loss of processed filaggrin monomer in the epidermis and a weakening of the epidermal barrier[46,56].

The flaky tail (*ft/ft*) mouse model has been widely used as a model of filaggrin deficiency. In 1958 the mutation, termed *ft*, arose spontaneously in a model already exhibiting a recessive hair phenotype termed *matted* (*ma*) (Fig.3).

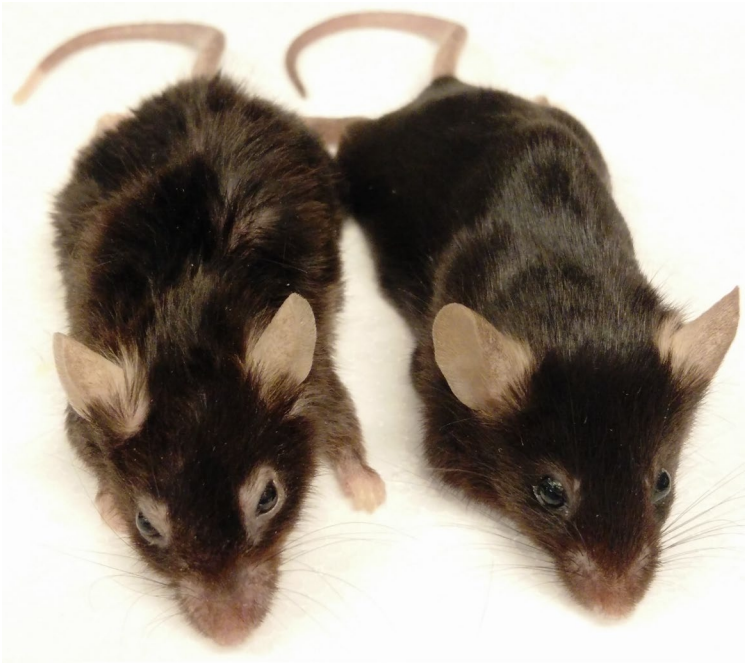

Figure 3. The flaky tail phenotype. *Adult flaky tail mouse (left) compared to adult C57Bl/6 mouse (right). The flaky tail mouse has a characteristic hair phenotype and develops spontaneous skin inflammation, especially visible around nose and eyes.*

The loci of *ft* and *ma* are closely linked on mouse chromosome 3[57,58], and therefore not likely to be separated spontaneously. Thus, the mouse strain has remained a mix of these two mutations. Furthermore, to complicate matters, the *ft/ft* mice are on a mixed background. The *ft* mutation in the *ft/ft* mouse model has been identified as a 1-basepair deletion (5303delA) causing a frameshift[59]. The result is expression of a truncated profilaggrin of approximately 220 kDa instead of the normal high molecular-weight profilaggrin, which is above 400 kDa[60]. The truncated form of profilaggrin is not processed into functional filaggrin monomers, which results in almost complete loss of filaggrin in the epidermis of *ft/ft* mice[59]. The *ft/ft* mice are characterized by dry, flaky skin, disorganized scales on the tail and by paw constrictions. Furthermore, the *ft/ft* mice exhibit an altered SC, increased transepidermal water loss (TEWL), develop spontaneous dermatitis and has increased responses to nickel[59–61]. However, an engineered *Flg*$^{-/-}$ mouse strain exhibits normal TEWL and does not develop spontaneous dermatitis, though an altered SC and increased allergen penetration still occurs[62].

1.2.3 Mattrin and mattrin deficiency

As described above, the *ft/ft* mouse model is a double mutant. Two studies emerged simultaneously that described the *ma* mutation. They found that the ma mutation is a nonsense mutation in the *TMEM79* gene (or *matt* gene)[22,63]. The *TMEM79* gene encodes the protein 5-transmembrane protein 79 (Tmem79 or mattrin), which is expressed in the trans-Golgi network of SG cells[22]. In this study, the authors show that mattrin co-localizes with the trans-Golgi, but not with lamellar bodies themselves. Furthermore, the lamellar body secretory pathway is dysfunctional in mattrin deficient mice, suggesting that mattrin is important for the function of this system, therefore the mutation results in altered SC formation and spontaneous dermatitis[22]. However, the exact function of mattrin has yet to be uncovered. Recently, it was established that mattrin is expressed in skin, cervix, prostate, tongue and somatosensory ganglia[64]. Furthermore, mattrin deficiency cause increased itching in these mice[64].

Not many studies have been carried out investigating *TMEM79* mutations in humans, but one of the studies that emerged describing the ma mutation in mice, also included a human part[63]. In this study, the authors sequenced *TMEM79* in AD patients negative for *FLG* mutations. They detected a missense SNP in the human *TMEM79* gene with and association to AD. Taken together, mutations in both *Flg* and *Matt* result in an altered formation of the SC, although these alterations are caused by faults in two different pathways.

1.2.4 The immune system of the skin

KCs are not described as immune cells themselves, but they play an important immunological role as the first line of defense. Thus, KCs express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs)[65] and NOD-like receptors (NLRs)[66] that are able to recognize PAMPs and DAMPs, which trigger the production and release of cytokines, chemokines and antimicrobial peptides (AMPs) that in turn activate immune cells within the skin[14,67,68]. The immune cells of the epidermis are a subset of antigen-presenting cells (APCs) termed Langerhans cells (LCs) and T cells[14,15,68]. In naïve mice, the primary subset of T cells in the epidermis is of the γδ T cell subset and is termed dendritic epidermal T cells (DETCs)[69]. The DETC subset found in murine epidermis are important for wound healing[70], tumor clearance[71] and skin homeostasis[72], and they also play a role in contact hypersensitivity[73,74]. The DETCs do not have a human equivalent, however, human epidermis contains both γδ T cells and αβ T cells with effector functions similar to those of DETCs[70]. Memory αβ T cells are the major T cell subset in adult human epidermis[16], where about half are $CD8^+$ and half are $CD4^+$ [75]. The dermis is more complex in its cellularity and harbors dermal dendritic cells (dDCs), macrophages, mast cells, innate lymphoid cells (ILCs), B cells, αβ T cells, γδ T cells[14,15,68].

1.2.5 Allergic contact dermatitis/contact hypersensitivity

Allergic contact dermatitis (ACD) is a common skin disease characterized by erythema, infiltration and possibly edema and blister formation in the affected area. ACD develops after repeated or prolonged exposure to contact allergens, which are most often metals, fragrances, hair dyes or preservatives[76] (Fig. 4).

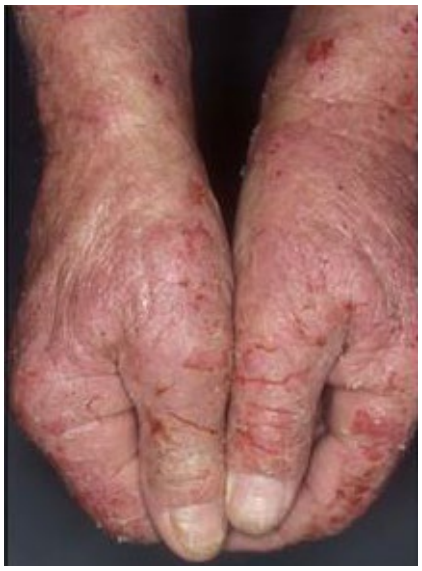

Figure 4. Example of severe contact allergy to chrome in a construction worker. *Image provided by the National Allergy Research Centre.*

A recent meta-analysis determined that 20.1 % of the general population is sensitized to at least one contact allergen with a higher prevalence found in women[77]. Although both KCs and skin-resident immune cells are important for the response in ACD, it is characterized as a T cell mediated disease[78–80]. ACD is immunologically classified as a type IV delayed-type hypersensitivity reaction and is divided into the sensitization phase and the elicitation/challenge phase.

The sensitization phase is the immunological process where the innate immune system reacts to the penetration of an allergen through the skin. The penetration of allergen results in activation of KCs, which results in expression of a myriad of inflammatory mediators, including IL-1β[81–83]. The innate response from KCs and local immune cells such as mast cells is followed by maturation and migration of skin-residing APCs (epidermal and dermal DCs) to the draining lymph node (dLN)[76,84–86]. Here, the allergen is presented in the context of an MHC class-I or class-II molecule to naïve allergen-specific $CD8^+$ and $CD4^+$ T cells, respectively, where after activation and clonal expansion of T cells occur[87,88]. Moreover, memory cells are generated, both circulating and skin-resident memory T cells[89]. The elicitation or challenge phase is initiated when the skin is exposed to the specific allergen again. During this phase, an unspecific innate response occurs, but an adaptive response with skin-resident memory T cells and recruited circulating memory T cells occurs as well, acting rapidly and inducing a strong reaction that causes the cellular damage and inflammation responsible for the clinical manifestations[90].

The experimental model of ACD is called contact hypersensitivity (CHS)[90]. Normally, the focus of ACD and CHS is what occurs locally in the skin. However, some studies have shown that treatment of skin with some contact allergens cause a systemic response with release of e.g. IL-6[91] and IFNγ[92]. Furthermore, the latter study also found changes in cell populations, such as IL-17A producing $CD8^+$ T cells, Tregs and iNKT cells, in non-draining lymph nodes[92]. These studies indicate that contact allergens generally affect the immune system. Interestingly, ACD is inversely associated with a number of immune diseases[93–96].

1.3 T cell development

T cell development takes place in the thymus, which is a bilobed lymphoepithelial organ located anterior to the heart[97]. To support different stages of development, the thymus is divided into

distinct microenvironments overall termed the cortex and the medulla. Several non-lymphoid cell populations, named thymic stroma, are responsible for creating these distinct environments. Thymic epithelial cells (TECs) constitute an important stromal subset and are divided into cortical (c)TECs and medullary (m)TECs[2]. Besides TECs, the thymic stroma contains endothelial cells, fibroblasts and different types of dendritic cells (DCs). The vast majority of cells in the thymus are developing T cells (thymocytes) but thymus-residing B cells and macrophages are present as well.

1.3.1 Thymic epithelial cells

The developmental stages of TECs are not yet fully understood. Over the years, different models have been used to describe the development of TECs. Previously cTECs and mTECs were thought to arise from different progenitor cells, even from different germ layers. However, it is well established that cTECs and mTECs differentiate from bipotent thymic epithelial progenitor cells (TEPs) that are present within the embryonic[98–101] and postnatal thymus[102]. Furthermore, it has been shown in several different studies that progenitors expressing cTEC markers, such as CD205, β5t and IL-7, could give rise to both cTECs and mTECs[103–106], forming the serial progression model of embryonic TEC development described by Alves et al. (2014)[107]. In this review, the authors suggest that cTECs differentiate from these progenitors by default in the absence of NFκB-activation. Both mTEC lineage specification and differentiation, however, requires activation of the NFκB pathway via TNF receptor family members RANK, CD40 and LTβR[108–110].

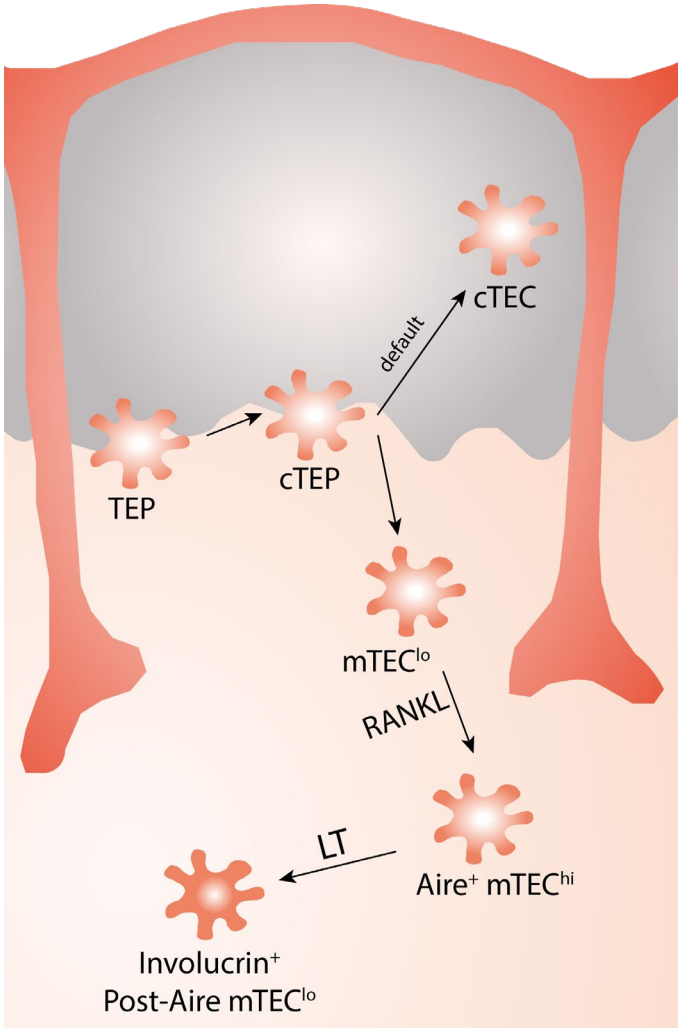

Figure 5. Steps of TEC development. *In the embryo, cTECs and mTECs arise from a bipotent thymic epithelial progenitor (TEP) and cTECs may become so by lack of NFκB activating stimuli. In the adult mouse, mTECs probably arises from committed mTEC progenitors. The mTEC differentiation program requires activation of TNF family members, where RANK:RANKL interactions are required for the shift from mTEC^{lo} cells to Aire^{+} mTEC^{hi} cells and LT:LTβR interactions are required for the post-Aire mTEC^{lo} subset that expresses involucrin. Figure inspired by*[2,3,107,111–113].

In adult mice, the molecular markers of TEC progenitors has not been defined yet[3], though some studies point to continued development of cTECs and mTECs from a common progenitor present up to 8 weeks of age[114–116]. Looking at the mTEC compartment, the maintenance and regeneration of cells seems to take place from lineage-restricted cells in the adult mouse[103,104].

Thymic crosstalk or lymphostromal interactions is the term used to describe the requirement of TECs for thymocytes and vice versa during development[117]. During early fetal stages, the thymus is not yet organized into cortex and medulla. Around E11 to E12, the earliest lymphoid progenitors

seed the thymus, which initiates organisation of the thymus[118]. Regarding cTECs, the stimuli gained from immature thymocytes modulates cTEC expression of Notch ligands and IL-7, which are important factors in early T cell development[119,120]. Thymic crosstalk in the medulla is described in more detail. Developing thymocytes express ligands for the TNF receptor family members RANK, CD40 and LTβR expressed by mTECs[108–111,121]. Ligation of these receptors initiates signalling via the NFκB pathway that activates a transcriptional program needed for mTEC differentiation[2]. Remarkably, these signals to mTECs are provided by different developing thymocytes in the fetal and adult stages of thymus development. The first T cell progenitors to seed the thymus and provide RANK ligand (RANKL) are the invariant $V\gamma3^+$ DETC progenitors[112] (Garman nomenclature[122]). Later, $ROR\gamma t^+$ innate lymphoid cells (ILCs), mature single positive thymocytes and iNKT cells provide one or more of these TNF receptor family ligands[3]. RANK:RANKL interactions induce expression of the Auto-immune regulator and Skint-1, the selecting ligand for development of $V\gamma3^+$ DETC. Therefore, the $V\gamma3^+$ subset prepare their own maturation and the development of conventional αβ T cells[112].

Aire is a key transcription factor for the process promiscuous gene expression, which allows mTEC expression of proteins otherwise restricted to distinct tissues (tissue-restricted antigens, TRAs) and thus negative selection of auto-reactive thymocytes[123]. Although mTECs are therefore capable of reflecting almost the entire coding genome, only 1-3 % of individual mTECs express a given TRA at a given time[124–129]. In adult mice, mTECs are divided according to their expression of MHC-II and CD80 into MHC-II^{low} $CD80^{low}$ ($mTEC^{lo}$) and MHC-II^{high} $CD80^{high}$ ($mTEC^{hi}$) subsets, where the $mTEC^{lo}$ subset was believed to be a pool of precursors of the $mTEC^{hi}$ subset[130,131]. The shift from $mTEC^{lo}$ to $mTEC^{hi}$ is driven by signals that include RANK and CD40 and is characterized by the expression of Aire and a range of TRAs[108,109,121]. Notably, a range of studies have suggested that the $Aire^+$ $mTEC^{hi}$ subset has further differentiation potential beyond Aire expression, and that the late stage of differentiation involves expression of the keratinocyte SC component involucrin[113,132–135]. Differentiation to this post-Aire $mTEC^{lo}$ stage was determined to be dependent on lymphotoxin (LT) on positively selected thymocytes ligating the mTEC LTβR[111]. Recently, two thorough studies investigating mTEC heterogeneity emerged simultaneously[136,137]. One study used fate mapping of Aire to identify four populations of mTEC subsets including a pre-Aire and post-Aire subset. Bulk RNA sequencing of identified mTEC subsets showed further heterogeneity with the post-Aire subset containing transcriptional signatures of cornified epithelium, but also a signature of the mucosal tuft cell. Interestingly, the tuft cell signature was present in both the pre-Aire and post-Aire subset, whereas the cornified

epithelium signature was only present in the post-Aire subset[136]. The thymic medulla harbours great heterogeneity, and probably to a much higher extend than currently known.

In humans, the thymic medulla contains Hassall's corpuscles (HCs), which are distinct, closely packed fully keratinized cells that contain filaggrin[97]. HCs are not as evident in mice, but are described in some reports as rare or small in size[138,139]. However, the recent studies mentioned above really implies that the HCs or similar structures are present in mice. The function of HCs is still not completely clear, although an *in vitro* study has demonstrated that human HCs are a potent source of TSLP and may indirectly via a dendritic cell subset induce differentiation of nTregs[140]. The important role of TEC in the development of conventional T cells is well-known. Lately, their critical role in the development of non-conventional T cells is becoming clear.

1.3.2 Lineage fate and development of conventional T cells

Progenitors that become either αβ or γδ T cells arrive in the thymus as CD4 and CD8 double negative (DN) cells[141]. Thymic endothelial cells express different adhesion molecules including P-selectin[142], enabling lymphocyte progenitors to adhere to the vessels of the thymus, while thymic epithelial cells (TECs) express chemokine ligands CCL21, CCL25 and CXCL12[143,144]. These molecules attract T cell progenitors into the thymus. The earliest T cell progenitors go through four DN stages, DN1-DN4, characterized by expression of CD44 and CD25. DN1 cells are $CD44^{+}CD25^{-}$, DN2 are $CD44^{+}CD25^{+}$, DN3 are $CD44^{-}CD25^{+}$ and DN4 are $CD44^{-}CD25^{-}$[145] (Fig. 6).

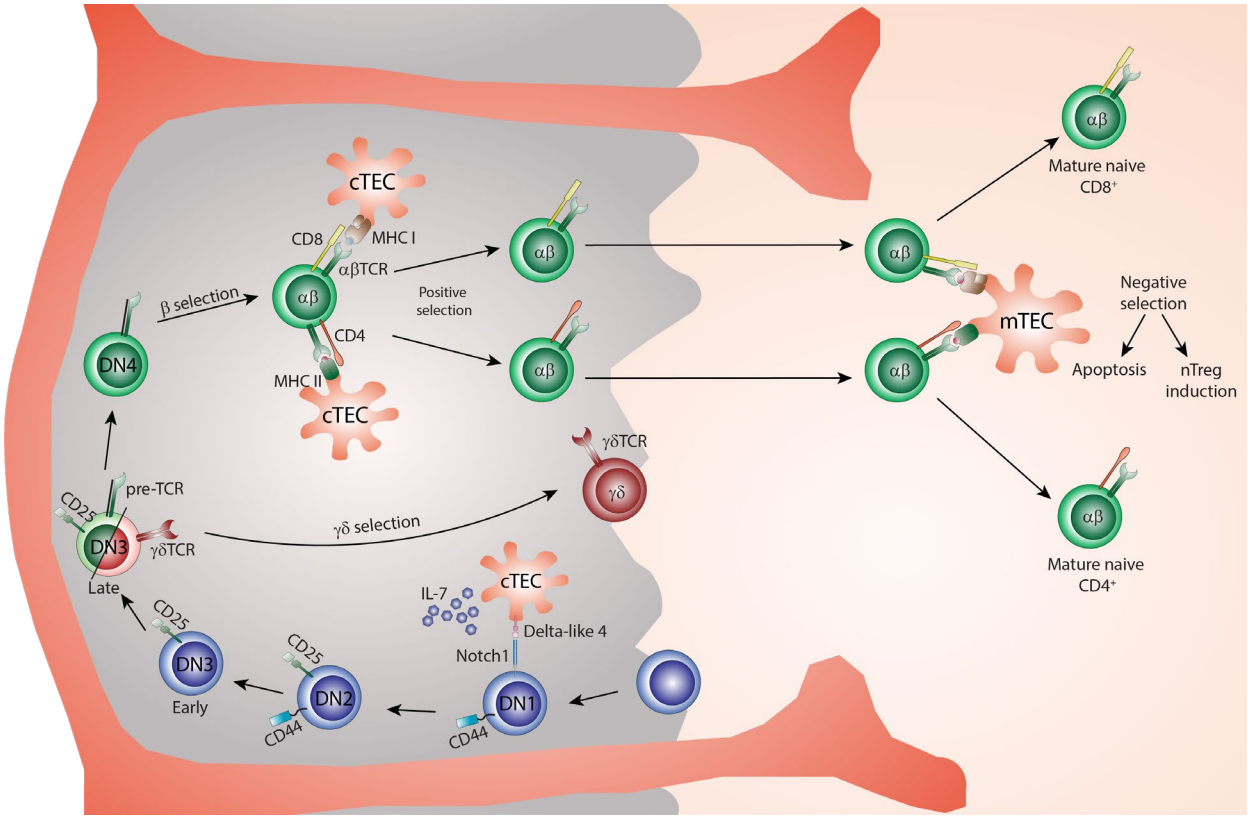

Figure 6. Lineage fate and development of conventional αβ T cells in the thymus. *T cell progenitors entering the thymus become committed to the T cell lineage by IL-7 and ligation of Notch1 with Delta-like 4 on cTECs. This initiates the V(D)J recombination of the TCRβ, TCRγ and TCRδ loci, and cells receiving weak signals through a functional pre-TCR by late DN3 commit to the αβ T cell lineage, proliferate and become CD4⁺CD8⁺ thymocytes. Now double positive, thymocytes rearrange their TCRα locus and are subsequently positively selected for recognition of MHC I or MHC II and become CD8⁺ or CD4⁺, respectively. Thymocytes that do not recognize MHC molecules die by apoptosis. Following positive selection, single positive thymocytes migrate to the thymic medulla, where they undergo negative selection. Negatively selected thymocytes die by apoptosis or become nTregs. Thymocytes that survive become mature naïve cells ready to egress. Illustration inspired by*[1,146].

Once settled in the thymus, the DN1 progenitors are met with IL-7 and the Notch ligand Delta-like 4 (DL4) expressed by cTECs. IL-7 and signals through Notch induces proliferation and differentiation of T lymphoid progenitors[147,148]. At DN2, commitment of progenitor cells to the T cell lineage initiates somatic rearrangement of the V(D)J segments in the TCRβ, TCRγ and TCRδ chain gene loci. From late DN3, the αβ and γδ lineages diverge. If a functional γδTCR is generated before a functional β-chain, it will commit to the γδ lineage. If instead the thymocytes produce a functional pre-TCR, a β-chain paired with a surrogate α-chain, it will progress towards the αβ lineage. There has been a lot of debate about the lineage commitment of αβ and γδ T cells, however, the signal strength model is the most commonly accepted one. This model posits that

weak integrated signals through the TCR promote adoption of the αβ lineage fate, whereas strong signals promote adoption of the γδ fate, irrespective of the TCR complex type[149,150]. If thymocytes fail to produce a functional TCR, they will die by neglect. In DN3, expression of a functional pre-TCR leads to arrest of β-chain rearrangement putting the cells in the DN4 stage[151]. While γδ-committed progenitors stay DN, β-committed progenitors express CD4 and CD8 co-receptors and are termed double positive (DP). The β-selected thymocytes go through a proliferative burst, and subsequently, rearrangement of the α-chain is started. Thus, a proliferating clone with a successful β-chain can be paired with different α-chains to increase the diversity of the αβTCR repertoire further. At this stage, a large number of DP thymocytes reside in the cortex. Following the formation of the TCR, the DP αβ thymocytes go through positive and negative selection.

Positive selection is a process that rescues DP thymocytes from undergoing apoptosis. Signalling through the TCR by interactions with self-peptide:MHC complexes on cTECs mediates the rescue of thymocytes that would otherwise have a lifespan of 3-4 days. The TCR is thereby tested for its ability to detect self-MHC molecules, and through positive selection developing thymocytes become either MHC class I-restricted or MHC class II-restricted expressing the CD8 or CD4 co-receptor, respectively and are now termed single positive (SP).

Engagement of the TCR by a peptide in the context of an MHC molecule induces the expression of chemokine receptor CCR7. The ligands for CCR7, CCL19 and CCL21, are highly expressed on mTECs, and CCR7 is essential for the migration of positively selected thymocytes into the medulla[152,153]. Besides CCR7, engagement of the TCR also induces production of several TNF superfamily cytokines including RANKL and CD40L. RANK is expressed by mTECs, and RANKL provided by positively selected thymocytes is crucial in the optimisation of the thymic medulla by inducing mTEC growth and maturation, while CD40L plays a supplementary role in the formation of the thymic medulla[108,121]. In the medulla, thymocytes undergo negative selection. As mentioned earlier the mTECs are unique in the sense that they are able to promiscuously express peptides from peripheral tissues, and thereby enable deletion of self-reactive clones[154]. The self-antigens produced by mTECs can be presented directly on mTECs or indirectly by neighbouring dendritic cells that are attracted to home near the mTECs partly by the chemokine XCL1 produced by mTECs in an Aire-dependent manner[155,156]. Some self-reactive T cells are not deleted, but are instead rendered regulatory and make up the pool of $CD4^+$ Forkhead box P3 $(Foxp3)^+$ nTregs. Both mTECs and thymic DCs are key regulators of nTreg development[157,158]. Thymocytes that survive negative selection begin expression of the transcription factor Kruppel-like factor (KLF) 2[159], which in turn induces expression of S1P1 and other molecules. S1P1 is the

receptor for S1P, which is present in abundance in the circulation and therefore causes the egress of mature T cells.

1.3.3 Development and effector pre-programming of γδ T cells

The development of γδ T cells is complex and large gaps in the understanding of developmental events still exist. γδ T cells are commonly characterized either by their Vγ-chain usage or by their effector function. Regarding their Vγ-chains, γδ T cells develop in distinct waves in the embryonic thymus and sequentially leave the thymus and seed distinct anatomical locations[160]. The first T cell wave to develop around embryonic day 13 are the unique $V\gamma3^+V\delta1^+$ cells, the DETCs[161], which seed the epidermis and primarily produce IFNγ. Shortly after, a wave of $V\gamma4^+V\delta1^+$ cells occurs, which seed the epithelial tissues of tongue, peritoneum, reproductive tract and dermis and primarily produce IL-17[162,163]. Finally, beginning development close to birth at E17 and continuing throughout life, $V\gamma2^+$ cells followed closely by $V\gamma1.1^+$ cells start occurring. $V\gamma2^+$ cells seed lymph nodes and dermis, whereas $V\gamma1.1^+$ cells seed liver and lymph nodes[164]. The development of $V\gamma3^+$ and $V\gamma4^+$ cells is restricted to the embryonic thymus, and their effector cytokines are strictly divided, whereas the $V\gamma1.1^+$ and $V\gamma2^+$ subsets have less strict effector functions though most $V\gamma1.1^+$ cells produce IFNγ and most $V\gamma2^+$ cells produce IL-17[164–166]. Less is known about the development of $V\gamma1.2^+$ and $V\gamma5^+$ cells, but they are present in the adult thymus in small numbers[160]. Considering the effector functions of γδ T cells, they are primarily divided into IL-17-producing γδ T cells, IFNγ-producing γδ T cells and IL-4- and IFNγ-producing γδ NKT cells that gain their effector function in the thymus[164,167]. Interestingly, specialization to the IL-17 producing fate seems to only occur from E16 to birth[165], although exceptions to this finding has appeared including recognition of cognate antigen[168] and TCR stimulation in the presence of IL-1β and IL-23[169–171]. A subset of apparently uncommitted, naïve-like γδ T cells also develop from the neonatal period and forward, which possibly gain their effector function in the periphery[168,170,172]. As seen in $CD4^+$ T cells, the key transcriptional regulator of IL-17 expression in γδ T cells is *Rorc* (encoding RORγt), while it is *Tbx21* (encoding Tbet) for IFNγ expression[173].

As described above, the most commonly appreciated model of lineage fate adoption is the signal strength model. Interestingly, signal strength through the TCR also plays an important role in differentiation of γδ T cells with discrete effector functions[174–178], although commitment to a γδ effector fate is reinforced by both TCR-dependent and –independent mechanisms. Thus, it appears that antigen-experienced γδ T cells become IFNγ-producing, whereas antigen-naïve γδ T cells

become IL-17-producers[174,176,179]. To this end, strong TCRγδ signalling seems to be required to induce the transcription factors Erg2 and Erg3 that supresses the IL-17 pathway[174,178,180] (Fig. 7). Furthermore, Egr2 and Egr3 regulate Id3, which is required for INFγ expression[181]. Id3 induce the transcription factors ThPOK and PLZF, which are necessary for development of γδ NKT cells, which express the highest level of IFNγ[176,178,182]. However, recently the tyrosine kinase Syk that activates the PI3K/Akt pathway downstream of TCR signalling was proven necessary for the development of IL-17-producing γδ T cells[183]. Other studies, where TCR signal strength has been modulated (via Zap70 or CD3γ/δ), show that defects in signal strength also affects IL-17 producing γδ T cells, but the outcome is different for the $V\gamma2^+$ and $V\gamma4^+$ subsets, which imply that different Vγ-subsets require different TCR signalling strength and molecules[178,184].

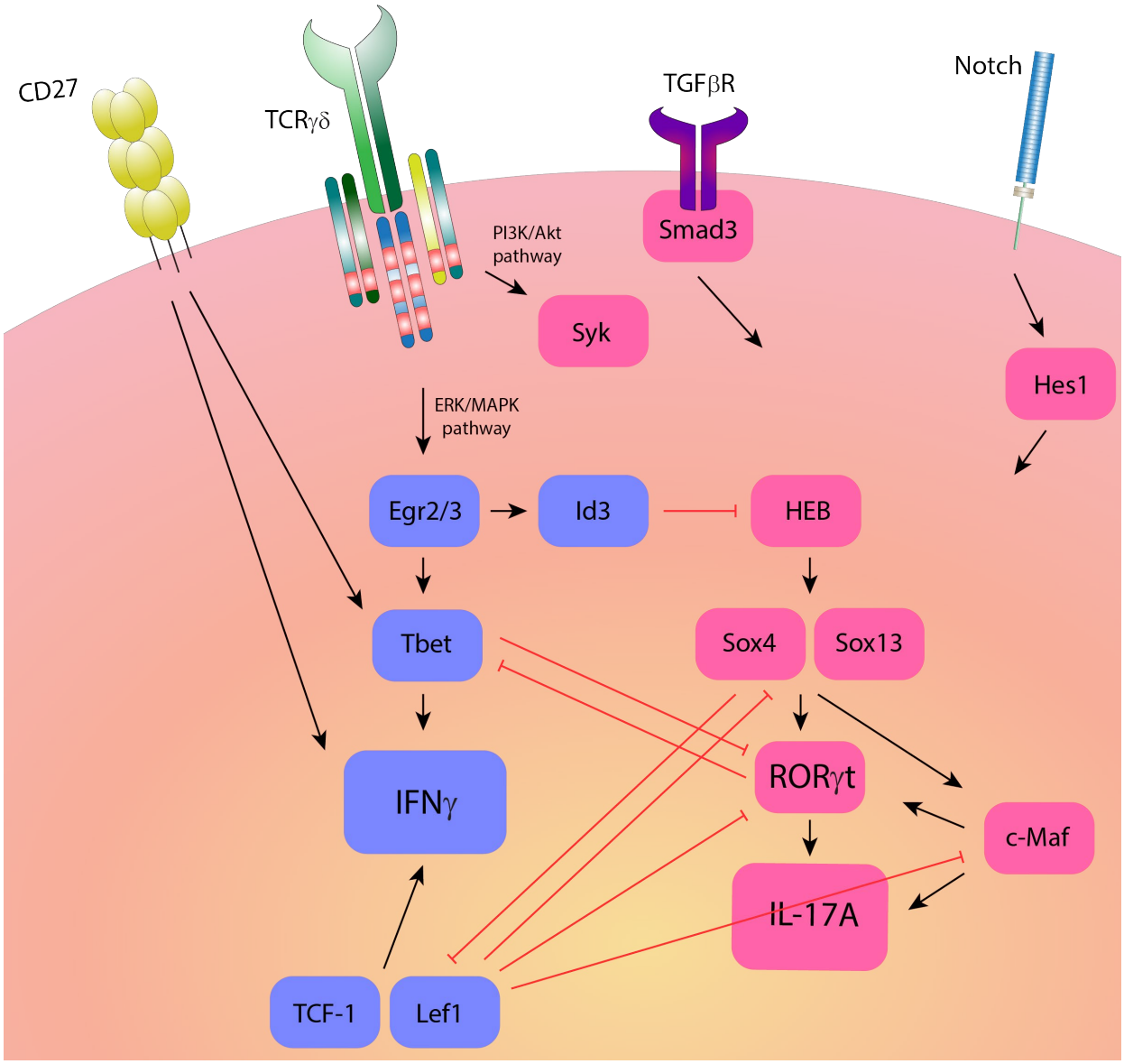

Figure 7. Factors in effector pre-programming of γδ T cells. *The effector fate of γδ T cells depend both on TCR-dependent and -independent mechanisms. Factors that induce a Type 1/INF-γ program are blue and factors that induce a type 17/IL-17A program are pink. Strong signals through the TCR leads to activation of the Egr2/3/Tbet/IFNγ axis that in turn inhibits factors in the Sox/RORγt/IL-17A axis. Furthermore CD27:CD70 interactions and Wnt-activation of TCF-1 and Lef1 promote the IFNγ program. Weak TCR signals promote the IL-17A program. Moreover, TGFβ/Smad3 and Notch/Hes1 activation promotes the IL-17A program. Activation of the IL-17A program in turn inhibits the IFNγ program rendering γδ T cells either IL-17A producers or IFNγ producers and not both. Inspired from*[164,185].

Regarding TCR-independent mechanisms, thymic stromal derived signals for IFNγ-producing effector fate includes CD27:CD70 interaction, which also lead to upregulation of the LTβR[179], and Wnt-activation of the transcription factors TCF1 and Lef1[186]. For the IL-17-producing effector fate, signals include Notch-induced Hes1 and TGFβ/Smad3[187,188]. The transcriptional networks that control effector pre-programming are complex, and beside the factors mentioned above, HEB and its downstream targets Sox4 and Sox13 are important for the IL-17 producing Vγ2$^+$ subset[186,189], while the transcription factor PLZF, interestingly, also is essential for the IL-17 producing Vγ4$^+$ subset[190]. Recently, the transcription factor c-Maf was identified as a universal and essential regulator of all IL-17-producing γδ T cells[191]. The fact that the Sox4/Sox13/RORγt/IL-17 axis is inhibited by the Egr2/Egr3/Id3 axis and by TCF1 and Lef1, and that TCF1 and Lef1 are counteracted by Sox4 and Sox13[186] underpins a pre-programming mechanism that renders individual cells either IL-17 or IFNγ producing and not both.

A lot of mystery still surrounds the development and effector pre-programming of γδ T cells. Especially the IL-17 producing γδ T cells wake some discussions as to whether they fit into the signal strength model. Some researchers have difficulties reconciling the fact that strong TCR signals are needed for lineage fate, but weak or absent signals are needed for IL-17 expression fate[167], even though some studies suggest that the lineage fate and effector fate of γδ T cells occur in two separable steps while they are both dependent on TCR signal strength[191,192]. Furthermore, a subset of γδ precursors in the thymus are capable of IL-17 expression with a still open TCR-δ locus[165]. Interestingly, Sumaria et al. 2019[167] propose that an IL-17 committed program might be established in a subset of progenitors before the expression of the TCRγδ and subsequently weak or alternative signaling through the TCR would fully manifest these to the IL-17 transcriptional program.

To complicate matters, IL-17 produced by αβ T cells inhibits development of IL-17-producing γδ T cells, which probably explains why IL-17-producing γδ T cell development is restricted to the embryonic thymus[165].

Although the development of γδ T cells is less clear than that of αβ T cells, the role of mTECs in the development of different non-conventional T cell subsets is becoming clear. For example, the mTEC compartment plays an important role in the development of both $V\gamma3^+$ DETC, iNKT cells, nTregs and natural Th17 cells[111,157,193–196].

1.3.4 Recent thymic emigrants

Leaving the thymus, conventional αβ T cells are still not fully mature and go through an approximate three week stage of being recent thymic emigrants (RTEs). The RTEs gradually up- (CD45RB, QA2, CD28, IL-7Rα) or down regulate (TCR/CD3, CD24) different surface markers, which distinguishes them from mature naïve T cells. The RTEs are functionally distinct with reduced proliferation capacity and reduces cytokine production, and their functional and phenotypic maturation requires access to secondary lymphoid organs[197].

Objectives

The overall aim of this PhD thesis was to investigate if epidermis-associated markers expressed in the thymus were able to affect T cell development, and thereby inflammation status in the periphery. Furthermore, if systemic inflammation initiated in the epidermis could affect the thymus, and thereby T cell development. The aims of the individual studies were:

Manuscript I: Preliminary data indicated that the filaggrin deficient *ft/ft* mice had an increased population of splenic IL-17A producing γδ T cells. As γδ T cells largely gain their effector program already in the thymus, we speculated that filaggrin possibly play a role in the thymus affecting T cell development. The objective of this study was therefore to investigate if filaggrin is expressed in mouse thymi, and if it affects the development of the IL-17A producing γδ T cells.

Manuscript II: The *ft/ft* mice used in manuscript I are double mutants and on a mixed background. To ensure the conclusions drawn in the first study where in fact a result of filaggrin deficiency, we backcrossed *ft/ft* mice onto a C57Bl/6 background and separated the two mutations leaving us with the two mouse models $Flg^{ft/ft}$ and $Matt^{ma/ma}$ with filaggrin deficiency and mattrin deficiency, respectively. The objective of this study was therefore to investigate the role of both filaggrin and mattrin on T cell development and phenotype in the periphery.

Manuscript III: In the two first studies we investigated how deficiency of epidermal proteins affect the thymus, and thereby the peripheral T cell subsets (inside-out). We know from other reports that stress and inflammation is able to affect the thymus and T cell development. Therefore, the objective of this study was to investigate if allergen-induced skin-inflammation can affect T cell development (outside-in).

Manuscript I

Increased Production of IL-17A-Producing γδ T Cells in the Thymus of Filaggrin-Deficient Mice

frontiers in Immunology

ORIGINAL RESEARCH
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Increased Production of IL-17A-Producing γδ T Cells in the Thymus of Filaggrin-Deficient Mice

*Mia Hamilton Jee[1,2], Jeanne Duus Johansen[2], Terkild Brink Buus[1], Trine Hilkjær Petersen[1], Anne-Sofie Østergaard Gadsbøll[1], Anders Woetmann[1], Niels Ødum[1], Jacob Pontoppidan Thyssen[2], Andrea Jane White[3], Graham Anderson[3], Carsten Geisler[1] and Charlotte Menné Bonefeld[1]**

[1] Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, [2] National Allergy Research Centre, Department of Dermato-Allergology, Copenhagen University Hospital Gentofte, Hellerup, Denmark, [3] Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom

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Francisco Sanchez-Madrid, Universidad Autonoma de Madrid, Spain

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Daniel J. Pennington, Queen Mary University of London, United Kingdom
Ashutosh Chaudhry, Memorial Sloan Kettering Cancer Center, United States

***Correspondence:**
Charlotte Menné Bonefeld
cmenne@sund.ku.dk

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Mutations in the filaggrin gene (*Flg*) are associated with increased systemic levels of Th17 cells and increased IL-17A production following antigen exposure in both humans and mice. In addition to Th17 cells, γδ T cells can produce IL-17A. The differentiation of γδ T cells to either IFNγ or IL-17A-producing (γδT17) cells is mainly determined in the thymus. Interestingly, it has been reported that filaggrin is expressed in the Hassall bodies in the human thymic medulla. However, whether filaggrin affects γδ T cell development is not known. Here, we show that filaggrin-deficient flaky tail (*ft/ft*) mice have an increased number of γδT17 cells in the spleen, epidermis, and thymus compared to wild-type (*WT*) mice. We demonstrate that filaggrin is expressed in the mouse thymic medulla and that blocking the egress of cells from the thymus results in accumulation of Vγ2$^+$ γδT17 cells in the thymus of adult *ft/ft* mice. Finally, we find increased T cell receptor expression levels on γδ T cells and increased levels of IL-6 and IL-23 in the thymus of *ft/ft* mice. These findings demonstrate that filaggrin is expressed in the mouse thymic medulla and that production of Vγ2$^+$ γδT17 cells is dysregulated in filaggrin-deficient *ft/ft* mice.

Keywords: *ft/ft* mice, filaggrin, γδ T cells, IL-17, thymus, development

INTRODUCTION

Filament aggregating protein (filaggrin) is a major structural skin protein that assists in the formation of the epidermal barrier. Filaggrin is a degradation product from the large pro-protein profilaggrin (>400 kDa). During terminal differentiation of keratinocytes, profilaggrin is carried apically, dephosphorylated and degraded into an N-terminal peptide, several filaggrin monomers and a C-terminal peptide (1, 2). The flaky tail (*ft/ft*) mouse is commonly used as a model of filaggrin deficiency exhibiting spontaneous development of eczematous skin lesions (3). The increased skin inflammation in filaggrin-deficient mice correlates with increased levels of IL-17A (4, 5).

Abbreviations: DETC, dendritic epidermal T cells; Flg, filaggrin gene; ft, flaky tail; TCR, T cell receptor; TEC, thymic epithelial cells; TSC, thymic stromal cells; WT, wild type; γδT17, 17-producing γδ T cells.

Accordingly, a strong reduction of skin inflammation is seen in IL-17A/filaggrin double-deficient mice compared to mice only deficient in filaggrin (6). In addition to the local IL-17A-driven skin inflammation, a systemic OVA-specific IL-17A response can be induced in filaggrin-deficient mice by exposure of the skin to OVA (3, 4). In line with this, we have recently shown that filaggrin deficiency is associated with an increase in the numbers of IL-17-producing T (Th17) cells in both humans and mice (7). Whether filaggrin deficiency affects other IL-17A-producing cell subsets is currently unknown.

γδ T cells can be divided into IFN-γ or IL-17-producers. The master transcriptional regulators of IFNγ and IL-17-producing γδ T (γδT17) cells are the T-box transcription factor (T-bet) and retinoic acid receptor-related orphan receptor-γt (RORgt), respectively (8). Whereas γδ T cells leave the thymus as naïve T cells and gain their effector function upon priming in peripheral lymphoid tissues, the effector fate of γδ T cells is mainly programmed in the thymus (9–12). The γδT17 cells can be divided into two groups: natural γδT17 cells, which are programmed for IL-17 production during their development in the thymus, and inducible γδT17 cells, which are primed for IL-17 production after leaving the thymus (9–13).

γδ T cells develop in distinct waves characterized by different Vγ segment usage (14). γδ T cells expressing Vγ1.1, Vγ3, or Vγ5 segments [Garman nomenclature (15)] primarily become IFN-γ producing cells, while γδ T cells expressing Vγ4 primarily become IL-17-producing cells. Interestingly, γδ T cells expressing the Vγ2 segment can develop into either IFNγ or IL-17-producing cells. Different signaling pathways determine whether a γδ T cell in the thymus will become IFNγ or IL-17 producing. Both T cell receptor (TCR)-dependent and TCR-independent signaling pathways are involved in γδ T cell development. Different subsets of thymic epithelial cells (TEC) provide the microenvironments needed for the development of T cells. Interestingly, it has been reported that terminally differentiated TEC in the Hassall's corpuscles in the human thymic medulla express filaggrin. The important role of TEC in the development of conventional T cells is well described, but the role of TEC in the development of γδ T cells is less clear. However, it is believed that TEC provide distinct ligands or selecting molecules modulating the thymic programming of γδ T cells. Strong TCR signaling induces development of IFNγ producing cells, whereas missing or weak TCR signaling leads to development of γδT17 cells (9, 10, 16). In addition to TCR signaling, signaling *via* costimulatory receptors and cytokine receptors also affects γδ T cell development (11, 17–19). Signaling *via* CD27 seems to play an important role in the differentiation of γδ T cells in thymus as $CD27^+$ γδ T cells differentiate into IFNγ-producing cells, whereas $CD27^-$γδ T cells become IL-17 producing (11). Finally, the cytokine environment in the thymus regulates the differentiation of γδ T cells. TGFβ, IL-1, IL-23, and IL-6 seem to mediate the development of IL-17-producing γδ T cells (17).

In the present study, we investigated whether the production of γδ T cells is affected in filaggrin-deficient *ft/ft* mice. We found a fivefold increase of splenic and epidermal γδT17 cells in *ft/ft* mice compared to wild-type (WT) mice. This increase of γδT17 cells was associated with an enhanced production of γδT17 cells in the thymus. In addition, we found that filaggrin is expressed in the thymus medulla of WT mice and that filaggrin expression is reduced in the thymus of *ft/ft* mice. Further analyses showed that the increased number of γδT17 cells was primarily contained within the $V\gamma2^+$ subset. Finally, we found higher TCR expression levels on γδ thymocytes and higher levels of IL-6 and IL-23 in the thymus of *ft/ft* mice compared to *WT* mice.

MATERIALS AND METHODS

Animal Model

Flaky tail mice (*a/a Tmem79^{ma} Flg^{ft}/J*, stock number 000281) (*ft/ft*) were purchased as cryopreserved embryos from the Jackson Laboratory and bred at our in-house animal facility. Age-matched, mixed gender C57Bl/6 mice were purchased from Janvier or Taconic Laboratories. Experiments were performed on the mice at the age of 8–12 weeks. The mice were housed in the specific pathogen free animal facility at the Department of Experimental Medicine, Panum Institute, in accordance with the national animal protection guidelines (license number 2012-15-2934-00663). C57Bl/6 mice were used as WT controls as *ft/ft* mice have previously been described to be outcrossed onto C57Bl/6 mice. However, *ft/ft* is not a strict congenic strain, but a semi-inbred strain (5). In some experiments, mice were treated with FTY720 (2.5 μg/ml) in their drinking water for six consecutive days.

Preparation of Single-Cell Suspensions

Single-cell suspensions from thymi, lymph nodes, and spleens were prepared by dissociating the organs on 70 μm cell strainers. The single cells were washed in RPMI medium (10% FBS, 0.5 IU/L penicillin, 500 mg/L streptomycin, 1% L-glutamine), and cell suspensions were adjusted to 2×10^7 cells/mL. Subsequently, 100 μL/well was plated in a round-bottomed 96-well plate. Single-cell suspensions from the epidermis were isolated from the ears. The ears were split into a dorsal and ventral part. The dorsal part was transferred to a 0.3% trypsin-GNK (2.94 g NaCL, 0.134 g KCl, 0.334 g glucose/dextrose per 1 g of trypsin) solution for 60 min at 37°C, 5% CO_2 with the dermis side down. The epidermis was peeled from the dermis and transferred to 0.3% trypsin-GNK with 0.1% DNase and left at 37°C for 10 min. Cells were filtered through a cell strainer, washed and plated overnight at 37°C, 5% CO_2 to allow re-expression of surface markers.

Staining and Flow Cytometry

Fc-receptors were blocked with anti-CD16/CD32. Surface markers on cells were stained with anti-CD3ε, -TCRγδ(GL3), -CD4, -CD8α, -CD24, -CD25, -CD44, -CD27, CD45RB, -CCR6, -Vγ1, -Vγ2, and -Vγ3 diluted in Brilliant Stain Buffer (BD Biosciences). Viability of cells was determined using Fixable Viability Dye (eFlour® 780) (eBioscience). When staining for intracellular cytokines, the cells were first stimulated with PMA (50 ng/ml), monensin sodium (4 μg/ml), and ionomycin (500 ng/ml) for 4 h and stained for surface markers. Following fixation and permeabilization with BD Cytofix/Cytoperm (BD Biosciences), the cells were stained for intracellular cytokines with anti-IL-17A and

anti-IFNγ antibodies. Data were collected on a BD LSRFortessa and analyzed with FlowJo Software.

Histology and Staining for Confocal Microscopy

Ears and thymi from *ft/ft* and C57Bl/6 mice were transferred to formaldehyde. Histology was performed by Nordic Biosite, Finland. Sections were stained with hematoxylin and eosin and with antibodies targeting filaggrin (Poly19058, BioLegend).

For confocal microscopy analyses, fresh thymi were imbedded in OCT compound (Sakura Fintek) and snap frozen on dry ice. The tissue was cut into 7 µm sections and fixed in acetone. The following antibodies were used for staining: rabbit anti-filaggrin (Poly19058, BioLegend), AlexaFluor 647 anti-mouse CD4 (GK1.5, BioLegend), and biotinylated anti-mouse CD8a (53-6.7, eBioscience). To detect the anti-filaggrin antibody, an AlexaFluor 555 donkey anti-rabbit IgG (Invitrogen) antibody was used. Biotinylated CD8 antibody was detected with Streptavidin conjugated to AlexaFluor 488 (Life Technologies). Purified rabbit polyclonal isotype control (Poly19058, Biolegend) was used as control to filaggrin stains. Sections were analyzed using a Zeiss LSM 880 confocal microscope.

Quantitative Real-Time PCR

Organs frozen in liquid nitrogen were disintegrated in a Precellys tissue homogenizer (Bertin Technologies) in 500–1,000 mL of TRI Reagent (Sigma Aldrich). For RNA extraction from thymic stromal cells (TSC), fresh thymi were cut into 6–8 pieces and thymocytes mechanically released by pipetting and changing of medium, and finally disintegrated as described above. Following centrifugation, the supernatant was mixed with 1-Bromo-3-chloropropane (Sigma Aldrich), samples were centrifuged and the upper phase recovered. RNA isolation was performed using the RNeasy Mini Kit 250 (Qiagen) according to manufacturer's specifications. RNA concentrations were measured using a Nanodrop 2000c spectrophotometer (Thermo Scientific), and RNA was diluted to a final concentration of 2 µg/µL. RNA was transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). Transcription of genes was measured by real-time PCR. Stock Taqman primer/probe sets with Taqman Universal Master Mix was processed in a Stratagene Mx3000P/Mx3005P (AH Diagnostics/Agilent Technologies), and the data was analyzed using MxPro software. Transcription of target genes was calculated relatively to GAPDH.

Protein Extraction

Extraction of protein was performed by lysing ears with lysis buffer (50 mM Tris Base, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100), and disintegrating the samples on a Precellys tissue homogenizer (Bertin Technologies). Subsequently, samples were spun down, and the supernatant was recovered. To purify TSC, thymi were cut into 6–8 pieces, and the thymocytes were mechanically released by pipetting up and down with a 1,000 µl pipette tip with the outermost end trimmed off. Following removal of media containing the thymocytes, fresh media was added and the release of thymocytes was repeated two times. TSC were lysed and protein extracted as described above.

ELISA

Protein lysates were adjusted to a concentration of 3.0 µg/µl following determination of concentration by Bradford assay. Concentrations of IL-6 and IL-23 were determined using Mouse IL-6 ELISA Ready-SET-Go and mouse IL-23 ELISA Ready-SET-Go kits (eBioscience) according to manufacturer's specifications.

Statistical Analysis

Differences between groups were evaluated by the two-tailed unpaired Student's *t*-test. The statistical analysis was performed using GraphPad Prism version 6.0, and a *p*-value below 0.5 was considered statistically significant. Statistical significance *p*-values are denoted as: *<0.05, **<0.01, ***<0.001, ****<0.0001.

RESULTS

ft/ft Mice Have Increased Numbers of γδT17 Cells in the Spleen and Epidermis

To investigate whether γδ T cells might be involved in the IL-17 driven immune responses in *ft/ft* mice, we examined the distribution of γδ T cells in the spleen of *ft/ft* and WT (C57Bl/6) mice. The percentages as well as the absolute numbers of γδ T cells were significantly increased in *ft/ft* mice compared to WT mice (**Figures 1A,B**). Next, we investigated whether the increase of γδ T cells in the spleen of *ft/ft* mice also resulted in an increased number of γδT17 cells by determining the number of γδ T cells expressing IL-17A. We found significantly increased percentages and absolute numbers of γδ T cells expressing IL-17A in the spleen of *ft/ft* mice compared to WT mice (**Figures 1C,D**). Interestingly, this increase was specific for IL-17A-producing cells as no significant differences were seen in the percentages or numbers of IFN-γ-producing γδ T cells between *ft/ft* and WT mice (**Figures 1E,F**). The elevated number of IL-17A-producing cells seen in *ft/ft* mice seemed to be specific for γδ T cells and $CD4^+$ T cells, as no differences in the frequencies of IL-17A-producing $CD8^+$ T cells or non-T cells were observed between *ft/ft* and *WT* mice (Figures S1–S3 in Supplementary Material). Expression of CD27, CD45RB, and CCR6 can be used to determine γδ T cell subsets that produce IL-17A or IFN-γ (11, 13, 20). We found significantly increased fractions of $CD27^-CD45RB^-$ or $CD27^-CCR6^+$ splenic γδ T cells in *ft/ft* mice (**Figures 1G,H**) in agreement with the observation described above. To determine if the accumulating γδT17 cells were restricted to a specific subset of γδ T cells in the spleen of *ft/ft* mice, we co-stained for IL-17A and Vγ1.1 or Vγ2. The increased fraction of $IL\text{-}17A^+$ cells seemed to be restricted to the $V\gamma2^+$ subset (**Figures 1I,J**). As *ft/ft* mice develop spontaneous skin inflammation (3), we next wanted to determine if elevated numbers of γδT17 cell also were found in the epidermis of *ft/ft* mice. We found a highly increased fraction of $V\gamma2^+$ T cells as well as an increased fraction of $V\gamma3^+$ T cells, the major T cell subset within epidermis, that were $IL\text{-}17A^+$ in the epidermis of *ft/ft* mice compared to *WT* mice (**Figures 1K,L**). Taken together, these data indicated that the peripheral γδ T cell population, including γδT17 cells, is significantly expanded in *ft/ft* mice compared to *WT* mice.

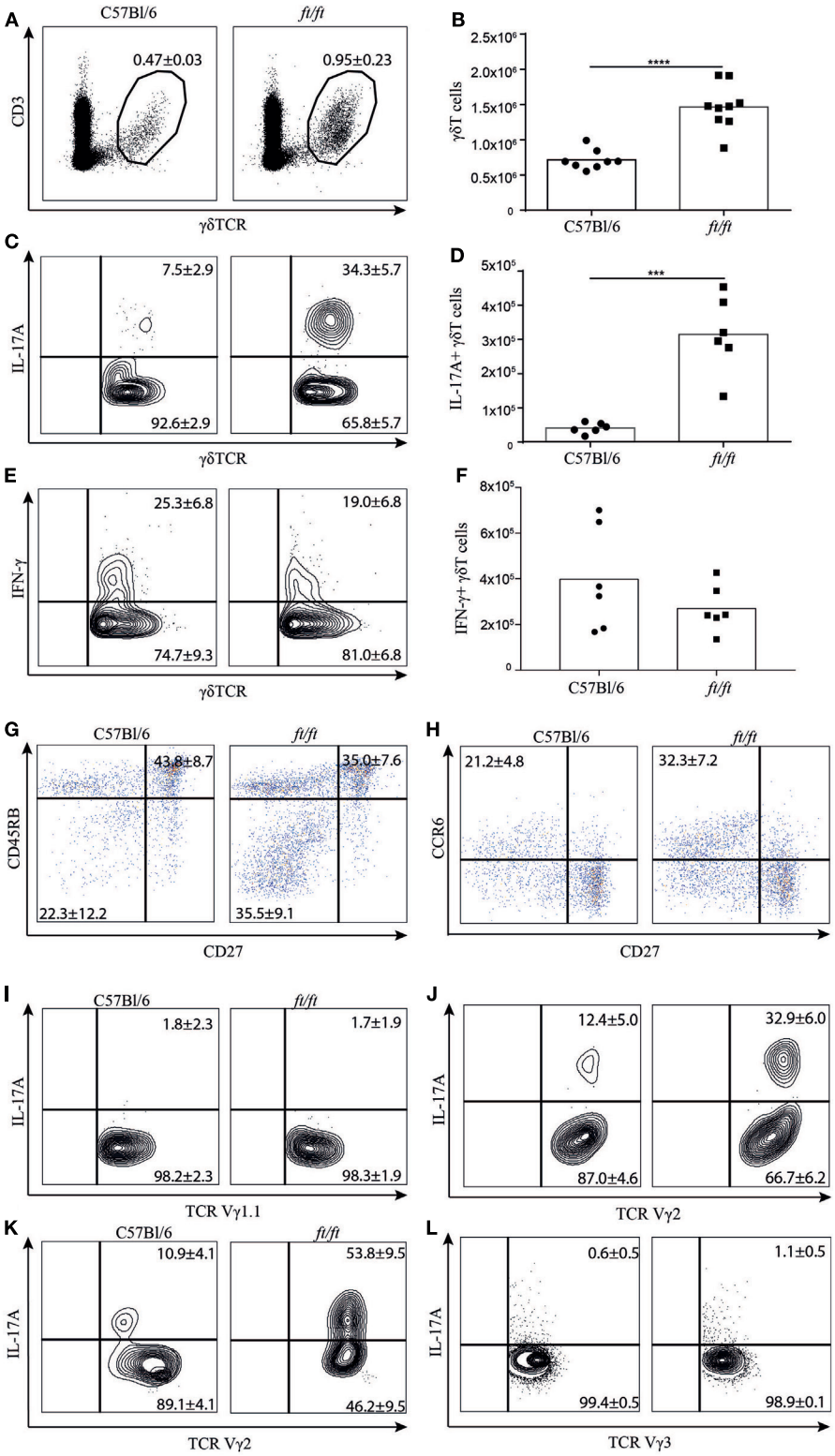

FIGURE 1 | Increased numbers of γδT17 cells in the spleen and epidermis of *ft/ft* mice. **(A–J)** Flow cytometric analyses of spleen cells from 8 to 12 weeks old C57Bl/6 and *ft/ft* mice. **(A)** Fraction of $CD3\varepsilon^+TCR\gamma\delta^+$ spleen cells. **(B)** Absolute numbers of $CD3\varepsilon^+TCR\gamma\delta^+$ spleen cells. **(C–F)** Fraction and absolute numbers of **(C,D)** IL-17$^+$ and **(E,F)** IFN-γ^+ $TCR\gamma\delta^+$ cells. **(G,H)** Dot plots showing CD27 versus CD45RB and CD27 versus CCR6 expression of $TCR\gamma\delta^+$ cells. **(I,J)** Plots showing IL-17 expression on TCR V$\gamma1.1^+$ or TCR V$\gamma2^+$. **(K,L)** Plots showing IL-17 expression on TCR V$\gamma2^+$ and TCR V$\gamma3^+$ cells from the epidermis. Data are representative of two to three independent experiments with three to four mice in each. The mean percentages ± SD are given for the relevant populations in the plots.

ft/ft Mice Have Increased Numbers of γδT17 Cells in the Thymus

To establish whether the increased number of γδT17 cells found in the peripheral lymphoid organs of *ft/ft* mice originated from natural γδT17 cells programmed in the thymus, we investigated the cellular distribution in the thymus of *ft/ft* and *WT* mice. We found no significant differences between *ft/ft* and *WT* mice in the fraction of double positive ($CD4^+CD8^+$), CD4 single positive and CD8 single positive cells or in the double negative ($CD4^-CD8^-$) 1–4 fractions ($CD44^+CD25^-$, $CD44^+CD25^+$, $CD44^-CD25^+$ and $CD44^-CD25^+$, respectively) (**Figures 2A,B**). However, *ft/ft* mice on average had a 20% increase in their total numbers of thymocytes as compared to *WT* mice (**Figure 2A**). Next, we analyzed the γδ T cell populations. We observed a significantly larger population of γδ T cells in the thymi of *ft/ft* mice compared to WT mice, which primarily was caused by the general increase in cell numbers in the thymi of *ft/ft* mice compared to WT mice (**Figures 2C,D**). Despite the similar fraction of total γδ T cells, we found a significant

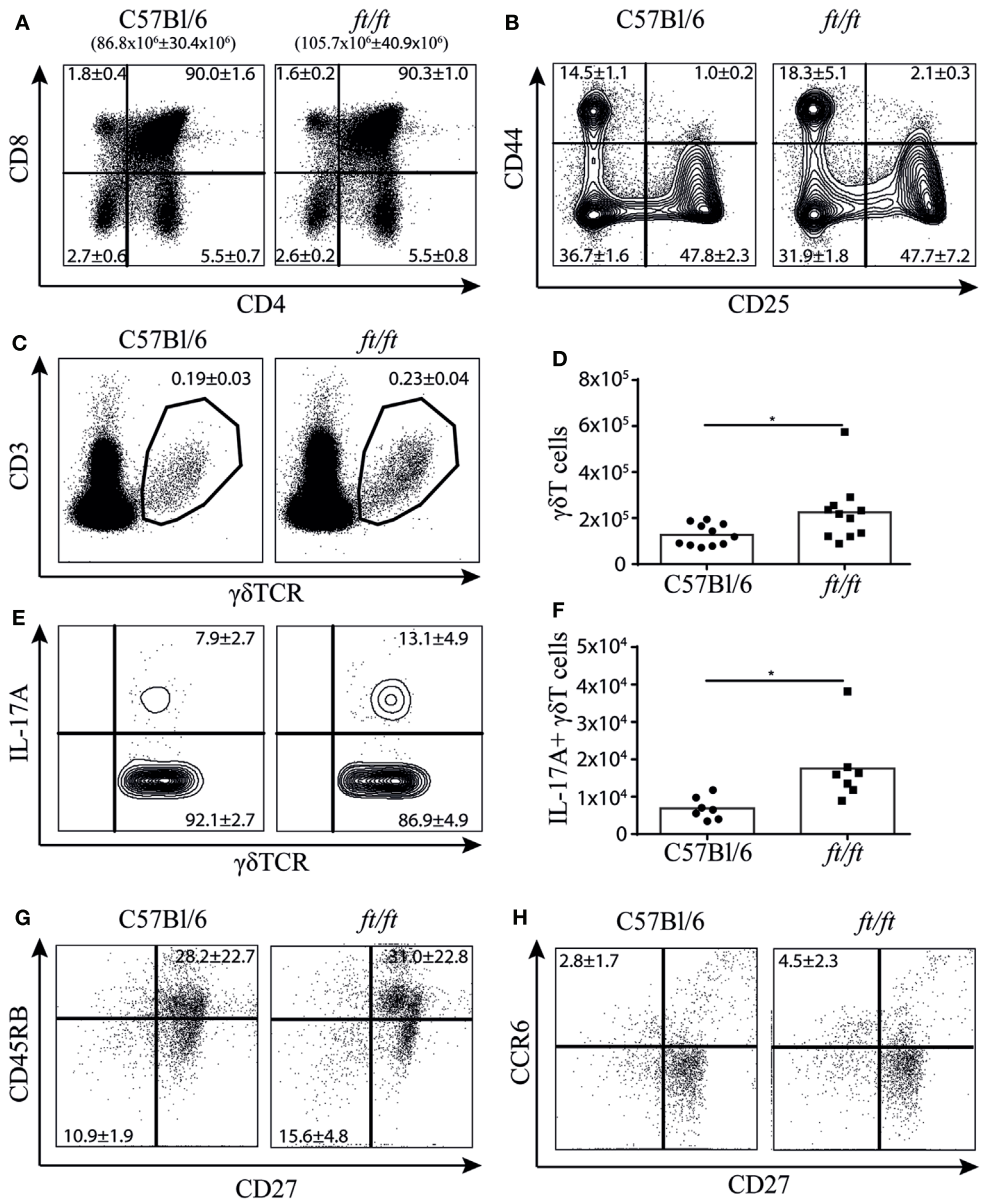

FIGURE 2 | Increased numbers of γδT17 cells in the thymus of *ft/ft* mice. **(A–H)** Flow cytometric analyses of thymocytes from 8 to 12 weeks old C57Bl/6 and *ft/ft* mice. **(A)** Plots showing CD4 and CD8 staining of total thymocytes. The total numbers of thymocytes are given above the plots. **(B)** Plots showing CD25 and CD44 staining on the double negative $CD4^-CD8^-$ thymocyte population. **(C,D)** Fraction and absolute numbers of $CD3\varepsilon^+TCR\gamma\delta^+$ thymocytes. **(E,F)** Fraction and absolute numbers of IL-17^+ $TCR\gamma\delta^+$ thymocytes. **(G,H)** Dot plots showing CD27 versus CD45RB and CD27 versus CCR6 expression of $TCR\gamma\delta^+$ thymocytes. Data are representative of two to four independent experiments with two to four mice in each. The mean percentages ± SD are given for the relevant populations in the plots.

increase in both the fraction and the total numbers of γδ T cells expressing IL-17A in *ft/ft* compared to *WT* mice (**Figures 2E,F**). In accordance with this, we found a significant increase in the fraction of $CD27^{-}CD45RB^{-}$ (**Figure 2G**) and $CD27^{-}CCR6^{+}$ γδ T cells (**Figure 2H**) in *ft/ft* compared to *WT* mice.

Filaggrin Is Expressed in the Thymic Medulla of Mice

Next, we speculated whether filaggrin is expressed in the thymus of mice and thereby could affect γδ T cell development. In humans, filaggrin is expressed in the Hassall's corpuscles (21, 22), but it is unknown whether filaggrin is expressed in the thymus of mice. To determine the expression and location of filaggrin in the thymus, we compared thymi of *ft/ft* mice and WT mice using immunohistochemistry. Skin was used as a positive control (**Figure 3A**). Interestingly, we found that filaggrin is expressed in small clusters of cells in the thymic medulla in WT mice, and to a lesser extend in the thymic medulla of *ft/ft* mice (**Figures 3B,C**). The *ft* mutation carried by the *ft/ft* mice is a frameshift mutation that results in the expression of a truncated profilaggrin and almost complete absence of filaggrin monomers in the epidermis of *ft/ft* mice (3). Thus, the mutation does not necessarily cause a decreased transcription of *Flg*. We found that filaggrin is transcribed in thymic stroma of both *ft/ft* and *WT* mice, but to a lesser degree than seen in skin (**Figure 3D**). Furthermore, we found an approximately threefold reduction in the transcription of filaggrin in the TSC of *ft/ft* mice compared to *WT* mice (**Figure 3D**). Taken together, these data show that filaggrin is expressed at the protein and RNA level in the thymic medulla of *WT* and *ft/ft* mice and that the expression is lower in *ft/ft* mice.

γδT17 Cells Continue to Be Produced After Birth in the Thymus of *ft/ft* Mice

It has been described that natural γδT17 cells normally are produced only during fetal stages (12, 23). To determine whether the increase of γδT17 cells in the thymus of *ft/ft* mice was a reminiscence from the fetal stage or was due to an ongoing development during adulthood, we analyzed mice treated with FTY720, an inhibitor of S1P-R1-mediated thymic egress (24). The total numbers of γδ T cells were significantly higher in both *ft/ft* and *WT* thymus following FTY720 treatment. However, in *ft/ft* mice, the accumulation of γδ T cells was significantly greater than in WT mice (**Figure 4A**). Consistent with previous studies (12, 23), adult *WT* mice did not accumulate γδT17 cells following FTY720 treatment (**Figure 4B**). However, in contrast to *WT* mice, we found that *ft/ft* mice accumulated γδT17 cells in the thymus when treated with FTY720 (**Figure 4B**). This difference was specific to γδT17 cells as $IFN\gamma^{+}$ γδ T cells accumulated to the same degree in *ft/ft* and *WT* mice (**Figure 4C**). To determine if the accumulating γδT17 cells were restricted to a specific subset of γδ T cells in the *ft/ft* thymi, we co-stained with Vγ1.1, Vγ2, and Vγ3. No significant accumulation of $V\gamma1.1^{+}$ or $V\gamma3^{+}$ γδT17 cells was detected (**Figures 4D,E**), whereas $V\gamma2^{+}$ γδT17 cells accumulated significantly (**Figures 4F,G**). As the accumulation of $V\gamma2^{+}$ γδT17 cells only accounted for approximately 50% of the total number of accumulated γδT17 cells in the thymus of *ft/ft* mice other γδT cell subsets, most likely $V\gamma4^{+}$ γδT17, probably also accumulated. Taken together, these experiments indicated that production of $V\gamma2^{+}$ γδT17 cells is dysregulated in *ft/ft* mice and that $V\gamma2^{+}$ γδT17 cells continue to be produced in the thymus of adult *ft/ft* mice.

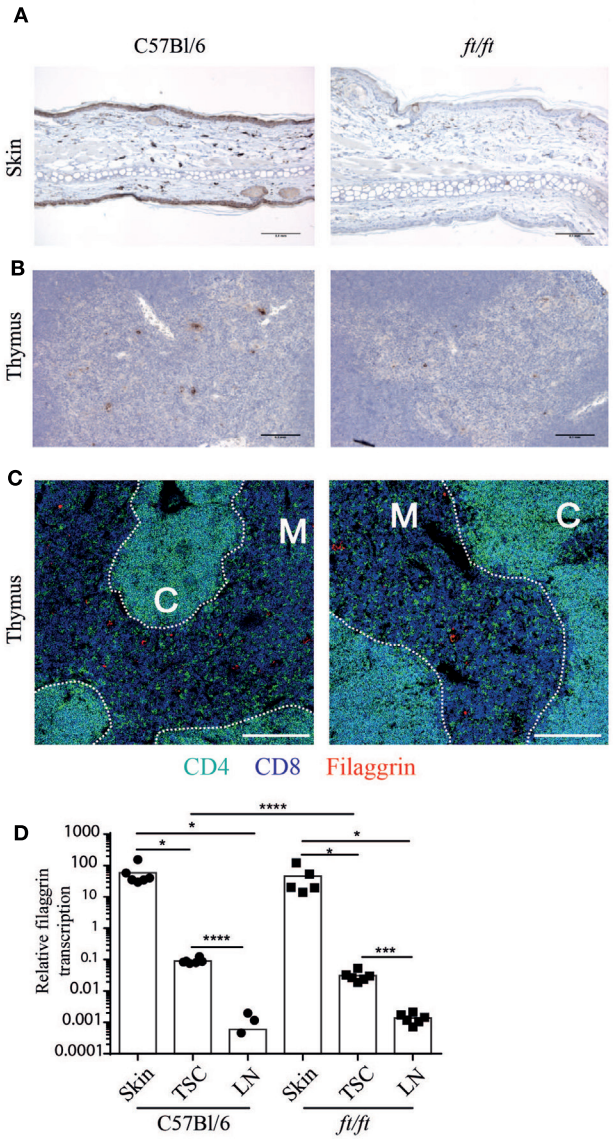

FIGURE 3 | Decreased filaggrin expression at the transcriptional level in the thymus of *ft/ft* mice. **(A–C)** Immunohistological analyses of ears and thymi from 8 to 12 weeks old C57Bl/6 and *ft/ft* mice. Sections of **(A)** ears and **(B)** thymi were stained with anti-filaggrin antibody. The sections are magnified ×200 and the bars indicate 0.1 mm. **(C)** Confocal microscopy images of thymi showing expression of CD4 (green), CD8 (blue), and filaggrin (red). The bars indicate 200 μm. **(D)** Relative profilaggrin transcription in the skin, thymic stromal cells (TSC), and lymph nodes of *ft/ft* and C57Bl/6 mice. The Ct-values of profilaggrin transcription were normalized to the transcription of GAPDH. The means ($n = 6$) are indicated by bars. For C57Bl/6 lymph nodes, three values of profilaggrin transcription were zero and are therefore not indicated with dots on the logarithmic scale. Expression of profilaggrin of TSC was compared to skin and lymph nodes for each group.

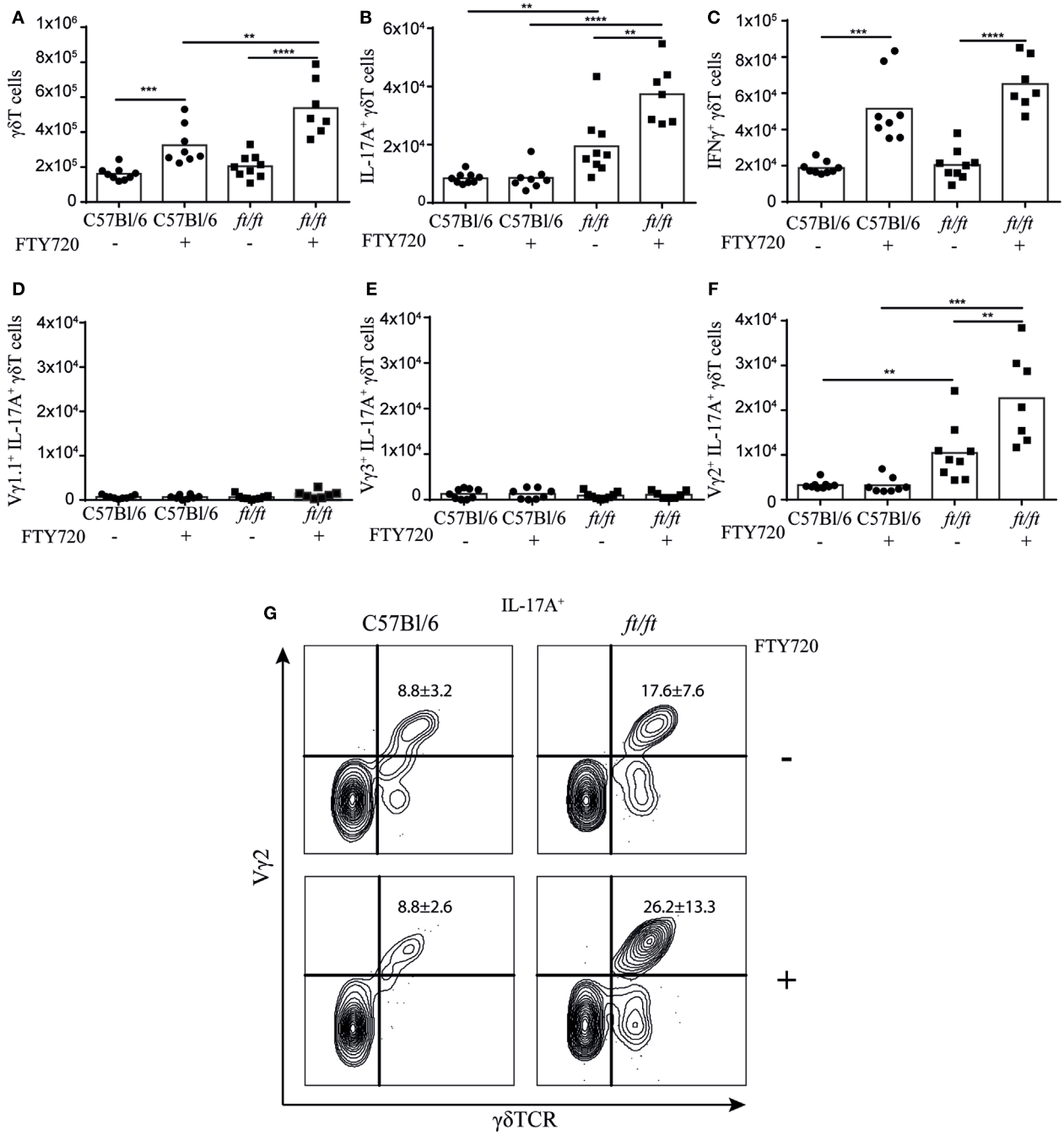

FIGURE 4 | *ft/ft* mice accumulate γδT17 cells in the adult thymus. Numbers of γδTCR cells and γδTCR subsets in the thymus of 8–12 weeks old *ft/ft* and C57BL/6 mice treated with FTY720 or untreated. Single-cell suspensions were stained with anti-TCRγδ, -TCRβ, -IL-17A, –IFN-γ. -Vγ1.1, -Vγ2, and -Vγ3. Percentages of each population were multiplied with the cell count of the parent gate to get the numbers presented. **(A)** Numbers of γδTCR cells. **(B)** Numbers of IL-17A$^+$ γδTCR cells. **(C)** Numbers of IFN-γ$^+$ γδTCR cells. **(D)** Numbers of Vγ1.1$^+$IL-17A$^+$ γδTCR cells. **(E)** Numbers of Vγ3$^+$IL-17A$^+$ γδTCR cells. **(F,G)** Numbers and percentage of Vγ2$^+$IL-17A$^+$ γδTCR cells.

Increased Levels of IL-6 and IL-23 in the Thymus of *ft/ft* Mice

The exact mechanisms determining the effector fate of γδ T cells in the thymus have yet to be fully uncovered, but antigen-naïve γδ T cells have been shown to produce IL-17, whereas antigen-experienced γδ T cells produce IFNγ (10). As the TCR expression level has been suggested to be a marker for whether the γδ T cells have encountered antigen or not (10), we determined

the TCR expression levels on thymic γδ T cells from *ft/ft* and *WT* mice. In accordance, with the increased number of γδT17 T cells, we found higher expression levels of both TCRγδ and CD3εon thymic γδ T cells in *ft/ft* mice compared to *WT* mice (**Figures 5A–D**). To further investigate possible mechanisms mediating the increased development of γδT17 cells in *ft/ft* mice,

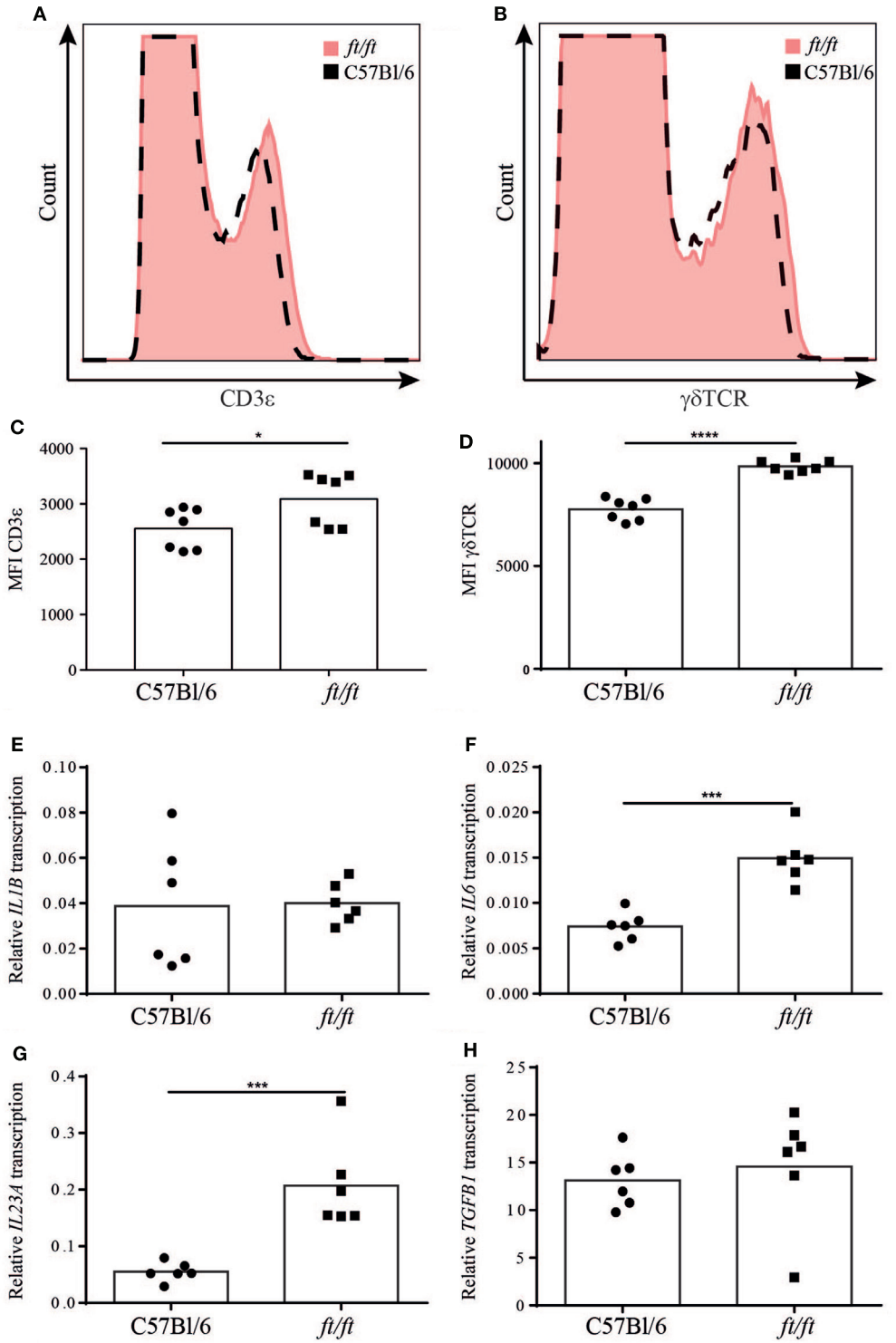

FIGURE 5 | Continued

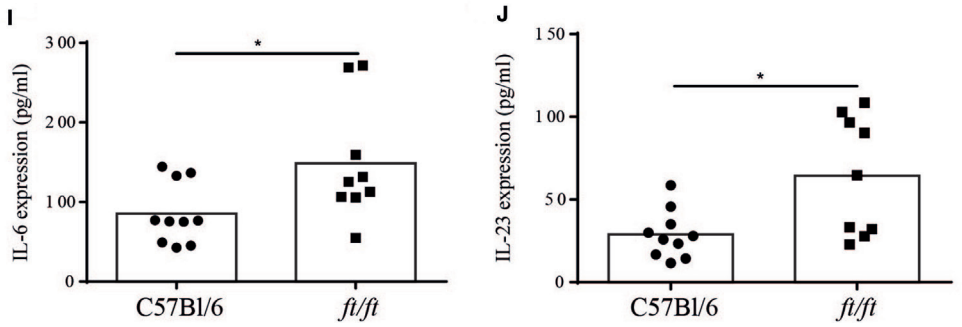

FIGURE 5 | Altered TCR expression and cytokine expression in *ft/ft* mice. Representative histograms of **(A)** CD3ε and **(B)** TCRγδ expression on thymocytes from 8 to 12 weeks old wild-type *(WT)* (dashed lines) and *ft/ft* (red filling) mice. Mean fluorescence intensity (MFI) of **(C)** CD3ε and **(D)** γδTCR expression. **(E–H)** Relative gene expression of *IL1B*, *IL6*, *IL23*, and *TGFB1* in the thymi from *ft/ft* and *WT* mice. The Ct-values of the specific gene transcription were normalized to the transcription of GAPDH. **(I,J)** Concentrations of IL-6 and IL-23 in thymic lysates from *ft/ft* and *WT* mice.

we analyzed the expression of factors known to be involved in the differentiation of γδ T cells and Th17 cells. Interestingly, we found a significantly increased transcription of IL6 and *IL23A*, but not of *IL1B* and *TGFB1* in *ft/ft* mice compared to *WT* (**Figures 5E–H**). Accordingly, we found significantly higher protein levels of IL-6 and IL-23 in *ft/ft* mice compared to *WT* (**Figures 5I,J**).

DISCUSSION

In this study, we show that adult *ft/ft* mice have an increased number of γδT17 cells in the thymus, spleen and epidermis compared to WT mice. Furthermore, we demonstrate that filaggrin is expressed by TSC in the thymic medulla of *WT* mice and that this expression is decreased at both the transcriptional and translational level in the thymus of *ft/ft* mice. Blocking thymic egress resulted in an accumulation of Vγ2$^+$ γδT17 cells in *ft/ft* mice, which was not seen in *WT* mice. Finally, we found an increased TCR expression level on thymic γδ T cells and an increased level of IL-6 and IL-23 in the thymi of *ft/ft* mice compared to *WT* mice.

The spontaneous skin inflammation found in *ft/ft* mice correlates with increased levels of IL-17A in the skin (4). In agreement with this, we found increased numbers of γδT17 cells in the epidermis of *ft/ft* mice compared to *WT* mice. Interestingly, we found that the majority of γδT17 cells in epidermis of *ft/ft* mice belonged to the Vγ2 subset, which are normally not present in the epidermis. In addition to the mutation in the *Flg* gene, *ft/ft* mice also have a mutation in the *Tmem79* gene. Therefore, we cannot exclude the possibility that the *Tmem79* gene plays a role in the increased number of γδT17 cells in the *ft/ft* mice. However, an increased level of IL-17A has been found in the skin of pure filaggrin-deficient mice supporting the importance of the Flg gene in γδT17 cell homeostasis (25).

Thymic crosstalk is the term used to describe the bidirectional need of TEC for development of T cells and of T cells for the development of TEC. In *WT* mice, γδT17 cells are only produced in the fetal thymus (12). In contrast, we show that γδT17 cells still are produced in the thymus of adult *ft/ft* mice. The mechanisms behind this are still unclear. However, distinct programs of thymus epithelial cell development exist in the fetal and adult thymus. Thus, the ability of the adult *ft/ft* thymus to continue to produce a γδ T cell subset typical of the embryonic thymus could indicate that the switch from fetal to adult programmes of TEC development are disturbed in *ft/ft* mice. Relevant to this, filaggrin expression in human thymus maps to Hassall's corpuscles, a product of mTEC terminal differentiation that is first evident in mice after birth (26). Thus, filaggrin may be required for an mTEC terminal differentiation programme that marks age-related changes in the thymic microenvironment, which then controls the ability of the thymus to support different programs of T cell development at specific developmental stages.

The γδTCR signaling strength determines which effector subset the thymic γδ T cells will commit to; strong γδTCR signaling results in development of IFN-γ producing cells and weak signaling in IL-17A producing cells (9–11, 16). A central regulator of TCR signaling in thymic γδ T cells is the TCR expression level (27). Mice with reduced TCR expression level on their thymic γδ T cells have reduced TCR signaling and increased development of γδT17 cells compared to WT mice (27). Based on this, one could suspect that the increased TCR expression level we find on thymic γδ T cells in the *ft/ft* mice compared to WT mice would result in an increased generation of IFN-γ producing γδ T cells. In contrast, we found increased levels of γδT17 cells in both the thymus and periphery of *ft/ft* mice. However, increased TCR expression levels are found on thymic γδT cells in mice where the TCR ligand is not expressed compared to mice where the TCR ligand is expressed and lack of TCR ligand expression correlated with increased development of γδT17 cells (10). Furthermore, it is well described that T cells down-regulate the TCR on their surface as part of T cell activation (28). It is therefore possible that the increased TCR expression found on thymic γδ T cells in the *ft/ft* mice is due to reduced expression of TCR ligand in these mice. Although our observations indicate that the development of Vγ2$^+$ γδT17 cells is dysregulated in *ft/ft* mice, we cannot formally exclude that the increased numbers and accumulation of Vγ2$^+$ γδT17 cells are caused by an increased thymic expansion of mature Vγ2$^+$ γδT17 cells in *ft/ft* mice that would normally be

restricted in some way by filaggrin in WT mice. However, we could conclude that the production of Vγ2$^+$ γδT17 cells in the thymus of *ft/ft* mice is increased.

In conclusion, in this study, we establish that filaggrin is expressed in the thymic medulla of *WT* mice, and this expression is decreased at both the transcriptional and translational level in *ft/ft* mice. Furthermore, we show that there is an enhanced production Vγ2$^+$ γδT17 cells in the thymus of filaggrin-deficient mice and that there is a general increase in the number of thymocytes. Therefore, we suggest that reduced expression of filaggrin in the thymus affects the production of γδ T cells, which leads to increased IL-17 polarization. Currently, mutations in *Flg* are primarily associated with skin disease, but our results support that they might also cause systemic alterations in the immune system. This is supported by the observation that both humans and mice with filaggrin deficiency have systemically elevated levels of Th17 cells. As 8–10% of the European population are carriers of a filaggrin mutation, it is important to uncover still unknown effects of this mutation, and it will be very interesting to analyze whether humans with filaggrin deficiency have elevated numbers of γδT17 cells and if so whether they represent natural or inducible γδT17 cells.

ETHICS STATEMENT

The mice were housed in the specific pathogen free animal facility at the Department of Experimental Medicine, Panum Institute, in accordance with the national animal protection guidelines (license number 2012-15-2934-00663).

AUTHOR CONTRIBUTIONS

MJ, CB, AG, AW, and TP performed the laboratory experiments. MJ, JJ, TB, AW, NØ, JT, AW, GA, CG, and CB conceived and designed the experiments. MJ, CG, and CB analyzed the data and wrote the paper. All authors revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00988/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Manuscript II

Mutations in genes encoding filaggrin and mattrin lead to defective T cell development and function

Mutations in genes encoding filaggrin and mattrin lead to defective T cell development and function

Mia Hamilton Jee[1,2], Julie Friis Weber[1], Veronika Mraz[1], Anders Boutrup Funch[1,2], Anne-Sofie Østergaard Gadsbøll[1], Andrea Jane White[3], Graham Anderson[3], Anders Woetmann[1], Niels Ødum[1], Jan Pravsgaard Christensen[4], Allan Randrup Thomsen[4], Jeanne Duus Johansen[2], Carsten Geisler[1] & Charlotte Menné Bonefeld[1,*]

[1]LEO Foundation Skin Immunology Research Center, Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark,

[2]National Allergy Research Centre, Department of Dermato-Allergology, Copenhagen University Hospital Herlev-Gentofte, Hellerup, Denmark,

[3]MRC Centre for Immune Regulation, Institute for Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom,

[4]Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

**Corresponding author Charlotte Menné Bonefeld,* cmenne@sund.ku.dk

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Abstract

Skin barrier defects caused by mutations in *Flg* encoding filaggrin and *Matt (Tmem79)* encoding mattrin are known risks factors for developing atopic dermatitis. Filaggrin and mattrin are expressed in keratinocytes in the granular layer of the epidermis and both are important for formation of stratum corneum. Mutations in *Flg* have been associated with a systemic increase in the levels of Th17 cells and increased IL-17A-production after antigen exposure in mice and humans. The flaky tail mouse is a double mutant, harboring mutations in both *Flg* and *Matt*, and has been shown to develop increased numbers of IL-17A-producing $V\gamma2^+$ γδ T cells. Having separated the mutations, we report that the mutation in *Matt*, and not the *Flg* mutation, results in an IL-17A-skewed T cell profile in both thymus, spleen and epidermis. Interestingly, the mutation in *Flg* results in an IFNγ-skewed profile in thymus and spleen. Furthermore, transcription of the keratinocyte markers *KRT10* and *LOR* encoding cytokeratin-10 and loricrin, respectively, are down-regulated in the thymus from mice with individual *Flg* and *Matt* mutations. Finally, mice with a mutation in either *Flg* or *Matt* have an altered response to the contact allergen DNFB and an increased viral load following upper airway infection with Sendai virus. Taken together, our data suggest that both filaggrin and mattrin play a role in the development of keratinocyte-like thymic epithelial cells in the thymus, which in turn affects T cell development and immune responses in peripheral tissues.

Introduction

Defects in the physical and chemical skin barrier are associated with an increased risk of developing inflammatory skin diseases. Especially loss-of-function mutations in the filaggrin gene (*FLG*), encoding the protein filaggrin, increase the risk of developing icthyosis vulgaris and atopic dermatitis (AD)[1,2]. Around 10 % of the Northern European population are heterozygous carriers of an *FLG* mutation and 0.1 % are homozygous carriers[3,4]. Approximately 30 % of European patients with AD harbor an *FLG* mutation[5]. Another protein important for the physical and chemical skin barrier is mattrin. Mattrin is a 5-transmembrane protein highly expressed in the trans-Golgi apparatus of the keratinocytes in stratum granulosum, and it has been suggested to play a role in the formation of the stratum corneum[6,7]. Mutations in *Matt (Tmem79)*, encoding mattrin, are also a predisposing risk factor for developing AD[6].

Filaggrin-deficient flaky tail mice spontaneously develop eczematous skin lesions and have commonly been used as a model for filaggrin deficiency. We have recently shown that filaggrin normally is expressed in the medulla of the thymus in mice[8]. Other studies have found that a keratinocyte-like subset of medullary thymic epithelial cells (mTEC) express a variety of markers normally associated with differentiating keratinocytes, such cytokeratin-10, involucrin, loricrin and mattrin[9,10]. In general, mTEC orchestrate negative selection of self-reactive T cells. Impressively, mTEC are able to present antigens normally restricted to peripheral tissues, because a subset of mTEC promiscuously express peripheral genes, in part due to expression of the Auto-immune regulator (Aire)[11]. The mTEC compartment is divided into MHC-II^{low}CD80^{low} mTEC^{lo} and MHC-II^{high}CD80^{high} mTEC^{hi} subsets. The shift from mTEC^{lo} to mTEC^{hi} is characterized by the expression of Aire and several tissue-restricted antigens. Continued mTEC differentiation is characterized by downregulation of MHC-II, CD80 and Aire, and results in the formation of post-Aire mTEC^{lo} subsets[12]. It is within the post-Aire mTEC^{lo} subset that the keratinocyte-like mTEC subset is found[9]. In

addition to playing a role in conventional αβ T cell development, mTEC also play an essential role in the development of non-conventional T cells like γδ T cells[10,13–17].

We have recently described that flaky tail mice have a defect in T cell development resulting in an increased production of IL-17A-producing γδ T cells that correlates with an increased number of IL-17A-producing γδ T cells in spleen and epidermis[8]. Taken together, these observations suggest that the spontaneous skin inflammation found in the flaky tail mouse is not mediated only by defects in the skin barrier but also by dysregulated T cell development. The flaky tail mouse, however, is a double mutant with a mutation termed *ft* in *Flg* and a mutation termed *ma* in *Matt*[6,18,19]. Interestingly, in studies where the two mutations were separated, it was found that isolated mutations in both *Matt* and *Flg* resulted in an altered formation of the stratum corneum of epidermis. The $Flg^{ft/ft}$ mutants did not develop spontaneous skin inflammation, whereas the $Matt^{ma/ma}$ mutants did[6,19]. If and how the isolated *ft* and *ma* mutations affect T cell development and systemic and epidermal inflammation is not known. To elucidate the effect of the individual *ft* and *ma* mutation on T cell development, T cell subset composition and peripheral immune responses, we separated the *ft* and *ma* mutations and backcrossed the mice onto a C57Bl/6 wild-type (WT) background to generate congenic strains. We report that the mutation in *Matt* but not in *Flg* results in an IL-17A-skewed T cell profile in both the thymus, spleen and epidermis, whereas the mutation in *Flg* results in an IFNγ-skewed profile in the thymus and spleen. Furthermore, transcription of the keratinocyte markers *KRT10* and *LOR* encoding cytokeratin-10 and loricrin, respectively, are down-regulated in the thymus from mice with isolated *Flg* and *Matt* mutations. Finally, mice with a mutation in either *Flg* or *Matt* have an altered response to the contact allergen DNFB and an increased viral load following upper respiratory tract infection with Sendai virus.

Materials and Methods

Animal Model

Flaky tail mice (*a/a Tmem79^{ma} Flg^{ft}/J*, stock number 000281) were purchased as cryopreserved embryos from the Jackson Laboratory and bred at our in-house animal facility. As flaky tail is not a strict congenic strain, the mice were crossed with C57Bl/6J mice to generate the heterozygous *Matt$^{ma/+}$Flg$^{ft/+}$* mice. The mutations were then separated, and mice were backcrossed to the congenic C57Bl/6J background for five generations. C57Bl/6 mice were used as wild-type control. Experiments were performed on female mice aged 8-12 weeks unless otherwise stated. Mice were housed in specific pathogen free conditions at the animal facility at The Department of Experimental Medicine, Panum Institute, in accordance with the national animal protection guidelines (license number 2018-15-0201-01409).

Mouse Genotyping

DNA was extracted from the tip of the mouse tail, and PCR was performed with primers targeting *Flg;* forward 5'-CAT CTC CAG TCA GGG CTG ACC-3' and reverse 5'-GCT GCC TGT GGC CGG ACT CG-3', and with primers targeting *Matt;* forward 5'-GGT TTC TCT TCA TTC TGC TG-3' and reverse 5'-AAG GCA ATG GAT TCA ACA C-3'. The PCR products were digested with the restriction enzymes AccI and CviQI (BioNordika Denmark) for *Flg* and *Matt* testing, respectively. The digested fragments were separated on an agarose gel by electrophoresis.

Preparation of Single-Cell Suspensions

Thymi and spleens were dissociated on 70 µm cell strainers. The single cells were washed in RPMI medium (10 % FBS, 0.5 IU/L penicillin, 500 mg/L streptomycin, 1 % L-glutamine) and adjusted to 2 x 10^7 cells/ml. Cells were plated in a 96-well round-bottomed plate with two million cells per

well. Epidermal single cells were isolated from mouse ears. Ears were split into a dorsal and ventral part with forceps. The dorsal part was transferred to a 0.3 % trypsin-GNK (2.94 g NaCl, 0.134 g KCl, 0.334 g glucose/dextrose per 1 g of trypsin) solution for 60 minutes at 37°C, 5 % CO_2 with the epidermis side up. Using forceps, the epidermis was peeled from the dermis, and treated in a 0.3 % trypsin-GNK solution with 0.1 % DNase at 37°C for 10 minutes. The cell suspensions were filtered through a cell strainer and plated overnight at 37°C, 5 % CO_2 to allow re-expression of surface markers.

Staining and Flow Cytometry

Fc-receptors were blocked with unlabeled anti-CD16/32. Surface markers were stained with anti-TCRβ (H57-597), -CD4 (GK1.5/RM4-5), -CD8a (53-6.7), -CD25 (PC61), -CD44 (IM7), -TCRγδ (GL3), -CD45RB (16A), -Vγ1.1 (2.11), -Vγ2 (UC3-10A6) and -Vγ3 (536) diluted in Brilliant Stain Buffer (BD Biosciences). Fixable Viability Dye (eFluor780) (eBioscience) was used to determine viability of cells. When staining for intracellular cytokines, cells were stimulated with PMA (50 ng/mL), monensin sodium (4 µg/mL and ionomycin (500 ng/mL) for 4 hours and subsequently stained for surface markers. Following fixation and permeabilization with BD Cytofix/Cytoperm (BD Biosciences), the cells were intracellularly stained with anti-IL-17A (TC11-18H10.1) and –IFNγ (XMG1.2). Data were collected on a BD LSRFortessa and analyzed using FlowJo 10.6.1 Soft-ware.

Immunofluorescence Microscopy of Epidermal Ear Sheets

Ears were excised, and ear sheets were prepared by separating the dorsal from the ventral side. The dorsal side of separated ears were floated dermis-side down in 3.8 % ammonium thiocyanate in PBS for 13 minutes at 37°C. Subsequently, epidermis was peeled from the dermis and the epidermal

sheets were fixed in Zamboni's fixative with 2 % PFA for 15 minutes. Epidermal sheets were blocked in 5 % goat serum in PBS for 1.5 hours at room temperature and stained with rat anti-mouse CD8α. Next, epidermal sheets were labelled with goat anti-rat Alexa Flour 555 antibody and thereafter post-fixed in Zamboni's fixative with 1 % PFA and blocked in 5 % goat serum in PBS. Epidermal sheets were then stained with rat anti-mouse CD4 Alexa Flour 488 and hamster anti-mouse TCRγδ (GL3) Alexa Flour 647 and DAPI. Finally, epidermal sheets were mounted in Pro-Long Diamond Antifade Mountant (Thermo Fischer) and cured overnight at room temperature. Digital images were acquired with a 40x Fluar numerical aperture (NA) 1.30 objective (Zeiss) on a Zeiss Axio Observer.Z1/7 that includes 1x Yokogawa (CSU-X1) spinning disc confocal; 405-, 488-, 561-, and 639-nm lasers; and an Orca Fusion camera. The system was controlled by Zen Lite; Zen Blue version. Z-stacks were captured in 10 randomly chosen positions per slide. At least three mice per strain were examined.

Quantitative Real-Time PCR

Mouse organs were frozen in liquid nitrogen. Organs were disintegrated in a Precellys tissue homogenizer (Bertin Technologies) in TRI Reagent (Sigma Aldrich). Isolation of RNA was performed using the RNeasy Mini Kit 250 (Qiagen) according to manufacturer's protocol. The concentration of RNA in the samples was measured using a Nanodrop 2000c spectrophotometer (Thermo Scientific), and the RNA was subsequently diluted to 2 µg/µL. RNA was transcribed into cDNA using the High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific) according to manufacturer's protocol. Transcription of target genes was measured by real-time qPCR using stock Taqman primer/probe sets with Taqman Universal Master Mix. Samples were processed in a LightCycler 480 II (Roche). Fold change was calculated relative to GAPDH using $2^{(-\Delta\Delta CT)}$.

Sensitization with Contact Allergen

Mice were painted (25 µl on each ear) with either olive oil and acetone (OOA 1:3, vehicle) or 0.15 % dinitrofluorobenzene (DNFB) in OOA for 3 consecutive days. Ear thickness was measured before allergen painting and on day 6 and 13 after sensitization. The mice were challenged on day 21 and ear thickness was measured 0, 6, 12, 24, 48, 72, 96, 144, 192, 240 and 336 hours after challenge.

Inoculation of Mice with Recombinant Sendai Virus

A recombinant Sendai virus with the firefly luciferase gene (luc) inserted into the M-F gene junction[20] was kindly provided by Dr. Charles J. Russell, St. Jude Children's Research Hospital, Memphis, Tennessee, USA. On day one, mice were anesthetized with isoflurane (Baxter) and intranasally (i.n.) inoculated with 30 µl of PBS containing 7000 PFU of virus. Mice were imaged every 24 hours for 9 days, and 15 minutes before each imaging session, mice were injected intraperitoneally (i.p.) with luciferin (Perkin Elmer) at a dose of 10 mg/ml in 300 µl and anesthetized with isoflurane 3 minutes prior to imaging.

In Vivo Imaging System (IVIS)

In vivo images were obtained using an IVIS SpectrumCT (Perkin Elmer) and analyzed using Living Image 4.3.1 software (Perkin Elmer). To quantify bioluminescence, we manually defined regions of interest (ROI) around nose, throat and lung. The average radiance (photons/s/cm^2/steradian), which is defined as the number of photons that leave a cm^2 of tissue and radiate into an angle of one steradian, was used to graph bioluminescence. "Negative" mice that did not appear positive with virus, although they had been positive the day before or after, were removed from the data set on the "negative" day. These probably occur if the i.p. injected luciferin is spread poorly on a given day.

Statistical Analyses

Differences between the studied groups were analyzed using the two-tailed unpaired Student's *t*-test. For statistical analyses in figure 7, multiple t-tests were performed and significance was determined using the Holm-Sidak method with $\alpha=0.05$ (adjusted p-value for multiple testing). The statistical analyses were executed using GraphPad Prism version 8.3.1 and a p-value below 0.05 was considered significant. Statistically significant differences are denoted *<0.05, **<0.01, ***<0.001. ****<0.0001. Outliers were identified using the Robust regression and Outlier removal test (ROUT) set to 1 % in GraphPad Prism.

Results

The spontaneous skin inflammation in Matt$^{ma/ma}$ mice is associated with an increased number of IL-17A-producing epidermal T cells

Previously it has been shown that mice with an isolated *ma* mutation spontaneously develop skin inflammation, whereas mice with an isolated *ft* mutation do not[6,7]. To further characterize the immunological mechanisms behind these observations, we first separated the *ft* and *ma* mutations found in the flaky tail mouse by back-crossing flaky tail mice to wild-type (WT) C57Bl/6 mice as outlined in Figure S1. In accordance with previous observations, we found that *Matt$^{ma/ma}$* mice spontaneously developed a macroscopic skin affection with straggly fur and puffy nose and ears, whereas no macroscopic signs of inflammation were observed in the skin of *Flg$^{ft/ft}$* mice (Fig. 1A). To characterize the T cell subsets found in the epidermis of WT, *Flg$^{ft/ft}$* and *Matt$^{ma/ma}$* mice, we prepared single cell suspensions from the epidermis and analyzed them by flow cytometry. Consistent with previous reports Vγ3$^+$ dendritic epidermal T cells (DETC), constituted the vast majority of the T cells in the epidermis of WT mice, and the frequency of αβ T cells was < 1 % (Fig. 1B and C and Fig. S2 for gating strategy). A similar T cell subset distribution was found in the epidermis of *Flg$^{ft/ft}$* mice, although they had slightly fewer DETC than WT mice (Fig. 1B and C). In contrast, although Vγ3$^+$ DETC constituted a major part, a substantial number of other γδ T cells as well as CD4$^+$ and CD8$^+$ T cells was also found in the epidermis of *Matt$^{ma/ma}$* mice (Fig. 1B and C). Furthermore, an approximate 2-fold increase in the total amount of epidermal T cells was found in *Matt$^{ma/ma}$* mice compared to WT and *Flg$^{ft/ft}$* mice (Fig. 1B).

To visualize the localization and composition of T cells in the epidermis, we prepared epidermal ear sheets and stained them for TCRγδ, CD4 and CD8 (Fig. 1D). In WT and *Flg$^{ft/ft}$* mice, we found no CD4 and CD8 cells in accordance with the flow cytometry data. In contrast, we found both CD4 and CD8 T cells in the *Matt$^{ma/ma}$* mice, although only in small numbers. Interestingly, the CD4 and

CD8 cells were located near hair follicles. Furthermore, the network of DETC seemed less dense in *Matt*$^{ma/ma}$ mice compared to WT mice.

We have recently shown that flaky tail mice have an increased fraction of IL-17A-producing Vγ2^{+} T cells and DETC in their epidermis compared to WT mice[8]. To determine whether this trait was associated with either the *ft* or the *ma* mutation, we purified epidermal cells from WT, *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice, stimulated them *in vitro* with PMA and ionomycin and subsequently analyzed them for expression of IL-17A and IFNγ. Surprisingly, the IL-17A phenotype was linked to the *ma* mutation and not to the *ft* mutation, as we found a highly increased number of IL-17A-producing γδ and CD4^{+} T cells in the epidermis from *Matt*$^{ma/ma}$ mice and not in WT and *Flg*$^{ft/ft}$ mice (Fig. 2A-D). The majority of IL-17A-producing T cells in the epidermis from *Matt*$^{ma/ma}$ mice belong to γδ T cell subsets, but they were neither Vγ3^{+} DETC, Vγ1.1^{+} nor Vγ2^{+} (Fig. 2B). In contrast to the *Matt*$^{ma/ma}$ mice, no alterations in the number of T cells producing IL-17A and IFNγ were found in the epidermis of *Flg*$^{ft/ft}$ mice compared to WT mice (Fig. 2A-D).

T cells from the spleen of Flg$^{ft/ft}$ and Matt$^{ma/ma}$ mice are skewed towards an IFNγ and an IL-17A/IFNγ profile, respectively

We have previously shown that flaky tail mice have highly increased numbers of γδ T cells and IL-17A-producing γδ T cells in the spleen[8,21]. Furthermore, we have found an approximately 2-fold increase in the frequency of IL-17A-producing CD4^{+} T cells in the blood from humans with mutations in the filaggrin gene compared to control individuals[21]. These observations suggested that filaggrin deficiency might result in an IL-17A-skewed T cell profile. However, our observations depicted in figure 1 and 2 in the present study indicated that a deficiency in mattrin rather than filaggrin might cause the IL-17A-skewed profile seen in the flaky tail mice. To investigate this further, we analyzed the T cell profile in the spleen from WT, *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice. As seen in the

flaky tail mice, we found increased frequencies and absolute numbers of γδ T cells belonging to the Vγ1.1, Vγ2, Vγ3 and other Vγ subsets in the spleens of *Matt*$^{ma/ma}$ mice compared to WT mice (Fig. 3A and B). We did not detect any differences in the number of αβ T cells in the spleen between the three mouse strains (Fig. S3). Next, we determined the numbers of IL-17A- and IFNγ-producing T cells in the spleen after *in vitro* stimulation with PMA and ionomycin. We found significantly increased numbers of IL-17A-producing T cells in *Matt*$^{ma/ma}$ mice compared to WT mice and *Flg*$^{ft/ft}$ mice (Fig. 3C). Furthermore, both *Matt*$^{ma/ma}$ and *Flg*$^{ft/ft}$ mice had a significantly increased number of IFNγ-producing T cells compared to WT mice with *Flg*$^{ft/ft}$ mice having the highest numbers (Fig. 3D).

The ft and ma mutations affect T cell development and skews T cells toward an IFNγ- and an IL-17A-dominated profile, respectively

In general, CD4$^+$ and CD8$^+$ T cells acquire their effector function in the periphery following their encounter with specific antigens. In contrast, a large fraction of γδ T cells are programmed during their development in thymus to be either IL-17A- or IFNγ-producing[22–24]. Interestingly, within the last decade it has been clear that a minor fraction of CD4$^+$ and CD8$^+$ T cells with an innate phenotype develops in thymus[17,25–27]. These innate CD4$^+$ and CD8$^+$ T cells are programmed to produce specific cytokines already during their development in the thymus like the γδ T cells. We have recently shown that flaky tail mice have an increased number of IL-17A-producing thymocytes[8]. To determine whether the *ft* and *ma* mutations affected T cell development, we purified cells from thymus of WT, *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice and analyzed them by flow cytometry. We found an increased fraction and number of double positive CD4$^+$CD8$^+$ thymocytes in both *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice (Fig. 4A and B). On average, *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice had a 65-85 % increase in their total numbers of thymocytes compared to WT mice (Fig. 4A). Furthermore, both *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$

mice had a significantly larger number of γδ and αβ thymocytes (Fig. 4C and D). The larger population of αβ thymocytes was primarily due to an increased number of single-positive $CD4^+$ thymocytes in both $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice (Fig. 4B). Next, we determined the amount of IL-17A- and IFNγ-producing thymocytes after *in vitro* stimulation with PMA and ionomycin of the thymocytes from WT, $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice. In line with the conditions in the epidermis and spleen, $Matt^{ma/ma}$ mice had a significant increase in IL-17A-producing γδ and $CD4^+$ thymocytes compared to WT and $Flg^{ft/ft}$ mice, and a significant increase in IL-17A-producing $CD8^+$ thymocytes compared to WT mice (Fig. 4F). In contrast, $Flg^{ft/ft}$ mice had a significant increase in IFNγ-producing γδ and $CD4^+$ thymocytes compared to WT mice and $Matt^{ma/ma}$ mice and a significant increase of IFNγ-producing $CD8^+$ thymocytes compared to WT mice (Fig. 4G). Taken together, these data show that mutations in filaggrin and mattrin affect T cell development and indicate that the *ft* mutation skews T cell development towards an IFNγ-dominated profile whereas the *ma* mutation skews T cell development towards an IL-17A-dominated profile.

The ma and the ft mutations result in a reduced transcription of keratinocyte-like mTEC markers

We have recently shown that flaky tail mice have reduced filaggrin expression in the thymic medulla compared to WT mice[8]. Furthermore, recent expression data suggest that mattrin is expressed in the keratinocyte-like subset of the post-Aire $mTEC^{lo}$ subset[9]. However, if and how the *ma* and *ft* mutations affect the expression of keratinocyte-like mTEC markers are not known. To study this, we first determined the transcription levels of *Flg* and *Matt* in whole thymus from WT, $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice. As expected, we found a strong reduction in the transcription level of *Flg* in $Flg^{ft/ft}$ mice and of *Matt* in $Matt^{ma/ma}$ mice (Fig. 5A). Interestingly, an approximate 75 % reduction in the expression of *Matt* was seen in $Flg^{ft/ft}$ mice and vice versa of *Flg* in $Matt^{ma/ma}$ mice (Fig. 5A), which could suggest that filaggrin and mattrin are co-expressed in the same subsets of TEC. Subsequently,

we determined the transcription levels of *KRT10* encoding cytokeratin-10*, IVL* encoding involucrin and *LOR* encoding loricrin. The transcription of *KRT10* and *LOR* was significantly downregulated in $Flg^{ft/ft}$ mice, whereas only *KRT10* was downregulated in $Matt^{ma/ma}$ mice and *IVL* transcription was unaffected by the mutations (Fig. 5B).

To investigate whether deficiency of filaggrin and mattrin influenced the earlier steps of mTEC development, we determined the transcription of *AIRE*. Notably, *AIRE* transcription was significantly down-regulated in both $Flg^{ft/ft}$ mice and $Matt^{ma/ma}$ mice, with the largest decrease in $Flg^{ft/ft}$ mice. During mTEC development RANK:RANKL interactions induce the expression of both Aire and Skint-1, the selecting ligand for $V\gamma3^+$ DETC[28]. As we found fewer DETC in $Flg^{ft/ft}$ mice (Fig. 1C) and more IL-17A-producing DETC in $Matt^{ma/ma}$ mice (Fig. 2B), we consequently determined the transcription of Skint-1 in thymus from WT, $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice. Remarkably, Skint-1 transcription was decreased in both $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice with the biggest reduction in $Flg^{ft/ft}$ mice (Fig. 5C).

$Flg^{ft/ft}$ mice have increased inflammatory responses to the contact allergen DNFB

We have recently shown that flaky tail mice have an increased inflammatory skin response to contact allergens[29]. To investigate how $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice respond to the experimental contact allergen dinitrofluorobenzene (DNFB), we sensitized WT, $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice on their ears with DNFB in olive oil:acetone (OOA) or with pure OOA as control for three consecutive days and challenged them after 21 days. The inflammatory response was determined by measuring ear-thickness before sensitization, on day 3, 6, 13 and 21 after sensitization and 6, 12, 24, 48, 72, 96, 144, 192, 240 and 336 hours after challenge (Fig. 6A). Ear-thickness measurements revealed that both $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice had increased ear-thickness compared to WT mice already before sensitization (Fig. 6B-D). Sensitization with DNFB induced an increased ear-thickness in all three mouse

strains. An enhanced response peaking at day 6 was found in *Flg*$^{ft/ft}$ mice compared to WT mice (Fig. 6B and C). In contrast, the response in *Matt*$^{ma/ma}$ mice peaked already at day 3 and was comparable in size to the changes found in WT mice (Fig. 6B and D). Upon challenge, the response peaked after 24 hours for all three mouse strains (Fig. 6B-D). Whereas a significantly increased response was found in *Flg*$^{ft/ft}$ mice, only a minor response was seen in M*att*$^{ma/ma}$ mice (Fig. 6C-D). Taken together, these results suggested that the skewed T cell profiles found in *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice affected the responses to DNFB, although the pre-existing skin inflammation as measured by the thickened ears in both *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice complicated drawing a valid conclusion.

Matt*$^{ma/ma}$ *and Flg*$^{ft/ft}$ *mice have increased virus titer upon infection with Sendai virus

To investigate immune responses in *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice independently of the pre-existing skin inflammation, we inoculated WT, *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice intra-nasally with a recombinant Sendai virus carrying a firefly luciferase gene and measured the virus load for 9 days by *in vivo* imaging. The virus load was quantified by bioluminescence in defined regions of interest (ROI) around nose, throat and lungs using an IVIS SpectrumCT (Fig. 7A and B). The virus load peaked between day 3 and 5 in the nose and throat of all three mouse strains (Fig. 7C and D). A 2-fold enhanced signal was detected in *Matt*$^{ma/mat}$ mice compared to WT mice in the nose at day 3 and 4 (Fig. 2C). Likewise, *Matt*$^{ma/ma}$ mice had a 2-fold increase of virus signal in the throat at day 3 and 4 compared to WT and *Flg*$^{ft/ft}$ mice (Fig. 7D). Interestingly, *Matt*$^{ma/ma}$ mice had a 2-fold increase of the virus signal in the lungs at day 3, 4 and 6 compared to WT (Fig. 2E). *Flg*$^{ft/ft}$ mice also had a significantly increased virus load in the lungs compared to WT mice at day 6 (Fig. 2E). These results suggest that the defective T cell development in *Flg*$^{ft/ft}$ mice and *Matt*$^{ma/ma}$ mice decreased the capacity to rapidly control Sendai virus induced infection in the respiratory tract.

Discussion

In this study, we isolated the *ft* and *ma* mutations found in the flaky tail mouse and demonstrated that the spontaneous, macroscopically visible skin inflammation and the accumulation of IL-17A-producing T cells in the epidermis seen in flaky tail mice are consequences of the *ma* mutation, in accordance with previous findings[6,19]. In addition, we found that $Matt^{ma/ma}$ mice have an increased number of IL-17A-producing T cells in their spleen and thymus and that $Flg^{ft/ft}$ mice have an increased number of IFNγ-producing T cells in their spleen and thymus. Furthermore, transcription of keratinocyte and mTEC markers was decreased in thymus from $Matt^{ma/ma}$ and $Flg^{ft/ft}$ mice, suggesting that both the *ma* and the *ft* mutations affected mTEC differentiation and thereby T cell development. A defective T cell development may underlie the observation that both $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice had an altered immune response to DNFB and Sendai virus.

Previous studies have shown that defects in the development of DETC result in spontaneous skin inflammation with a substantial increase in the number of αβ T cells infiltrating the epidermis[30,31]. In accordance, αβ T cells with dendritic morphology seem to replace DETC in TCRδ-deficient mice[32]. In the present study, we found defective T cell development of γδ and αβ T cells in both $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice. The *ft* mutation skewed T cell development towards an IFNγ profile, whereas the *ma* mutation skewed T cell development towards an IL-17A profile. It was recently shown that thymus-derived IL-17A-producing γδ T cells play a central role in skin inflammation[33]. In accordance, flaky tail mice have an increased development of IL-17A-producing γδ T cells and develop spontaneous skin inflammation with an IL-17A dominated profile[8,34]. Our study indicates that it is the *ma* mutation in flaky tail mice that is the main cause to the IL-17A skewed profile and the spontaneous skin inflammation.

Interestingly, a previous study found that skin inflammation can cause thymic atrophy and affect T cell development[35]. Although we found increased cellularity in the *Matt*$^{ma/ma}$ thymus, we find it likely that some of the immunological changes seen in the *Matt*$^{ma/ma}$ mice might be caused by the widespread skin inflammation that provide a feedback loop on T cell development in the thymus promoting the IL-17A skewed profile.

Whereas the vast majority of T cells residing in the epidermis of WT mice is Vγ3^{+} and capable IFNγ producers, the dermis homes both Vγ2^{+} and Vγ4^{+} T cells that are important sources of IL-17[36,37]. Interestingly, we found a large population of γδ T cells in the *Matt*$^{ma/ma}$ epidermis that accounted for almost all the IL-17A-producing γδ T cells but was neither Vγ1.1^{+}, Vγ2^{+} nor Vγ3^{+}. We suspect that these cells belong to the Vγ4^{+} subset for which unfortunately no commercially available antibodies exist. It could be suggested that the chronic skin inflammation seen in *Matt*$^{ma/ma}$ mice permits migration of these cells from the dermis to the epidermis.

In WT mice development of IL-17A-producing γδ T cells is limited to the embryonic thymus[38]. We have recently shown that flaky tail mice develop IL-17A-producing γδ T cells in the adult thymus. In the present study, we found an increased percentage and number of IL-17A-producing γδ T cells in adult *Matt*$^{ma/ma}$ thymus. Possible explanations could be that these cells are long term residents or that they represent recirculating cells. Alternatively, distinct programs for TEC differentiation could exist in the fetal and adult thymus and *Matt*$^{ma/ma}$ mice that may cause a disturbance in the programs. Thus, the capacity of the adult *Matt*$^{ma/ma}$ thymus to produce a subset characteristic of the fetal thymus could suggest that the switch from fetal to adult programs of TEC development is disturbed in the *Matt*$^{ma/ma}$ mice. Furthermore, it is known that the signaling strength through the γδ TCR deter-

mines effector function so that strong TCR signaling results in IFNγ-producing cells and weak signaling results in IL-17A-producing cells[22,24,39,40]. Consequently, we hypothesized that both the *ma* and *ft* mutations result in an altered thymic environment with altered antigen-presentation and T cell selection. In accordance, we found that the *ft* and *ma* mutations not only affected transcription of filaggrin and mattrin, but also affected the transcription of other keratinocyte markers. This suggested that filaggrin is not only a marker of terminal mTEC differentiation but is actually required for terminal mTEC differentiation. The expression of involucrin was not significantly decreased in $Flg^{ft/ft}$ mice, which might suggest that this marker is upstream of filaggrin in the differentiation process. In the epidermis, mattrin is expressed in the trans-Golgi apparatus membrane of stratum granulosum keratinocytes, and it is possibly needed for the assembly of lamellar bodies[19] that contain lipids that are extruded into the intercellular space during cornification[41,42]. Thus, mattrin and filaggrin are expressed in the same differential keratinocyte stages, and it is therefore conceivable that they are expressed in the same keratinocyte-like mTEC subset in the thymus. In support of an affected mTEC differentiation, we found significantly decreased transcription of AIRE and Skint-1 in $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice. Interestingly, Skint-1 is a thymic selecting ligand for the DETC[43]. By engaging Skint-1^{+} mTEC, Vγ3^{+} thymocytes differentiate to potential IFNγ-producing DETC while suppressing the IL-17-producing capacity.

Due to the defective T cell development and affected T cell phenotype in both $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice, we expected that these mice would have an altered response to antigens compared to WT mice. In line with this, we have recently shown that flaky tail mice have an increased response to contact allergens. The enhanced response was mediated by a combination of increased allergen penetration and the IL-1/IL-17A driven spontaneous inflammation seen in flaky tail mice[29]. As both $Flg^{ft/ft}$ and $Matt^{ma/ma}$ mice have a defect skin barrier but only $Matt^{ma/ma}$ mice develop spontaneous

IL-17A driven skin inflammation, we expected that both mouse strains would have an increased response to DNFB but that the highest response would occur in *Matt*$^{ma/ma}$ mice. Interestingly, this was not the case. *Flg*$^{ft/ft}$ mice had an increased response both during sensitization and elicitation. In contrast, *Matt*$^{ma/ma}$ mice had a different response with a faster peak response both during sensitization and challenge but with a milder/comparable response, especially during challenge, compared to WT mice. We suggest that the spontaneous inflammation seen in *Matt*$^{ma/ma}$ mice might affect the generation of tissue-resident memory T cells, which are central for the response to DNFB in the contact hypersensitivity model reported here[44].

Mutations in filaggrin have been shown to increase the risk of viral skin infections[34,45]. Interestingly, we found an increased viral load in the nose and throat of *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice compared to WT mice on day 3 and 4 after intranasal inoculation with Sendai virus. In addition, *Matt*$^{ma/ma}$ mice also had an increased virus load in the lungs compared to both *Flg*$^{ft/ft}$ and WT mice on day 3 and 4 after intranasal inoculation with Sendai virus. Production of IL-17A seems to mediate the increased risk of developing eczema vaccinatum upon cutaneous inoculation with vaccinia virus in flaky tail mice[34]. In the present study, the *ma* mutation seemed to be associated with increased risk of developing a more severe infection with Sendai virus compared to the *ft* mutation, most likely due to the IL-17A skewed T cell profile found in the *Matt*$^{ma/ma}$ mice.

Currently, mutations in *Flg* are associated with skin diseases. Yet, our data suggest that filaggrin is not just an important skin barrier protein, but also affects the thymic microenvironment and thus T cell development with an impact on peripheral immunity. Approximately 10 % of the European population are carriers of a filaggrin mutation, which makes it important to uncover still unknown

effects of the mutation. Furthermore, while filaggrin is well-studied in the epidermis, very few studies have investigated the importance of mattrin. We suspect that mutations in mattrin are common among patients with inflammatory skin conditions and maybe also in patients with increased systemic inflammation, however, future studies are needed to answer these questions.

Conflicts of interest

We declare no conflicts of interest.

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Figure legends

Figure 1. Increased numbers of epidermal αβ T cells in *Matt*$^{ma/ma}$ mice and decreased numbers of epidermal γδ T cells in *Flg*$^{ft/ft}$ mice. A. Images of C57Bl/6, *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice. **B.-C.** Flow cytometric analyses of epidermal cells from 8-12 week old mice. **B.** Fraction TCRγδ and TCRβ T cells (left) and absolute numbers (right) gated from single, viable cells. **C.** Numbers of CD4$^+$TCRβ$^+$, CD8$^+$TCRβ$^+$, Vγ1.1$^+$TCRγδ$^+$, Vγ2$^+$TCRγδ$^+$ and Vγ3$^+$TCRγδ$^+$ T cells. **D.** Confocal microscopy images of epidermal ear sheets stained for TCRγδ, CD4, CD8 and DAPI.

Figure 2. Increased numbers of epidermal IL-17-producing T cells in *Matt*$^{ma/ma}$ mice. A-D. Flow cytometric analyses of epidermal cells from 8-12 week old mice. **A.** Fraction (left) and number (right) of single, viable TCRγδ$^+$ cells producing either IL-17A or IFNγ. **B.** TCRγδ$^+$ cells further gated for individual Vγ-chains and subsequently IL-17A. Graphs depict numbers of TCRγδ$^+$Vγ1.1$^+$IL-17A$^+$, TCRγδ$^+$Vγ2$^+$IL-17A$^+$, TCRγδ$^+$Vγ3$^+$IL-17A$^+$ and other TCRγδ$^+$IL-17A$^+$ cells. The last category is calculated from TCRγδ$^+$IL-17A$^+$ cells that do not express Vγ1.1, Vγ2 or Vγ3. **C.** Fraction (left) and number (right) of single, viable TCRβ$^+$ cells producing either IL-17A or IFNγ. **D.** TCRβ$^+$ cells further gated for CD4 and CD8 and subsequently IL-17A and IFNγ. Graph depicts numbers of CD4$^+$TCRβ$^+$IL-17A$^+$, CD8$^+$TCRβ$^+$IL-17A$^+$, CD4$^+$TCRβ$^+$IFNγ$^+$, CD8$^+$TCRβ$^+$IFNγ$^+$ cells.

Figure 3. Increased numbers of splenic T cells producing IFNγ in both *Matt*$^{ma/ma}$ and *Flg*$^{ft/ft}$ mice. A-D. Flow cytometric analyses of splenic cells from 8-12 week old mice. **A.** Fraction (left) and number (right) of single, viable TCRγδ$^+$ or TCRβ$^+$ splenic cells. **B.** TCRγδ$^+$ cells further gated for individual Vγ-chains. Graphs depict numbers of TCRγδ$^+$Vγ1.1$^+$, TCRγδ$^+$Vγ2$^+$, TCRγδ$^+$Vγ3$^+$ and

other TCRγδ$^+$ cells. Numbers of other TCRγδ$^+$ cells are calculated from TCRγδ$^+$ cells that do not express Vγ1.1, Vγ2 or Vγ3. **C-D.** TCRγδ$^+$ cells from A. were further gated for IL-17A or IFNγ and TCRβ$^+$ cells were further gated for CD4 and CD8 and subsequently for IL-17A and IFNγ. **C.** Graphs depict numbers of TCRγδ$^+$IL-17A$^+$, CD4$^+$TCRβ$^+$IL-17A$^+$ and CD8$^+$TCRβ$^+$IL-17A$^+$ cells. **D.** Graphs depict numbers of TCRγδ$^+$IFNγ$^+$, CD4$^+$TCRβ$^+$ IFNγ$^+$ and CD8$^+$TCRβ$^+$ IFNγ$^+$ cells.

Figure 4. Increased numbers of thymic IL-17-producing T cells in *Matt$^{ma/ma}$* mice and increased numbers of thymic IFNγ-producing T cells in *Flg$^{ft/ft}$* mice. A-G. Flow cytometric analyses of thymic cells from 8-12 week old mice. Fraction (**A.**) and number (**B.**) of single, viable CD4$^+$, CD8$^+$, CD4$^+$CD8$^+$ (DP) and CD4$^-$CD8$^-$ (DN) thymic cells. Fraction (**C.**) and number (**D.**) of single, viable TCRγδ$^+$ or TCRβ$^+$ thymic cells. **E.** TCRγδ$^+$ cells further gated for individual Vγ-chains. Graphs depict numbers of TCRγδ$^+$Vγ1.1$^+$, TCRγδ$^+$Vγ2$^+$, TCRγδ$^+$Vγ3$^+$ and other TCRγδ$^+$ cells. Numbers of other TCRγδ$^+$ cells are calculated from TCRγδ$^+$ cells that do not express Vγ1.1, Vγ2 or Vγ3. **F-G.** TCRγδ$^+$ cells from C. were further gated for IL-17A or IFNγ and TCRβ$^+$ cells were further gated for CD4 and CD8 and subsequently for IL-17A and IFNγ. **F.** Graphs depict numbers of TCRγδ$^+$IL-17A$^+$, CD4$^+$TCRβ$^+$IL-17A$^+$ and CD8$^+$TCRβ$^+$IL-17A$^+$ cells. **G.** Graphs depict numbers of TCRγδ$^+$IFNγ$^+$, CD4$^+$TCRβ$^+$ IFNγ$^+$ and CD8$^+$TCRβ$^+$ IFNγ$^+$ cells.

Figure 5. Decreased transcription of keratinocyte-like mTEC markers in *Matt$^{ma/ma}$* and *Flg$^{ft/ft}$* mice. A-C. Quantitative real-time PCR on whole thymus samples on **A.** *Flg* and *Matt*, **B.** *KRT10*, *IVL* and *LOR* and **C.** *AIRE* and *Skint1*. Transcription is normalized to GAPDH.

Figure 6. *Flg$^{ft/ft}$* mice have an increased response to the experimental contact allergen DNFB. **A.** Mice were sensitized on the dorsal aspect of their ears with 0.15 % DNFB or OOA as control for

three consecutive days. On day 21, mice were challenged and ear thickness was measured as indicated with +. **B-D.** Graph depicts ear thickness of the sensitization (left) and challenge (right) phases for C57Bl/6, *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice treated with DNFB (red) or OOA (black).

Figure 7. Both *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$ mice exhibit increased viral loads following infection with Sendai virus. A. On day 0, mice were inoculated i.n. with 7000 PFU of recombinant Sendai virus with a firefly luciferase insert. On day 1-9 mice were injected i.p. with luciferin and following 15 minutes incubation mice were anesthetized and analyzed by IVIS. Bioluminescence was detected in regions of interest, ROIs (right). **B.** Visualization of bioluminescence in a given mouse from day 1-9. Bioluminescence in units of radiance in ROIs around nose (**C.**), throat (**D.**) and lung (**E.**). For statistical analyses multiple t-tests were performed and significance was determined using the Holm-Sidak method with α=0.05 (adjusted p-value for multiple testing). Statistically significant differences are denoted *<0.05, **<0.01, ***<0.001. Red stars denote significant differences between *Matt*$^{ma/ma}$ and WT, whereas green stars denote differences between *Flg*$^{ft/ft}$ and WT.

Figure S1. Strategy for backcrossing and separating mutations in flaky tail mice. Flaky tail mice were crossed with C57Bl/6 mice to create heterozygous flaky tail$^{ft/+\ ma/+}$ mice. Further backcrossing and genotyping of these mice was performed until we had heterozygous *Flg*$^{ft/+}$ and *Matt*$^{ma/+}$ backcrossed onto the C57Bl/6 background for 5 generations. *Flg*$^{ft/+}$ were then crossed *Flg*$^{ft/+}$ and *Matt*$^{ma/+}$ with *Matt*$^{ma/+}$ until we had homozygous *Flg*$^{ft/ft}$ *and Matt*$^{ma/ma}$ mice.

Figure S2. Gating strategy for epidermal T cells. A. Single, viable lymphocytes were further gated for TCR$\gamma\delta$ and TCRβ. TCR$\gamma\delta^+$ cells were directly gated for IL-17A and IFNγ, whereas TCRβ^+ cells were first gated for CD4 and CD8 and subsequently for IL-17A and IFNγ. **B.** Single,

viable lymphocytes were gated for TCRγδ and either directly for IL-17A and IFNγ or first for each Vγ-chain and subsequently for IL-17A and IFNγ.

Figure S3. Gating strategy and additional results from spleen. A. Single, viable lymphocytes were further gated for TCRγδ and TCRβ. TCRγδ$^+$ cells were directly gated for IL-17A and IFNγ, whereas TCRβ$^+$ cells were first gated for CD4 and CD8 and subsequently for IL-17A and IFNγ. **B.** Graphs depict number of single, viable CD4$^+$TCRβ$^+$ and CD8$^+$TCRβ$^+$ T cells. **C.** Plots show fractions of TCRγδ$^+$ (top row), CD4$^+$TCRβ$^+$ (middle row) and CD8$^+$TCRβ$^+$ (bottom row) T cells producing either IL-17A or IFNγ.

Figure S4. Gating strategy and additional results from thymus. A. Single, viable lymphocytes were further gated for CD4 and CD8 or TCRγδ and TCRβ. The CD4-CD8- population was further gated for CD25 and CD44, whereas TCRβ$^+$ cells were further gated for CD4 and CD8. **B.** Graphs depict numbers of DN1 (CD44$^+$CD25$^-$), DN2 (CD44$^+$CD25$^+$), DN3 (CD44$^-$CD25$^+$) and DN4 (CD44$^-$CD25$^-$) cells. C. Graphs depict total numbers of CD4$^+$TCRβ$^+$ and CD8$^+$TCRβ$^+$ cells. **D.** Plots show fractions of TCRγδ$^+$ (top row), CD4$^+$TCRβ$^+$ (middle row) and CD8$^+$TCRβ$^+$ (bottom row) T cells producing either IL-17A or IFNγ.

Figure 1

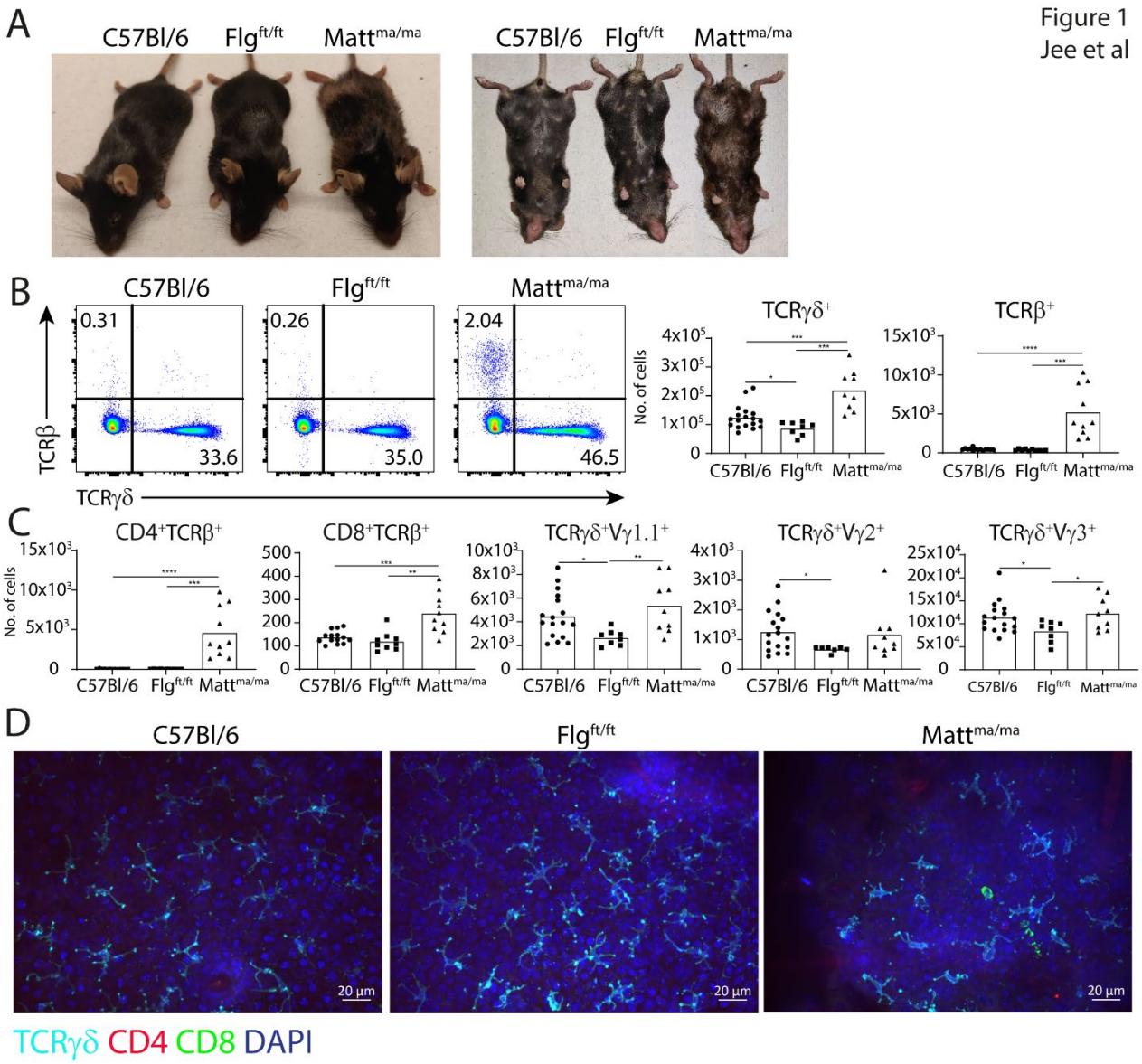
A
C57Bl/6
Flg^ft/ft
Matt^ma/ma
C57Bl/6
Flg^ft/ft
Matt^ma/ma
Figure 1
Jee et al
B
C57Bl/6
Flg^ft/ft
Matt^ma/ma
0.31
33.6
0.26
35.0
2.04
46.5
TCRβ
TCRγδ
TCRγδ+
TCRβ+
No. of cells
C
CD4+TCRβ+
CD8+TCRβ+
TCRγδ+Vγ1.1+
TCRγδ+Vγ2+
TCRγδ+Vγ3+
No. of cells
D
C57Bl/6
Flg^ft/ft
Matt^ma/ma
20 µm
TCRγδ CD4 CD8 DAPI

Figure 2

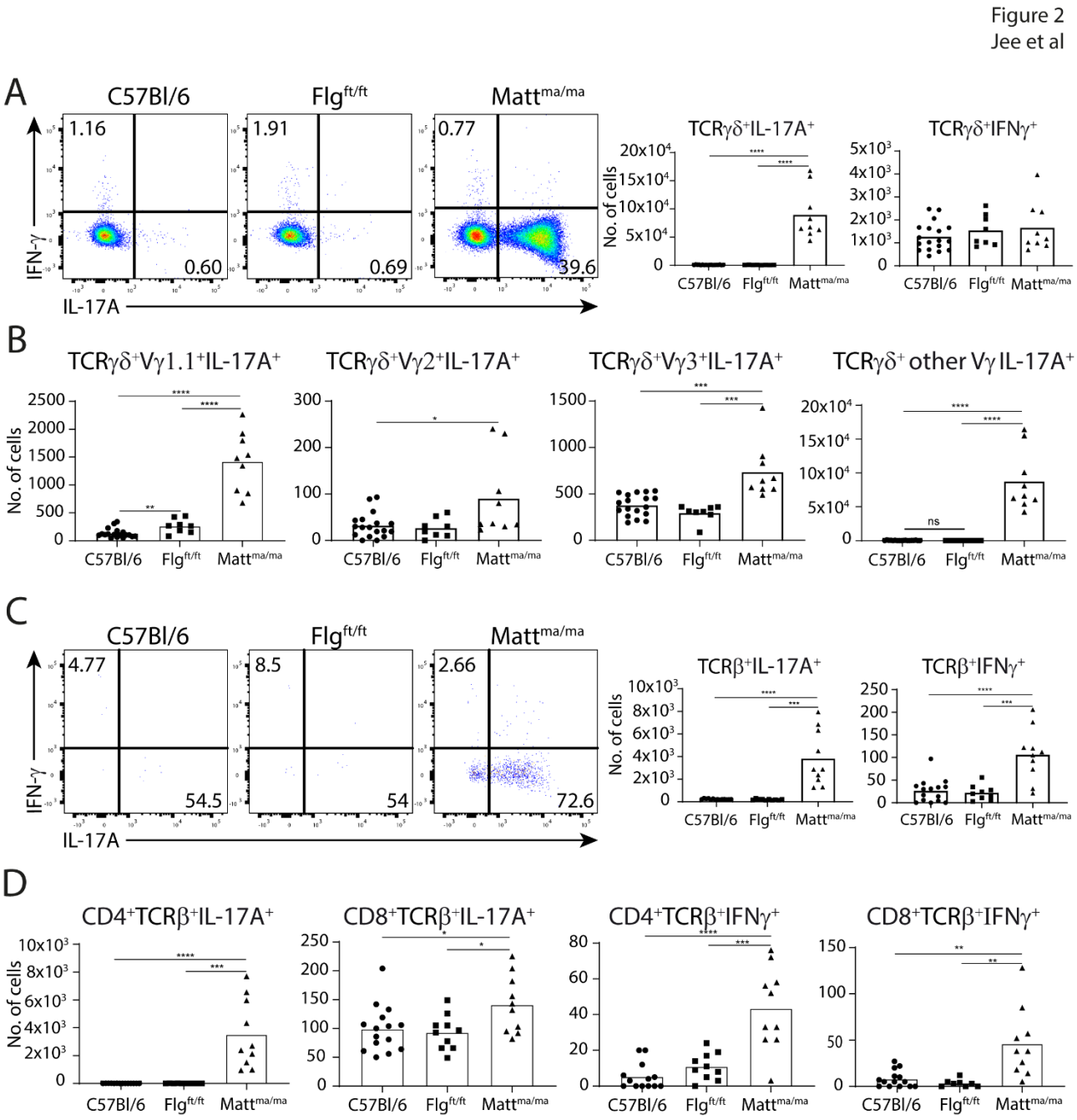
Figure 2
Jee et al
A
C57Bl/6
Flg^ft/ft
Matt^ma/ma
1.16
0.60
1.91
0.69
0.77
39.6
IFN-γ
IL-17A
TCRγδ+IL-17A+
TCRγδ+IFNγ+
No. of cells
B
TCRγδ+Vγ1.1+IL-17A+
TCRγδ+Vγ2+IL-17A+
TCRγδ+Vγ3+IL-17A+
TCRγδ+ other Vγ IL-17A+
C
4.77
54.5
8.5
54
2.66
72.6
TCRβ+IL-17A+
TCRβ+IFNγ+
D
CD4+TCRβ+IL-17A+
CD8+TCRβ+IL-17A+
CD4+TCRβ+IFNγ+
CD8+TCRβ+IFNγ+

Figure 3

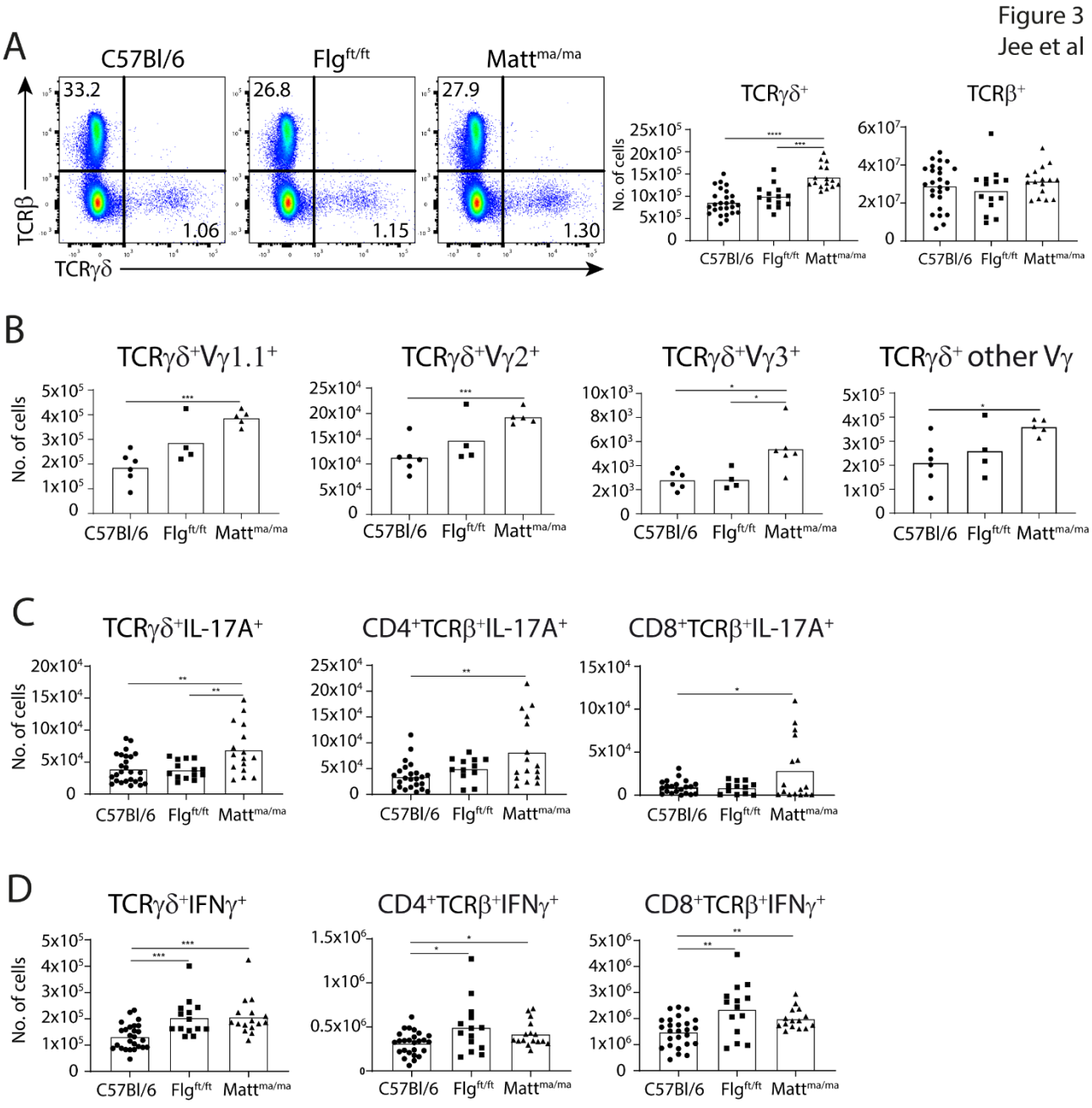
Figure 3
Jee et al
A
C57Bl/6
Flg^ft/ft
Matt^ma/ma
33.2
26.8
27.9
1.06
1.15
1.30
TCRβ
TCRγδ
TCRγδ+
TCRβ+
No. of cells
B
TCRγδ+Vγ1.1+
TCRγδ+Vγ2+
TCRγδ+Vγ3+
TCRγδ+ other Vγ
C
TCRγδ+IL-17A+
CD4+TCRβ+IL-17A+
CD8+TCRβ+IL-17A+
D
TCRγδ+IFNγ+
CD4+TCRβ+IFNγ+
CD8+TCRβ+IFNγ+

Figure 4

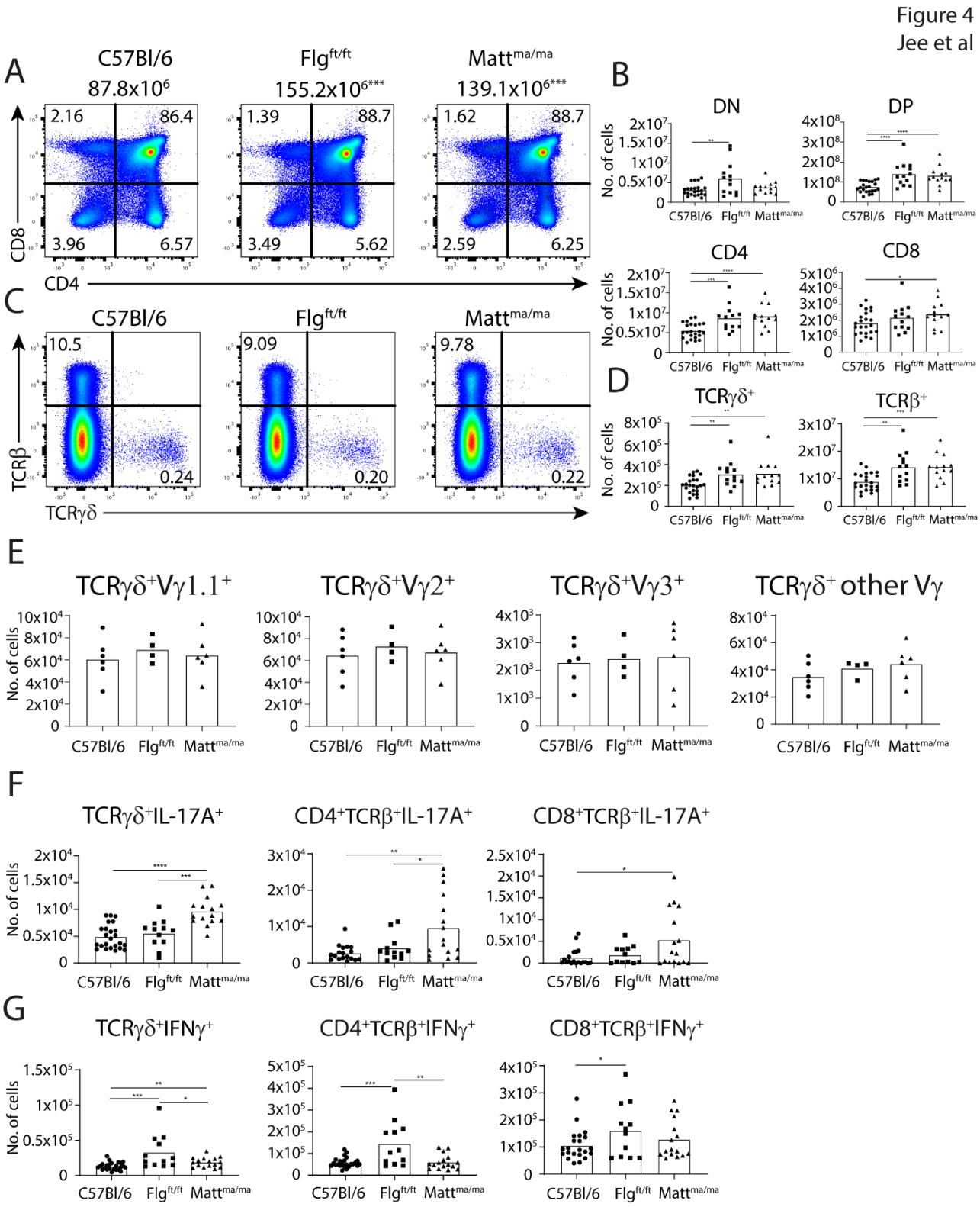
Figure 4
Jee et al
A
C57Bl/6
87.8x10⁶
Flg^ft/ft
155.2x10⁶***
Matt^ma/ma
139.1x10⁶***
2.16
86.4
3.96
6.57
1.39
88.7
3.49
5.62
1.62
88.7
2.59
6.25
CD8
CD4
B
DN
DP
CD4
CD8
No. of cells
C57Bl/6
Flg^ft/ft
Matt^ma/ma
C
C57Bl/6
Flg^ft/ft
Matt^ma/ma
10.5
0.24
9.09
0.20
9.78
0.22
TCRβ
TCRγδ
D
TCRγδ⁺
TCRβ⁺
No. of cells
E
TCRγδ⁺Vγ1.1⁺
TCRγδ⁺Vγ2⁺
TCRγδ⁺Vγ3⁺
TCRγδ⁺ other Vγ
No. of cells
F
TCRγδ⁺IL-17A⁺
CD4⁺TCRβ⁺IL-17A⁺
CD8⁺TCRβ⁺IL-17A⁺
No. of cells
G
TCRγδ⁺IFNγ⁺
CD4⁺TCRβ⁺IFNγ⁺
CD8⁺TCRβ⁺IFNγ⁺
No. of cells

Figure 5

Figure 5
Jee et al

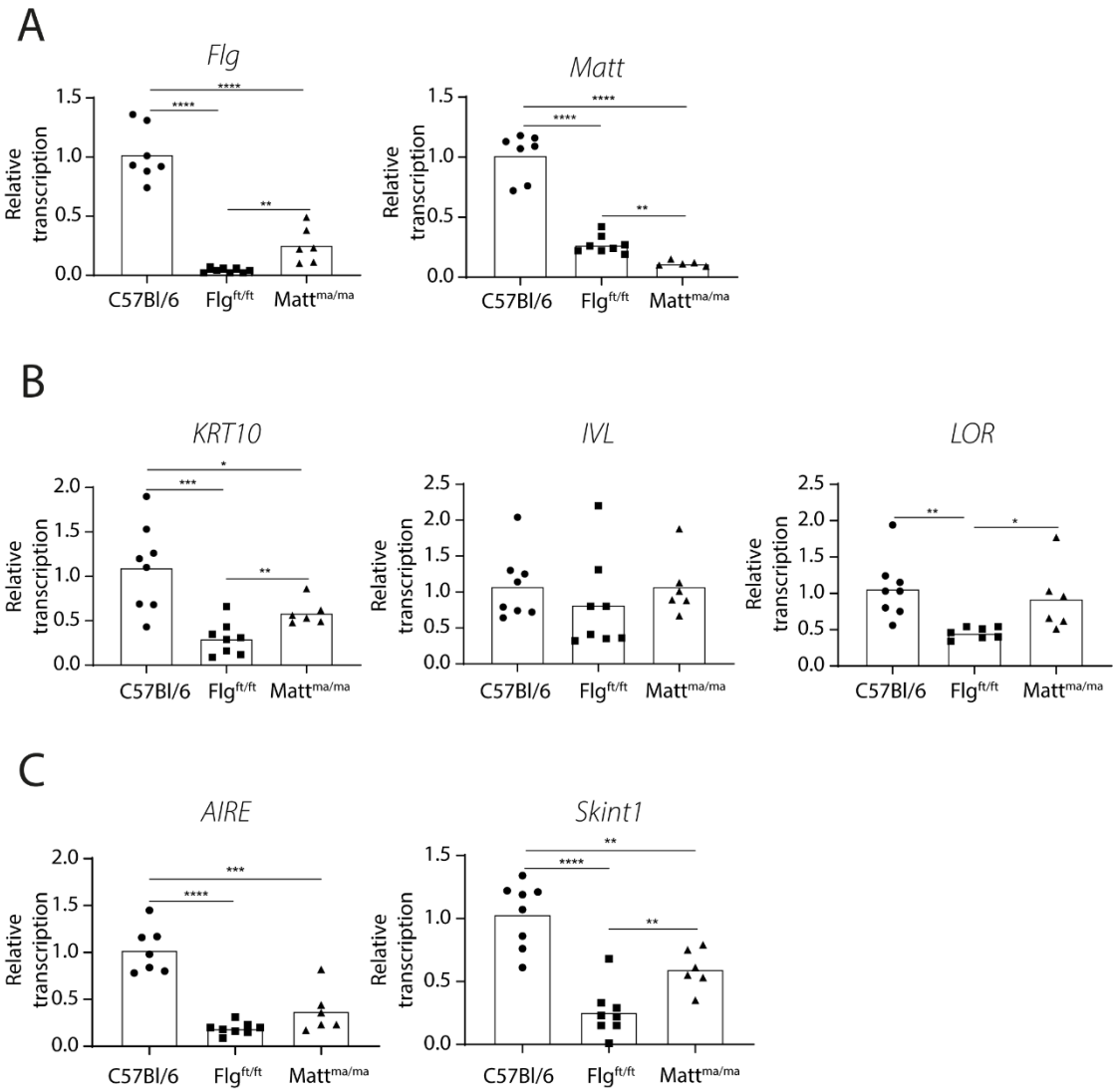

Figure 6

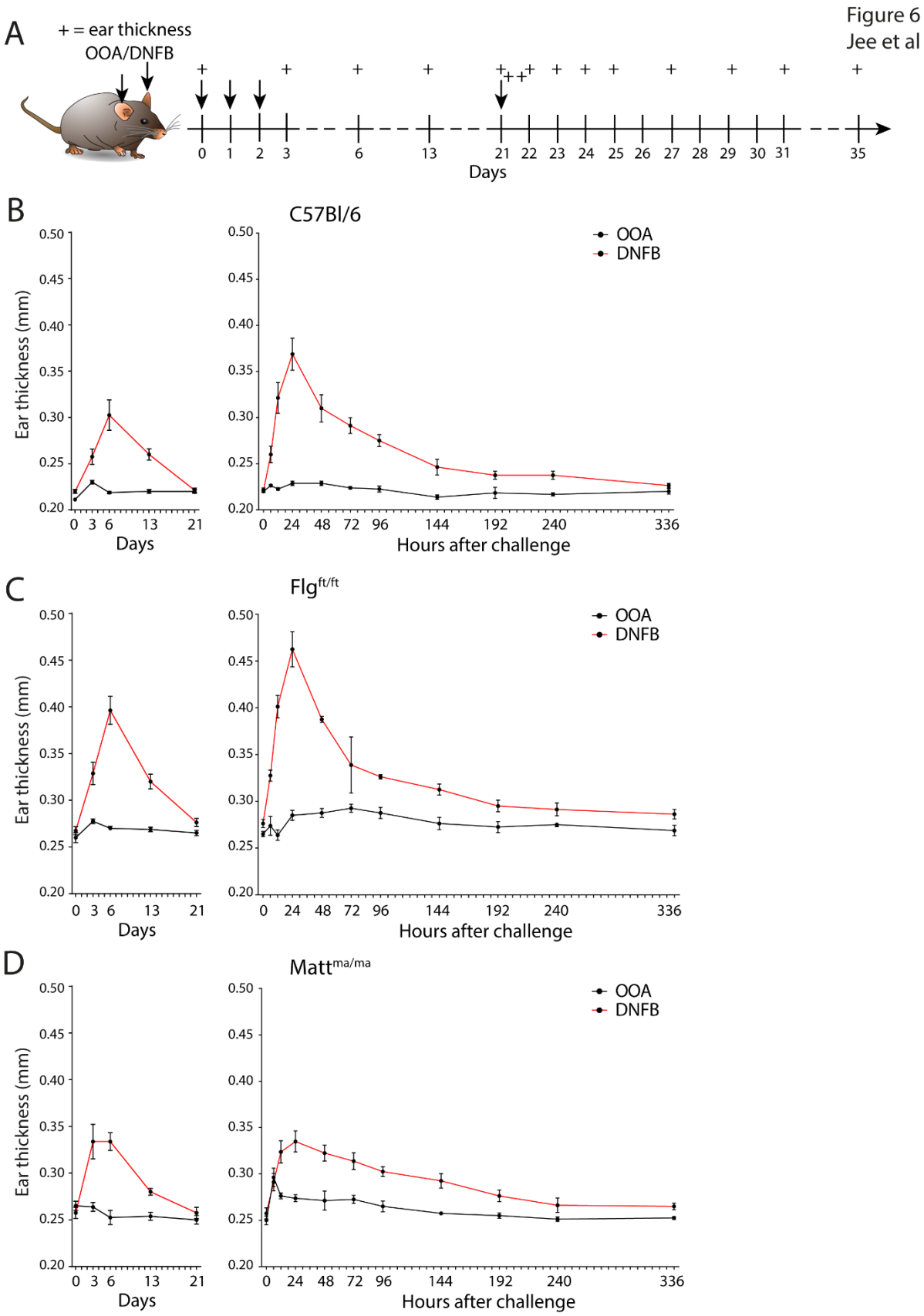
Figure 6
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A
+ = ear thickness
OOA/DNFB
0 1 2 3 6 13 21 22 23 24 25 26 27 28 29 30 31 35
Days
B
C57Bl/6
OOA
DNFB
Ear thickness (mm)
0.50 0.45 0.40 0.35 0.30 0.25 0.20
0 3 6 13 21
Days
0 24 48 72 96 144 192 240 336
Hours after challenge
C
Flg^ft/ft
OOA
DNFB
Ear thickness (mm)
Days
Hours after challenge
D
Matt^ma/ma
OOA
DNFB
Ear thickness (mm)
Days
Hours after challenge

Figure 7

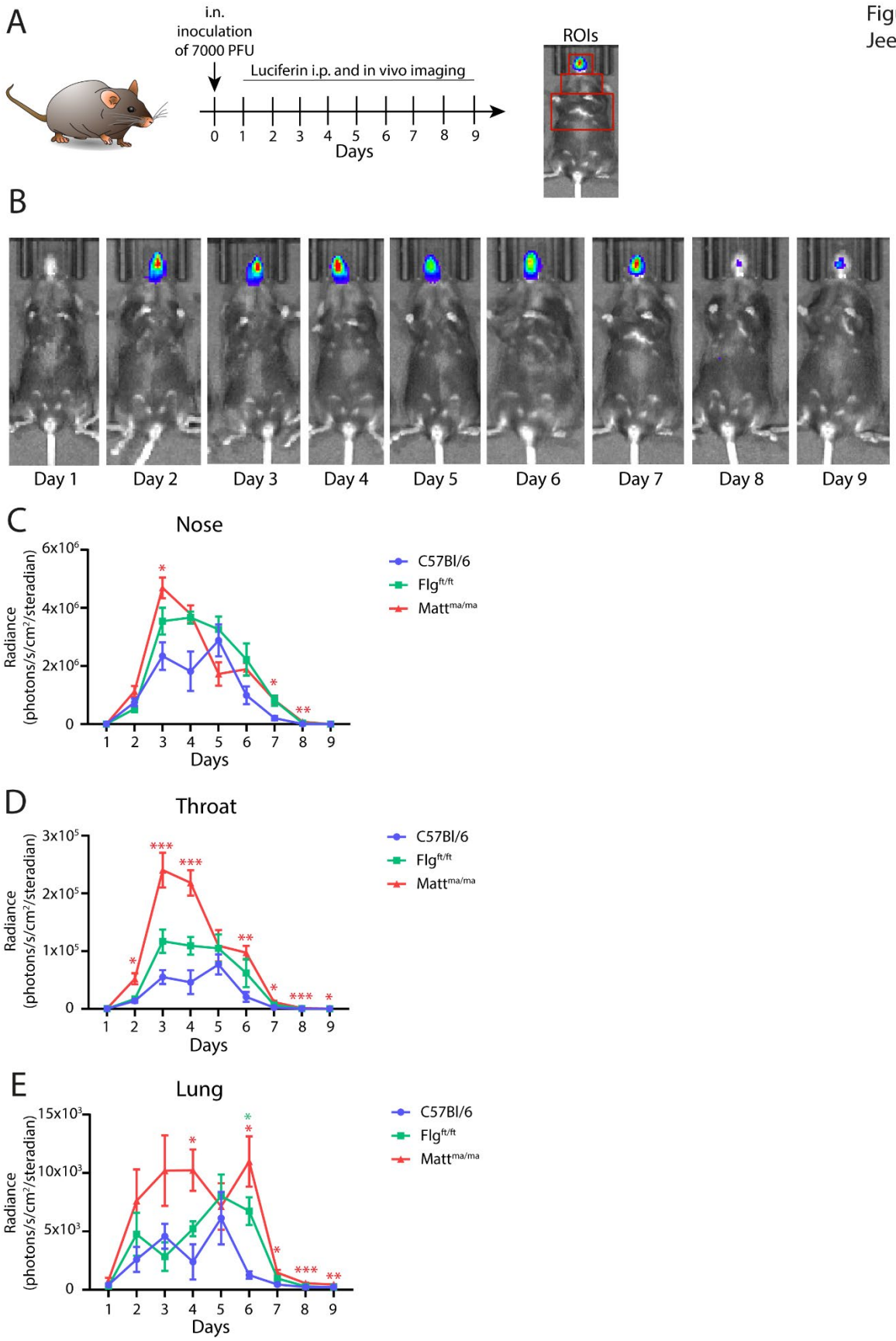

Figure 7
Jee et al

Figure S1

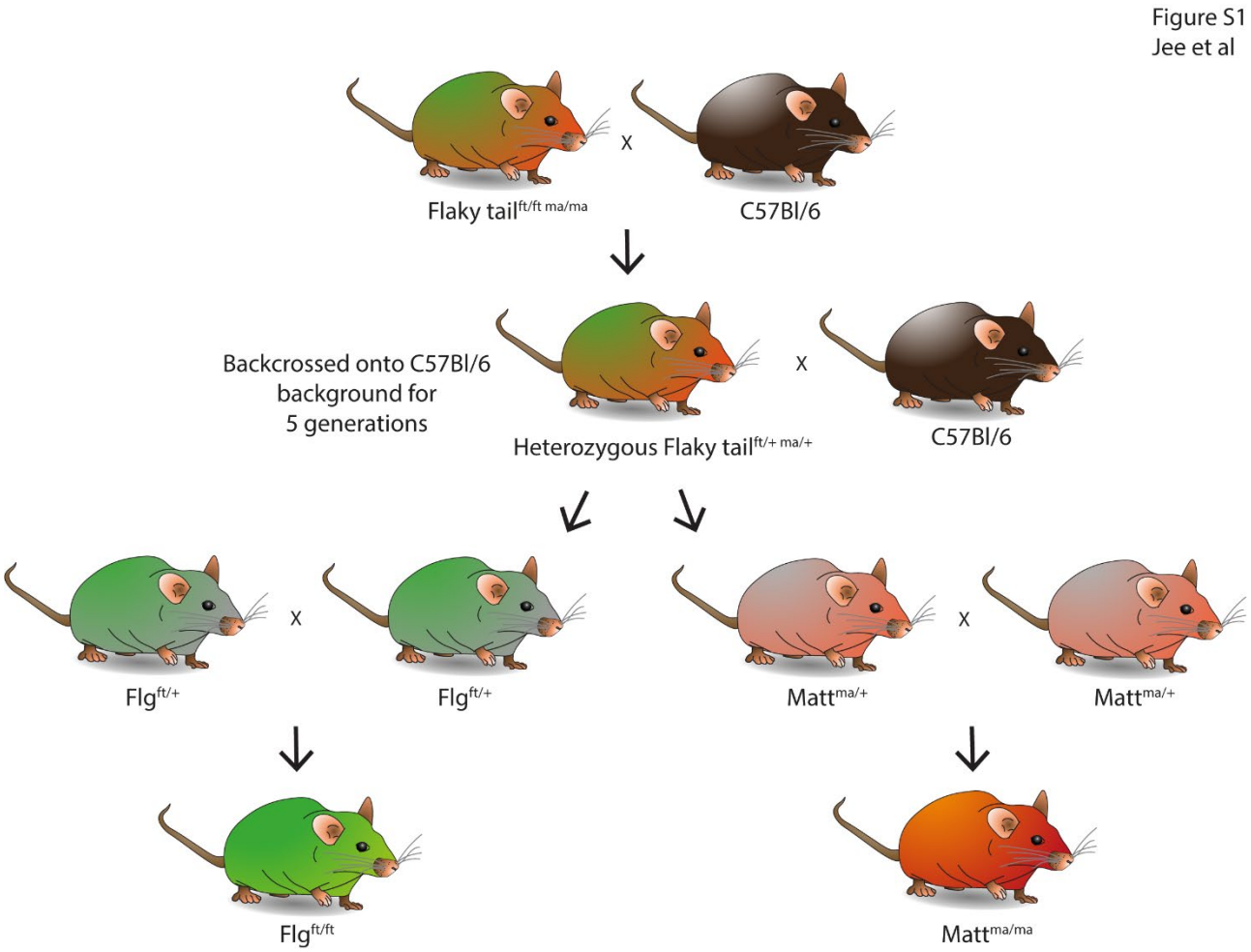
Figure S1
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Flaky tail ft/ft ma/ma
X
C57Bl/6
Backcrossed onto C57Bl/6
background for
5 generations
Heterozygous Flaky tail ft/+ ma/+
X
C57Bl/6
Flg ft/+
X
Flg ft/+
Matt ma/+
X
Matt ma/+
Flg ft/ft
Matt ma/ma

Figure S2

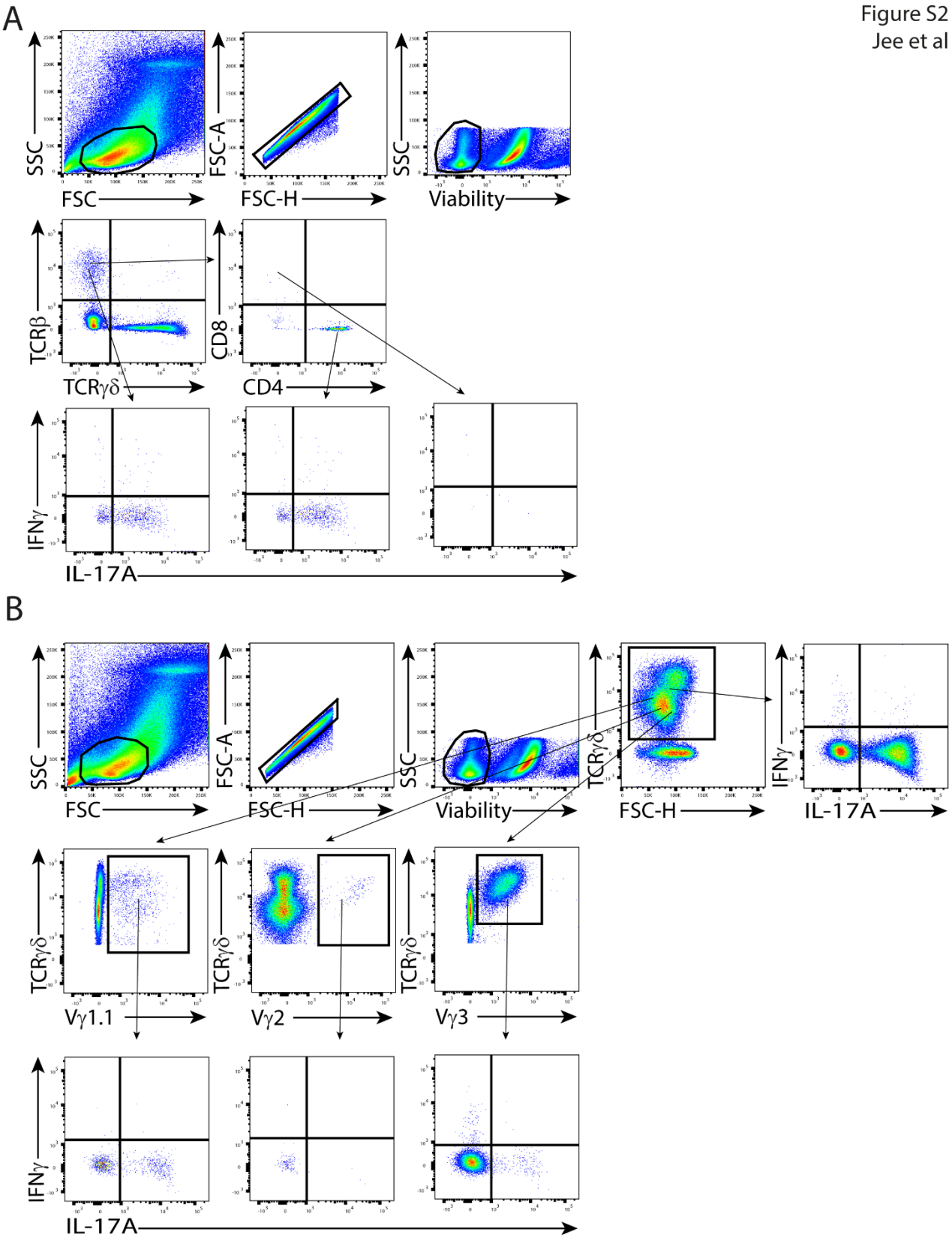

Figure S2
Jee et al

Figure S3

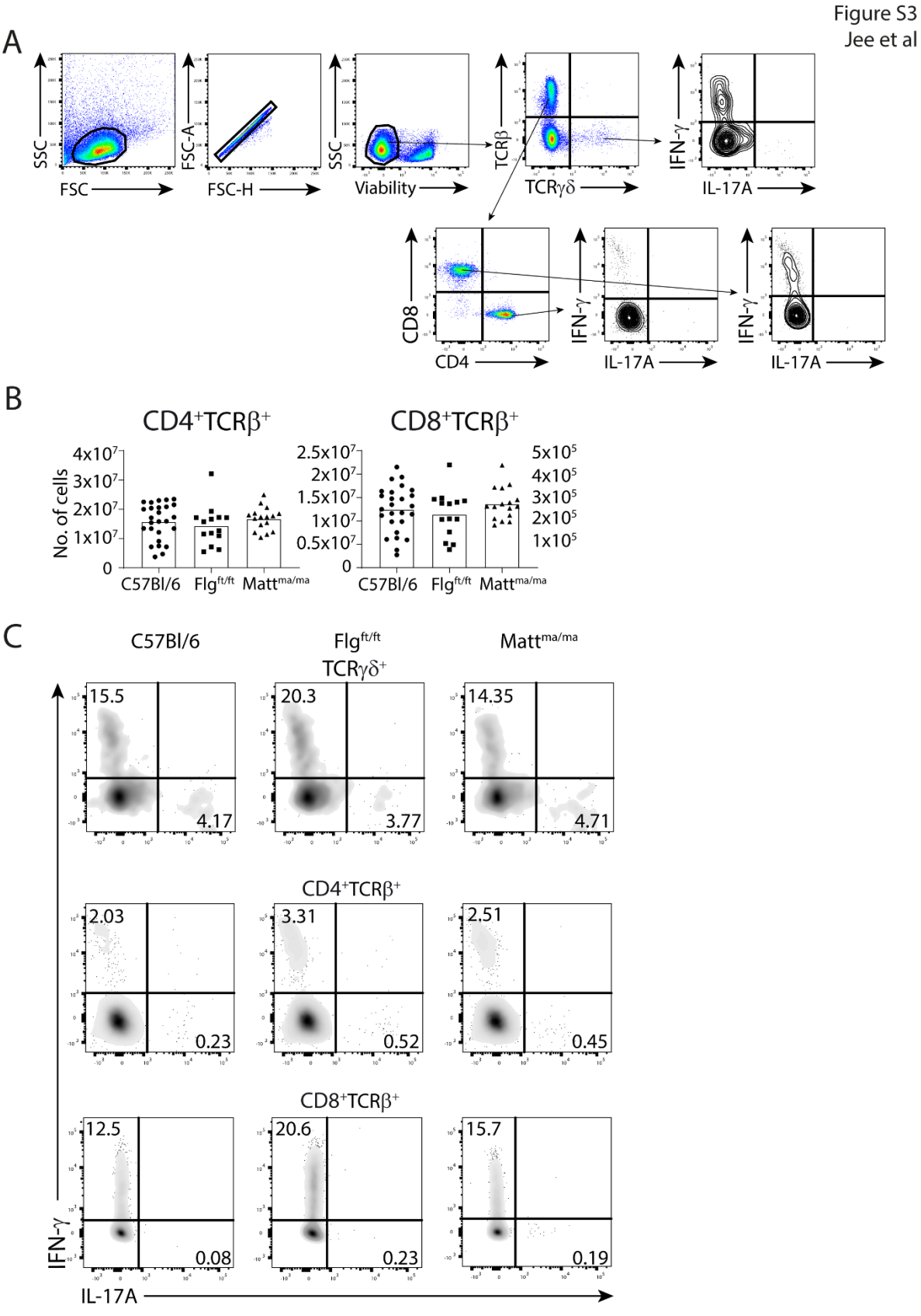
Figure S3
Jee et al
A
SSC
FSC
FSC-A
FSC-H
SSC
Viability
TCRβ
TCRγδ
IFN-γ
IL-17A
CD8
CD4
IFN-γ
IL-17A
IFN-γ
IL-17A
B
CD4+TCRβ+
CD8+TCRβ+
No. of cells
4x10^7
3x10^7
2x10^7
1x10^7
0
2.5x10^7
2x10^7
1.5x10^7
1x10^7
0.5x10^7
0
5x10^5
4x10^5
3x10^5
2x10^5
1x10^5
C57Bl/6
Flg^ft/ft
Matt^ma/ma
C57Bl/6
Flg^ft/ft
Matt^ma/ma
C
C57Bl/6
Flg^ft/ft
Matt^ma/ma
TCRγδ+
15.5
4.17
20.3
3.77
14.35
4.71
CD4+TCRβ+
2.03
0.23
3.31
0.52
2.51
0.45
CD8+TCRβ+
12.5
0.08
20.6
0.23
15.7
0.19
IFN-γ
IL-17A

Figure S4

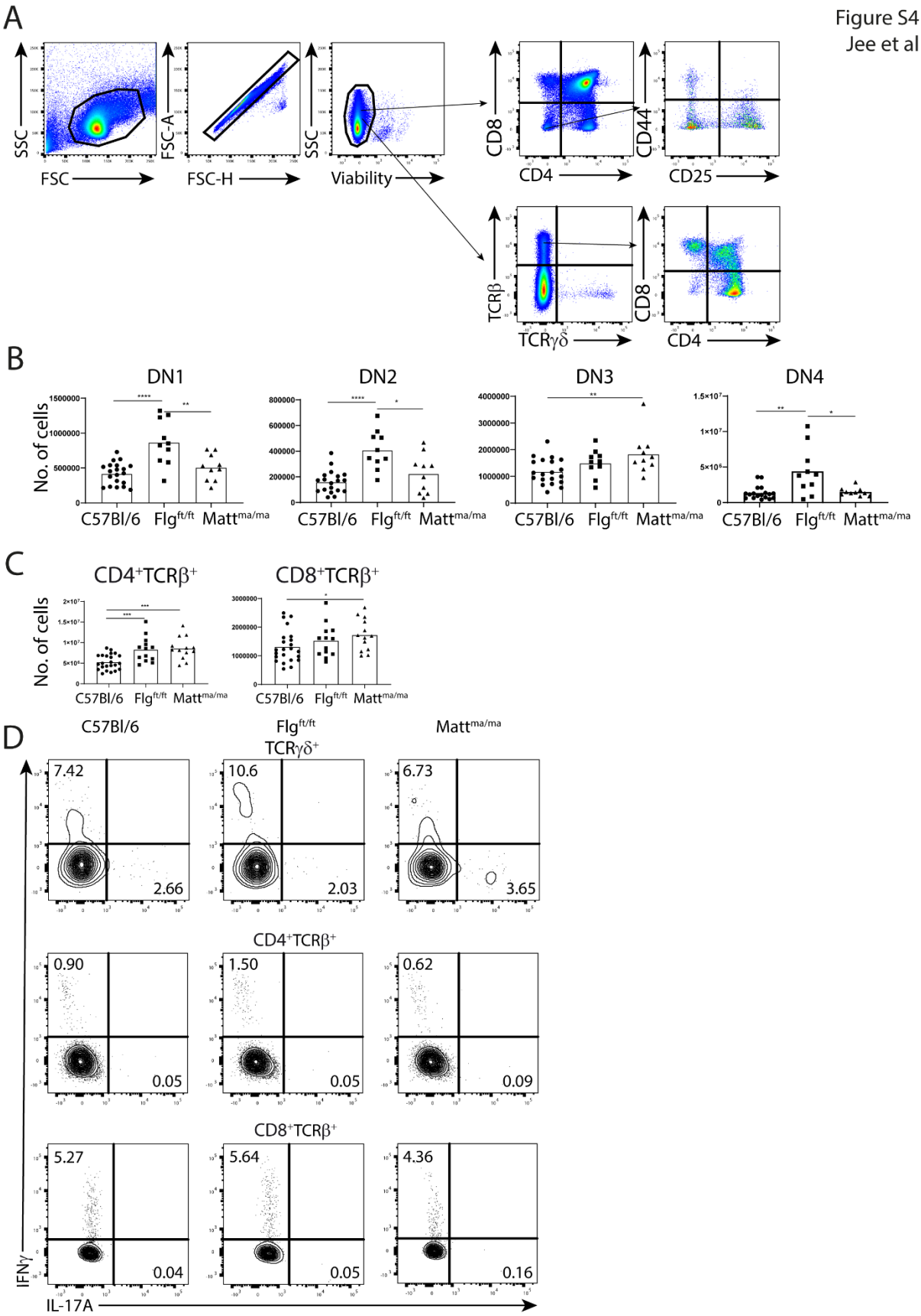

Figure S4
Jee et al

Manuscript III

Sensitization to contact allergens induces development and export of IL-17A-producing innate CD8$^+$ T cells from the thymus

Sensitization to contact allergens induces development and export of IL-17A-producing CD8$^+$ T cells from the thymus

Mia H. Jee[1,2], Gustav Ø. Jørgensen[1], Julie F. Weber[1], Anders Woetmann[1], Niels Ødum[1], Jeanne D. Johansen[2], Carsten Geisler[1], and Charlotte M. Bonefeld[1]

[1]The LEO Foundation Skin Immunology Research Center, Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
[2]Department of Dermatology and Allergy, National Allergy Research Centre, Copenhagen University Hospital Gentofte, Denmark

Corresponding author:
Charlotte Menné Bonefeld, The LEO Foundation Skin Immunology Research Center, Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark.
E-mail: cmenne@sund.ku.dk. Phone: +45 3532 7866.

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Abstract

Whereas conventional T cells develop and leave the thymus as naïve T cells and further differentiate to effector cells in the periphery, non-conventional innate-like T cells largely gain their effector functions in the thymus. Although γδ T cells make up a large part of the non-conventional T cells, several studies have described the existence of αβ T cell subsets with innate phenotypes in the thymus. It has been suggested that systemic infection and inflammation can drive an increased development of innate αβ T cells in thymus. However, it is not known whether local, limited inflammations affect the development of innate αβ T cells. In this study, we investigated if local skin inflammation induced by exposure to the experimental contact allergen 1-fluoro-2,4-dinitrobenzene (DNFB) affects the development of innate αβ T cells in thymus. We found that sensitization with DNFB on the ears increased the numbers of IL-17A-producing $CD8^+$ αβ T cells in the thymus. Furthermore, we found an increased number of IL-17A-producing $CD45RB^{lo}CD8^+$ αβ T cells in the spleen of mice sensitized to DNFB. In conclusion, this study indicates that local skin inflammation as seen in allergic contact dermatitis is able to affect T development.

Introduction

T cell development takes place in the thymus. Immature $CD4^-CD8^-$ lymphoid progenitors enter the thymus from the bone marrow via the circulation. The progenitors can develop into different lineages of T cells through diverse developmental pathways that are regulated by the thymic microenvironment[1]. The majority of T cells generated in the thymus are conventional αβ T cells. At the double positive (DP) stage the thymocytes express both CD4 and CD8 co-receptors. The DP thymocytes further develop into either $CD4^+$ or $CD8^+$ single positive αβ T cells, if they survive both the positive and negative selection that ensure recognition of self-MHC-molecules and deletion of autoreactive T cells, respectively[2]. The conventional $CD4^+$ or $CD8^+$ αβ T cells leave the thymus as naïve T cells that upon recognition of their specific antigen in secondary lymphoid organs become activated and differentiate to effector T cells.

In addition to conventional αβ T cells, more innate-like T cell lineages develop in the thymus. The best characterized of these are γδ T cells and NKT cells. In addition, it has been shown that both $CD4^+$ and $CD8^+$ αβ T cells with an innate phenotype develop in the thymus[3–6]. The development of innate $CD8^+$ αβ T cells was first described in different mouse strains deficient in T cell signaling molecules or transcription factors[5]. The innate $CD8^+$ αβ T cells were characterized by being $CD44^{high}$ and by their rapid production of IFN-γ upon T cell receptor (TCR) triggering, a characteristic normally attributed to memory $CD8^+$ αβ T cells[5]. Subsequently, innate $CD8^+$ αβ T cells were also found to develop in wild-type (WT) mice[6]. It was shown that 10% of $CD8^+$ αβ T cells that develop in WT mice have the innate phenotype[6]. In addition, innate "natural" $CD4^+$ αβ T cells that produce IL-17 (nTh17) develop and mature in the thymus of WT mice but at a lower frequency than innate $CD8^+$ αβ T cells[3,4]. It was recently shown that infection with *Trypanosoma cruzi* that induce a Th1-driven immune response, alters $CD8^+$ αβ T cell development leading to the development of $CD8^+$ αβ T cell with the innate phenotype. Interestingly, a similar phenotype could be induced in

mice treated with IL-12 and IL-18[7]. However, whether local immune responses affect T cell development is unknown.

Allergic contact dermatitis (ACD) is one of the most frequent inflammatory skin diseases with approximately 20% of the European population being sensitized to one or more contact allergens[8]. ACD is induced upon exposure of the skin to contact allergens like metals, fragrances or preservatives and is a T cell mediated response facilitated by both $CD4^+$ and $CD8^+$ αβ T cells[8,9]. The main effector cytokines produced by T cells in response to contact allergens are IFN-γ and IL-17A[10–14]. Innate immune responses in the skin play important roles in the initiation of ACD[15]. Interestingly, both IL-12 and IL-18 are produced by skin-resident cells in the response to contact allergens and both cytokines are central in the initiation of the response[16–19]. We therefore speculated whether sensitization to contact allergens might induce an altered T cell development with maturation of innate T cell subsets. To investigate this, we used a mouse model for ACD where mice were sensitized with the experimental contact allergen 1-fluoro-2,4-dinitrobenzene (DNFB). In this study, we found an increased number of IL-17-producing $CD8^+$ αβ T cells in the thymus of mice exposed to DNFB on their ears. Furthermore, we found an increased number of IL-17-producing $CD8^+$ αβ T cells with a recent emigrant phenotype in the spleen of these mice.

Materials and Methods

Mice

C57Bl/6J mice were purchased from Janvier Laboratories (Le Genest-Saint-Isle, France) and housed in specific pathogen free conditions at the animal facility at The Department of Experimental Medicine, The Panum Institute, in accordance with the national animal protection guidelines (license number 2018-15-0201-01409). Experiments were performed on animals aged 8-12 weeks. Mice were sensitized with the experimental allergen 1-fluoro-2,4-dinitrobenzene (DNFB) by painting each ear with 25 µl of 0.15 % DNFB in 1:3 olive oil:acetone (OOA) for three consecutive days (day 0-2). OOA was used as control. Mice were euthanized on day 6 and ear thickness was measured and thymus and spleen were harvested (Figure 1A). In some experiments, mice were treated with FTY720 (2.5 µg/ml) in their drinking water from day 0-7, while being painted with either DNFB or OOA from day 2-4 (Figure 2A).

Single-cell suspensions

Spleens and thymi were dissociated on 70 µm cell strainers, and subsequently single cells were washed in RPMI medium (10% FBS, 0.5 IU/L penicillin, 500 mg/L streptomycin, 1% L-glutamine) and adjusted to 2 x 10^7 cells/ml. Cells were plated in a 96-well round-bottomed plate with two million cells per well.

Staining and flow cytometry

Fc-receptors were blocked using anti-CD16/32. Surface markers were stained with anti-TCRβ (H57-597), -TCRγδ (GL3), -CD4 (GK1.5/RM4-5), -CD8a (53-6.7), -CD25 (PC61), -CD44 (IM7) and -CD45RB (16A) diluted in Brilliant Stain Buffer (BD Biosciences). Fixable Viability Dye

(eFluor780) (eBioscience) was used to determine viability of cells. For intracellular cytokine stainings, cells were stimulated with PMA (50 ng/ml), monensin sodium (4 µg/ml) and ionomycin (500 ng/ml) for 4 hours and subsequently stained for surface markers. Following fixation and permeabilization with BD Cytofix/Cytoperm (BD Biosciences), the cells were intracellularly stained with anti-IL-17A (TC11-18H10.1) and –IFN-γ (XMG1.2). Data were collected on a BD LSRFortessa and analyzed using FlowJo Software.

Statistical analysis

Differences between the studied groups were analyzed using the two-tailed unpaired Student's *t*-test. The statistical analyses were executed using GraphPad Prism version 8.3.1 and a p-value below 0.5 was considered significant. Statistically significant differences are denoted *<0.05, **<0.01, ***<0.001. ****<0.0001. Outliers were identified using the "Identify outliers" function in GraphPad Prism with a ROUT set to 1 %.

Results

No effect of overall T cell development is induced by sensitization with DNFB

It is well-established that contact allergens are capable of igniting severe local immune responses that are largely mediated by T cells[20]. Furthermore, some studies have shown that some contact allergens can trigger systemic immune responses[21,22]. To determine if sensitization with a contact allergen affects T cell development, we sensitized mice on their ears with DNFB in olive oil:acetone (OOA) or with pure OOA as control for three consecutive days (Figure 1A). After additional 3 days, we determined the local response as changes in ear-thickness. A 179 % increased ear-thickness was found in mice sensitized with DNFB compared to control mice (Figure 1B). Furthermore, we determined the distribution of different T cell lineages by flow cytometric analyses of single-cell suspensions from thymus. No overall differences in $CD4^+$, $CD8^+$, $CD4^+CD8^+$ (double positive, DP) or $CD4^-CD8^-$ (double negative, DN) populations were found between OOA and DNFB treated mice, neither in fractions nor total cell numbers (Fig. 1C-D). Furthermore, we found no differences in the overall populations of DN1-4 cells and γδ T cells (data not shown).

Epidermal sensitization with DNFB induces an increased number of IL-17A-producing innate $CD8^+$ αβ thymocytes

To determine if sensitization with DNFB could affect development of mature $CD4^+TCR\beta^{hi}$ and $CD8^+TCR\beta^{hi}$ thymocytes, we analyzed thymic single cells suspensions by flow cytometry. We did not detect any differences in fractions and numbers of these two subsets in mice treated with DNFB or OOA (Fig. 2A). Furthermore, thymocytes were stimulated with PMA and ionomycin *in vitro* to detect cytokine production. Still, no differences in fraction or cell numbers were found between DNFB- and OOA-treated mice concerning IFNγ-producing $CD4^+TCR\beta^{hi}$, $CD8^+TCR\beta^{hi}$ and IL-

17A-producing $CD4^{+}TCR\beta^{hi}$ thymocytes (Fig. 2B). Interestingly, we found a significantly higher fraction and number of IL-17A-producing $CD8^{+}TCR\beta^{hi}$ thymocytes in DNFB- compared to OOA-treated mice (Fig. 2C). To determine whether the increase in IL-17A-producing $CD8^{+}TCR\beta^{hi}$ thymocytes was due to increased development in the thymus or mediated by increased infiltration of IL-17A-producing $CD8^{+}TCR\beta^{hi}$ T cells generated in the periphery and subsequently recirculated to the thymus, we repeated the experiments in mice treated with FTY720 (Fig. 2D). FTY720 inhibits S1P-mediated egress of cells from the thymus and lymph nodes and thereby deplete circulation T cells[23,24]. Notably, the total number of IL-17A-producing $CD8^{+}TCR\beta^{hi}$ thymocytes increased to the same levels in mice treated with FTY720 as in control mice following DNFB treatment (Fig. 2D). Taken together, these data indicated that a population of innate IL-17A-producing $CD8^{+}$ T cells develop in the thymus following epidermal sensitization with DNFB.

Epidermal sensitization with DNFB induces more IL-17A-producing $CD8^{+}$ recent thymic emigrants in the spleen

After leaving the thymus, αβ T cells are still not fully mature and go through an approximate three week stage of being recent thymic emigrants (RTEs). The RTEs gradually up-regulate CD45RB, QA2, CD28 and IL-7Rα and down-regulate TCR/CD3 and CD24[25]. To investigate if epidermal sensitization affected the thymic output, we treated mice with DNFB on the ears for three consecutive days (Fig. 1A), harvested splenocytes and analyzed the expression of CD45RB with flow cytometry. We found significantly more $CD8^{+}TCR\beta^{hi}$ T cells with low CD45RB expression in DNFB-treated mice compared to OOA-treated mice (Fig. 3A). Furthermore, of these $CD8^{+}TCR\beta^{hi}CD45RB^{lo}$ T cells, a significantly higher number produced IL-17A after PMA and ionomycin stimulation *in vitro* (Fig. 3B). Taken together, these data indicate that sensitization to DNFB at a local skin area changes the thymic output of T cells.

Discussion

In this study we describe a population of IL-17A-producing $CD8^{+}TCR\beta^{hi}$ thymocytes that develop in response to sensitization to the contact allergen DNFB at a local skin area. Furthermore, we found that sensitization with DNFB resulted in a large increase of IL-17A-producing $CD45RB^{low}CD8^{+}$ T cells in the spleen, indicating an enhanced export of IL-17A-producing $CD8^{+}$ T cells from the thymus as a consequence of DNFB sensitization.

Acute thymic atrophy with decreased thymopoiesis can occur in a number of situations that include stress, infections or acute inflammation[26,27]. Yet, we did not find an overall change in the thymic cellularity of DNFB- compared to OOA-treated mice. Furthermore, we did not see a change in the double negative, double positive or single positive populations of the thymus (Fig. 1). This suggested that the dose of DNFB and the size of the affected skin area used in the present study did not lead to a systemic stress reaction and atrophy of the thymus. Generally, the innate subsets of $CD4^{+}$ and $CD8^{+}$ T cells have been associated with IL-17A- and IFNγ-production, respectively[3–6]. However, we found a small but significant population of IL-17A-producing $CD8^{+}$ αβ T cells that developed in the thymus following sensitization with DNFB. In accordance, both IFNγ- and IL-17A-producing $CD8^{+}$ T cells are implicated in contact hypersensitivity, and neutralization of IL-17A suppresses the response[13,14]. Therefore it is obvious to suspect recirculation from the periphery into the thymus. However, if this was the case it would also be expected that more IFNγ-producing thymocytes were found in mice sensitized with DNFB compared to mice sensitized to OOA. Moreover, treatment of the mice with FTY720 before and throughout sensitization inhibits both thymic egress, but also recirculation from secondary lymphoid organs. These data suggest that the increased number of IL-17A-producing $CD8^{+}$ αβ thymocytes in this study are newly developed innate T cells.

The fact that local inflammation in a limited skin area induced by sensitization with DNFB apparently is able to affect T development and T cell subset balance indicates that contact allergens might affects how we respond to infections or stress. As approximately 20 % of the population are sensitized to one or more allergens[8], further studies regarding how this affects our immune responses are highly relevant.

Conflicts of Interest

We declare no conflicts of interest

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Figure legends

Figure 1. Epidermal sensitization with DNFB does not alter overall DN, DP and SP stages. A. Experiment outline. Mice were painted with either OOA or DNFB from day 0-2 and euthanized on day 5, where thymus was harvested. **B.** Ear thickness was measured on day 5. **C-D.** Flow cytometric analyses of thymic single-cell suspensions from mice aged 8-12 weeks. Data obtained from 3 independent experiments with 4 mice per group. **C.** Average fractions of single, viable thymocytes further gated for CD4 and CD8. **D.** Graphs depict total numbers of DN, DP and SP thymocytes.

Figure 2. Increased number of IL-17A-producing $CD8^+$ T cells in the thymus of mice following epidermal DNFB treatment. A. Experiment outline. Mice were treated with FTY720 from day 0-7 and sensitized with DNFB or OOA on day 2-4. Mice were euthanized on day 7 and thymus was harvested. **B-D.** Flow cytometric analyses of thymic single-cell suspensions from mice aged 8-12 weeks. Mice were treated with either OOA or DNFB and with or without FTY720. Data obtained from 2 individual experiments with 4 mice per group. **B.** Single, viable cells were further gated for TCRβ and subsequently for CD4 and CD8. Graphs depict number of $CD4^+TCR\beta^+$ and $CD8^+TCR\beta^+$ thymic T cells. **C-D.** Populations from B. were further gated for IL-17A and IFN-γ. **C.** Number of $CD4^+TCR\beta^+IFN\text{-}\gamma^+$, $CD4^+TCR\beta^+IL\text{-}17A^+$ and $CD8^+TCR\beta^+IFN\text{-}\gamma^+$ T cells. **D.** Mean fraction (left) and total number (right) of $CD8^+TCR\beta^+IL\text{-}17A^+$.

Figure 3. Increased number of IL-17A-producing $CD8^+$ T cells in the spleen of mice following epidermal DNFB treatment. A-B. Flow cytometric analyses of single, viable splenocytes from mice treated with DNFB or OOA for three consecutive days (day 0-2) and euthanized on day 5. Data obtained from two individual experiments with 4 mice per group aged 8-12 weeks. **A.** Mean

fractions (left) and total numbers (right) of $CD8^+TCR\beta^+CD45RB^{lo}$ T cells. **B.** Mean fractions (left) and total numbers (right) of $CD8^+TCR\beta^+CD45RB^{lo}IL\text{-}17A^+$ T cells.

Figure 1

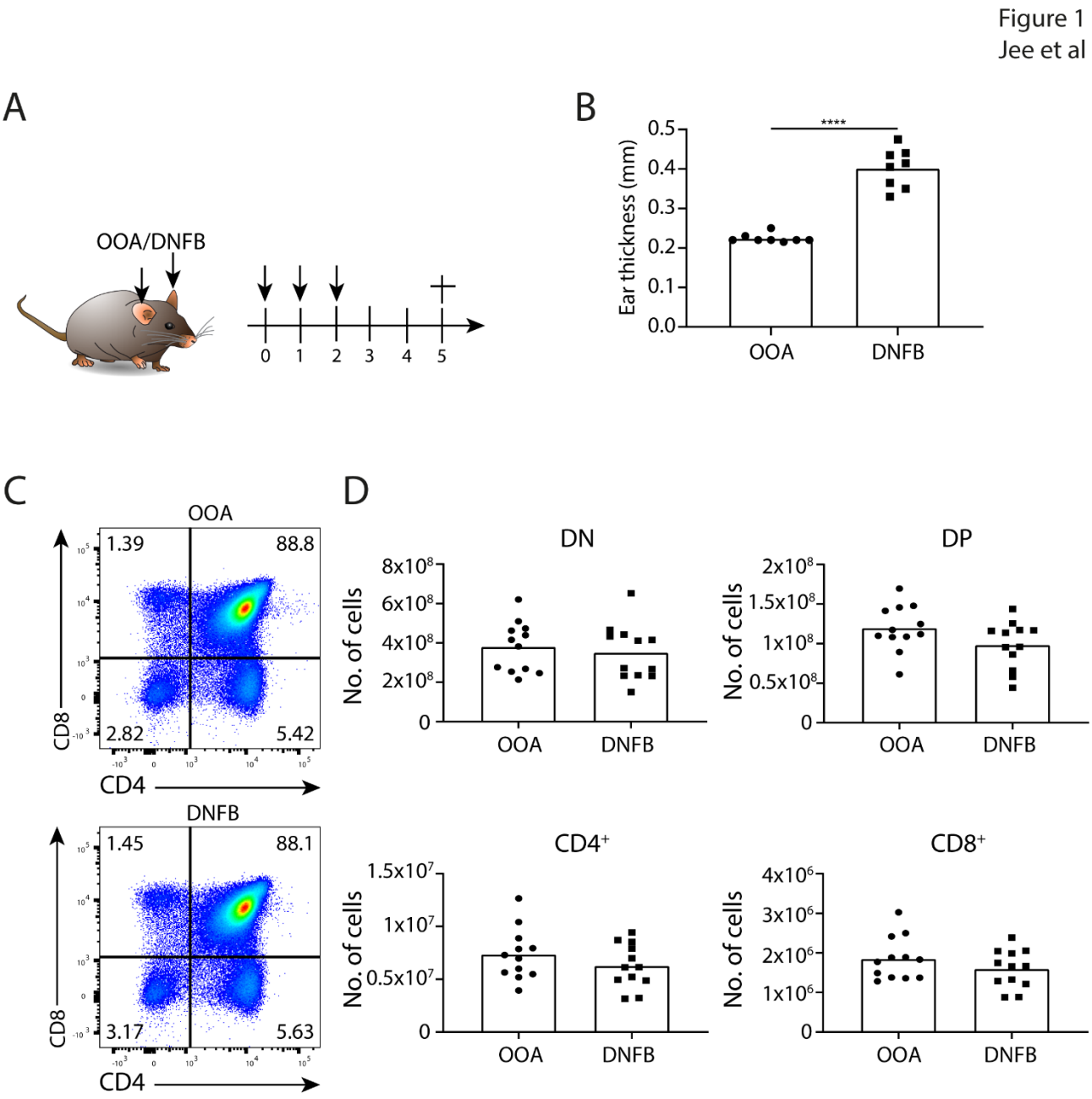
Figure 1
Jee et al
A
OOA/DNFB
0 1 2 3 4 5
B
Ear thickness (mm)
0.5
0.4
0.3
0.2
0.1
0.0
****
OOA
DNFB
C
OOA
1.39
88.8
2.82
5.42
CD8
CD4
DNFB
1.45
88.1
3.17
5.63
CD8
CD4
D
DN
No. of cells
8x10⁸
6x10⁸
4x10⁸
2x10⁸
0
OOA
DNFB
DP
No. of cells
2x10⁸
1.5x10⁸
1x10⁸
0.5x10⁸
0
OOA
DNFB
CD4⁺
No. of cells
1.5x10⁷
1x10⁷
0.5x10⁷
0
OOA
DNFB
CD8⁺
No. of cells
4x10⁶
3x10⁶
2x10⁶
1x10⁶
0
OOA
DNFB

Figure 2

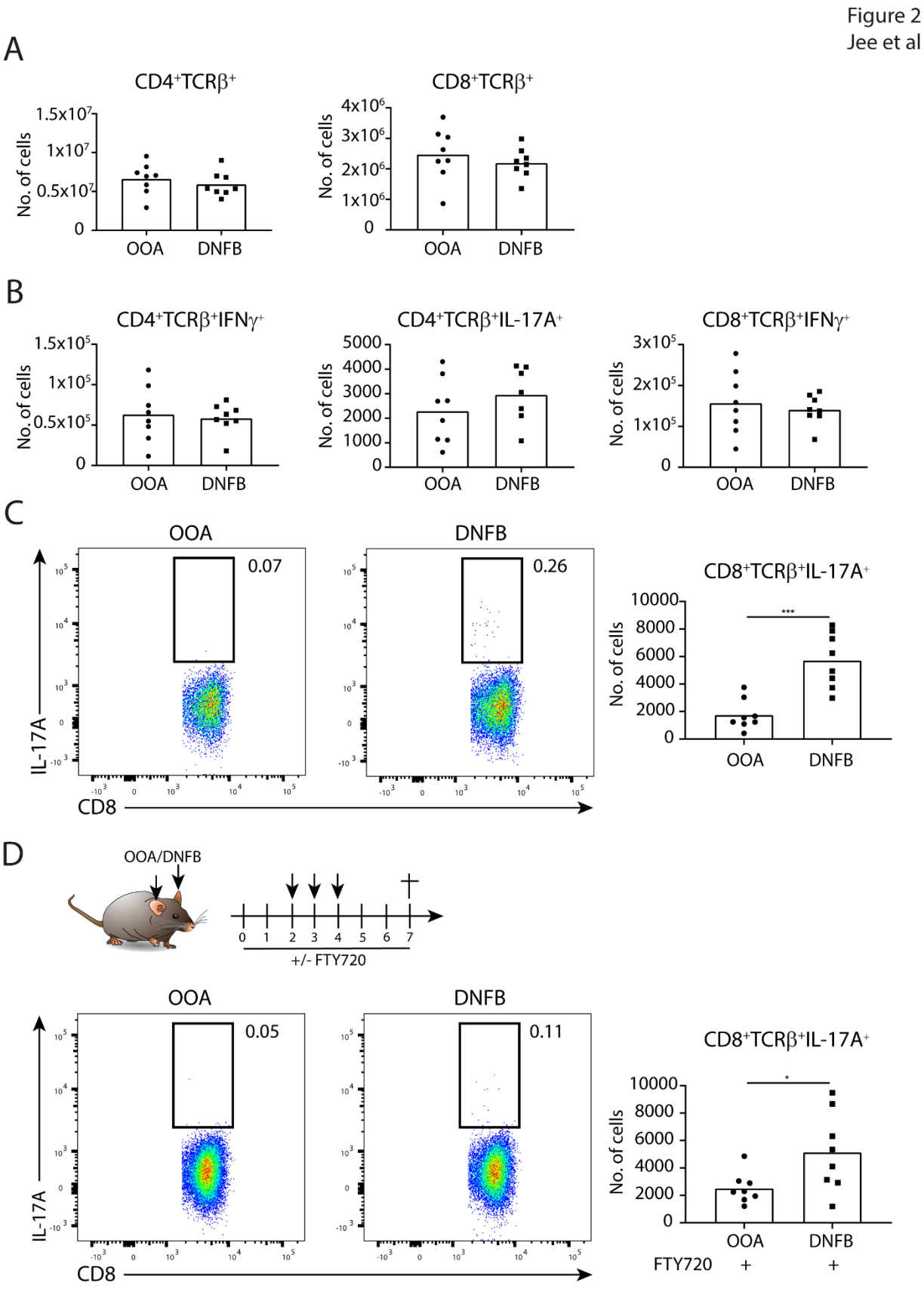
Figure 2
Jee et al
A
CD4+TCRβ+
No. of cells
1.5x10^7
1x10^7
0.5x10^7
0
OOA
DNFB
CD8+TCRβ+
4x10^6
3x10^6
2x10^6
1x10^6
B
CD4+TCRβ+IFNγ+
1.5x10^5
1x10^5
0.5x10^5
CD4+TCRβ+IL-17A+
5000
4000
3000
2000
1000
CD8+TCRβ+IFNγ+
3x10^5
2x10^5
1x10^5
C
OOA
DNFB
0.07
0.26
IL-17A
CD8
CD8+TCRβ+IL-17A+
10000
8000
6000
4000
2000
***
D
OOA/DNFB
0 1 2 3 4 5 6 7
+/- FTY720
0.05
0.11
*
FTY720 + +

Figure 3

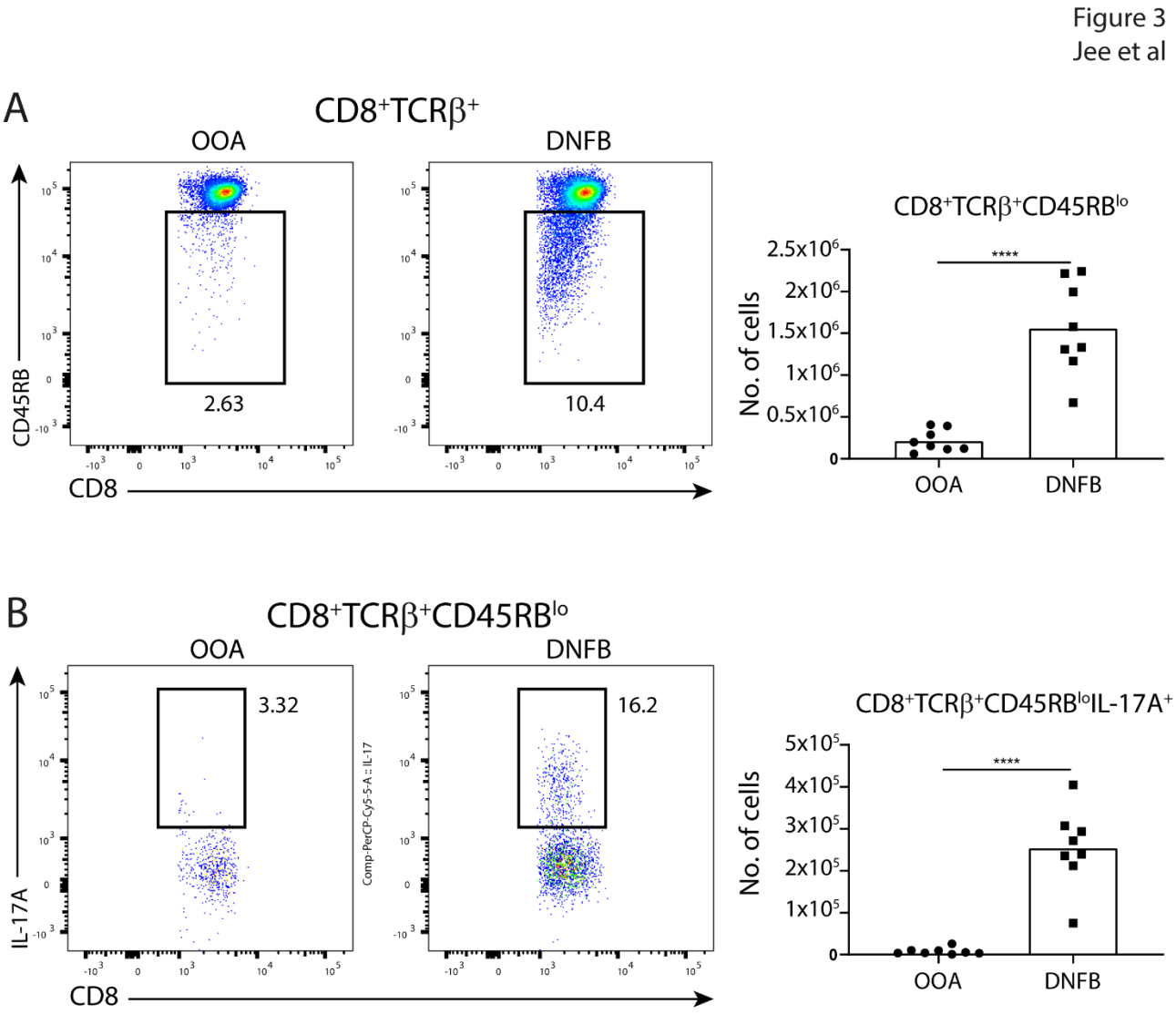
Figure 3
Jee et al
A
CD8+TCRβ+
OOA
DNFB
CD45RB
CD8
2.63
10.4
CD8+TCRβ+CD45RBlo
No. of cells
2.5x10^6
2x10^6
1.5x10^6
1x10^6
0.5x10^6
0
****
OOA
DNFB
B
CD8+TCRβ+CD45RBlo
OOA
DNFB
IL-17A
CD8
Comp-PerCP-Cy5-5-A :: IL-17
3.32
16.2
CD8+TCRβ+CD45RBloIL-17A+
No. of cells
5x10^5
4x10^5
3x10^5
2x10^5
1x10^5
0
****
OOA
DNFB

Manuscript Improvements

Manuscript II

To improve manuscript II for publication, we would like to include H&E stains of ears to see the inflammatory status of the epidermis in the three mouse strains. We also wish to enrich thymic single-cell suspensions for TEC by bead selection prior to repeating RNA extraction and qPCR, or preferably, sort them using FACS (see considerations on methodology). Moreover, we wish to include microscopy experiments on markers that we have found down regulated at the transcriptional level to visualize if they are affected at the translational level as well. To this end, we are using a form of 3D imaging of solvent-cleared organs (3DISCO) of whole thymus lobes. Furthermore, we want to include single-cell sequencing experiments to visualize alterations in TEC populations of the three mouse strains. Finally, some experiments in this manuscript have only been performed once and will need to be repeated.

Manuscript III

Data from study III suggest that local epidermal exposure to a contact allergen affects the development or maturation of T cells. However, more data are needed to establish these effects in detail. We suspect that the increased number of IL-17A-producing $CD8^+$ T cells found after DNFB treatment are of the innate type. Therefore, we will investigate the expression of CD44, CD122 and Eomes. Furthermore, IL-4 produced by NKT cells induce upregulation of Eomes[198]. Therefore, we would like to investigate the thymic cytokine environment in more detail and determine expression of key transcription factors. Numerous markers to distinguish RTEs exist[197], but the extra-thymic maturation of T cells is a gradual process and not all RTEs express set markers, and a large fraction of RTEs are excluded using surface markers and flow cytometry[199]. Thus, we would like to investigate RTEs using another model, for example RAG-GFP mice.

As we have only looked at one concentration of DNFB, it would be relevant to establish if the concentration of allergen correlates with number of IL-17A-producing $CD8^+$ T cells in the thymus. Furthermore, it would be relevant to know if the alterations can be induced with other allergens as well, or if they are allergen-specific.

Considerations on methodology

As discussed previously, the *ft/ft* mice used in study I are on a mixed background and furthermore harbour two mutations. To solve this problem and be able to look at the true effects of the individual mutations, we backcrossed the *ft/ft* mice onto a congenic C57Bl/6 background and separated the two mutations, creating two new strains used for study II.

The primary method used during this PhD has been flow cytometry, which is a widely used and robust method. However, it does have some limitations. Mostly these limitations have to do with the extraction of cells from various tissues, and some studies have shown that flow cytometry greatly underestimates the total number of cells compared to e.g. microscopy[200,201]. In fact, one of these studies showed that preparation of single cell suspensions from non-lymphoid tissues recovered as few as 2 % of memory CD8 T cells[201]. Even though that study focuses on tissue residency and CD8 T cells, one might fear that working with non-lymphoid tissues such as skin, the small number of cells extracted might alter the distribution of subsets, as some cells may be easier to extract or survive better in a single-cell suspension. These limitations should not cause the differences between the three mouse strains, but might underestimate the true total number of cells in all groups.

In manuscript II the qPCR experiments are performed on whole thymi (manuscript II, Fig. 5). As we find a large and significant increase in the cellularity of both $Flg^{ft/ft}$ and $Matt^{ma/ma}$ thymi, we suspect that some of the decrease found in transcription of genes expressed by subsets of mTECs, possibly results from a dilution of TECs in $Flg^{ft/ft}$ and $Matt^{ma/ma}$ thymi. However, if the results were only consequences of such dilution, we would expect all of the transcription data to look very similar across different genes. Nevertheless, we wish to perform RNA extraction and qPCR on either sorted TECs or bead selected TECs.

In manuscript II, we used a CHS model, where mice were sensitized on the ears for three consecutive days and challenged after 21 days. We chose to measure ear thickness at day 6 and 13 and to challenge at day 21 to ensure that the primary response had declined. Thus, the ear thickness following sensitization represents the innate response, whereas the ear thickness following challenge represents a secondary memory response (manuscript II, Fig. 6).

In manuscript II, we used a recombinant Sendai virus with a luciferase gene insert. To visualize viral load, luciferin needs to be injected i.p. 15 minutes before IVIS analysis. In these experiments, some mice could appear negative for the virus one day, but positive the day before or after. This problem possibly arises if the i.p. injected luciferin does not reach circulation and spreads throughout the body. Overall, this occurred only in few mice. However, we chose to eliminate results from negative mice on day 2-8 if they were positive the day before and after (and therefore we did not eliminate results from negative mice on day 1 and 9) based on the imaged radiance (manuscript II, Fig. 7B).

Finally, the use of mouse models to understand human immunology has both advantages and disadvantages. This subject in itself is huge. In brief, humans and mice share >90 % of the same genes and therefore discovery of new mouse genes can often be predicted in humans and the other way around[202]. Nevertheless, substantial differences, both genetic and physiological, exist between the two species. Studying complex human diseases in mouse models often results in conclusions that turn out not to hold true in humans[203]. Using mice in all three manuscripts, we are aware of disadvantages and will take them into account trying to understand disease mechanisms. Regarding filaggrin, mattrin and KC differentiation it seems that humans and mice are quite alike. However, immune cells and responses can be quite different between the species, and interestingly Beura et al.[204] recently showed that the immune system of experimental mice is similar to that of human infants, whereas “dirty” or antigen-experienced mice show much more resemblance to adult humans. These findings suggest that some of the differences between mice and humans are not just species differences, but also differences in antigen-experience. Thus, working with mice, antigens and the immune system, we should always consider our results in this regard and consider how to improve our models.

Discussion and perspectives

The studies of this thesis are all performed using mouse models, and losing yourself in details in the model sometimes leaves you asking what the purpose or perspectives were. Around 10 % of the Northern European population are heterozygous carriers and 0.1 % are homozygous or compound heterozygous carriers of an *FLG* mutation[46,56]. Therefore, uncovering so far unknown effects of filaggrin loss-of-function mutations are relevant. So far, reports investigating the role of filaggrin are obviously focused on effects in the epidermis. However, *FLG* mutations are also associated with increased risk of asthma and allergic rhinitis[205,206], which target organs where filaggrin is not expressed. It has been suggested that increased allergen penetration of the skin might explain these findings[207,208]. Another or additional explanation could be that decreased expression of filaggrin in the thymus skews the immune response in a certain direction as indicated in manuscript I and II. Regarding the *Matt* mutation, not many studies have investigated the effects of it. Nevertheless, in the study by Saunders et al.[63], the association between the *Matt* mutation and AD was discovered in a group of people with severe AD that did not have an *FLG* mutation. Therefore, it is likely that the *Matt* mutation could be the underlying cause in many cases of severe AD, where the patients have simply not been genotyped. In mice, the *Matt* mutation results in a more severe phenotype than the *Flg* mutation, which makes me speculate that disease penetrance is larger in humans with a *Matt* mutation compared to an *Flg* mutation. This leads to the next question: why do some filaggrin mutation carriers develop AD, whereas some do not (up to 60 %)[45]? The answer is probably a sum of genetics and environment (as with most diseases). The environment in the sense, that which infections or allergens we encounter probably schools our immune system, which in turn make individuals fight infections in different ways. AD is primarily a Th2-mediated disease, but Th17 and Th22 cells are also involved especially in infants and Asians[45,209]. If one or more infections have schooled your immune system in that direction, you might have increased risk of developing AD if other risk factors are present as well.

Interestingly, filaggrin mutations are quite common in the Northern European population with around 10 % mutation carriers. Although subjectively observed, the double mutant *ft/ft* mice (manuscript I) seemed less affected than the single mutant $Matt^{ma/ma}$ mice (manuscript II). Therefore, the filaggrin mutations may also have beneficial roles. In line with this, human filaggrin mutation carriers have increased levels of serum Vitamin D[210], which is possibly beneficial in Northern Europe.

In accordance with our findings that filaggrin is expressed in the thymic medulla (manuscript I) and that filaggrin deficiency seems to affect the transcription of other terminal differentiation markers (manuscript II), the translocation of the profilaggrin N-terminal into the nucleus has been observed to precede the transformation of KCs into corneocytes with loss of nucleus[211]. We speculate that profilaggrin might play a similar role in the thymus, and therefore affect the differentiation of HCs, or the similar mouse structure, in the thymus. These speculations are supported by the finding that other keratinocyte markers are downregulated in *Flg*$^{ft/ft}$ mice, which suggests that filaggrin is not only a marker of terminal differentiation, but also plays a role in the process itself. Not much is known about the function of terminally differentiated keratinocyte-like cells in the thymus. As mentioned earlier, one study by Watanabe et al.[140] showed that TSLP is produced by human HCs and that TSLP *in vitro* is able to indirectly induce differentiation of nTregs. Interestingly, in mice, another mTEC subset, the thymic tuft cells, produce IL-25 and are necessary for the thymic development and function of NKT2 cells. Furthermore, thymic tuft cells are required to provide tolerance toward tuft-restricted antigens[136]. mTECs are more heterogeneous than first assumed, and each subset may play a specific role in providing a supporting environment for one or more lymphocyte subsets and may be essential in providing tolerance towards their peripheral mirrors.

One mind boggling question arising from manuscript I and II is whether the changes found in the thymus and changes of T cell composition are mediated by internal structural thymic changes causing an altered output or systemic inflammation due to the barrier defects causing increased recirculation to the thymus. Immune cells of naïve mouse epidermis entail Langerhans cells and Vγ3^{+} DETC[14,15,68]. Upon viral infection[212] or allergen exposure[75], DETC are displaced by tissue resident CD8^{+} memory T cells. Therefore, the finding that *Matt*$^{ma/ma}$ mice harbor αβ T cells in their epidermis may indicate that they are not naïve. Our mice are SPF-housed and we speculate that otherwise harmless commensal microorganisms are able to prime *Matt*$^{ma/ma}$ mice due to continuous inflammation of their skin and therefore a leaky barrier. Previous reports have determined that stress and inflammation can affect the thymus and T cell development[213]. Therefore we suspect that the changes found in the *Matt*$^{ma/ma}$ thymus and periphery may be partially caused by widespread skin inflammation and partly caused by internal structural changes in the thymus. Interestingly, the *Flg*$^{ft/ft}$ mice are not macroscopically inflamed, and the epidermis data suggest that they are naïve, which makes me speculate that the different composition of T cells found in their thymi and spleens is caused by central structural changes in their thymi, and not by a defect

skin barrier with enhanced antigen penetration. If *Flg*$^{ft/ft}$ mice are thus antigen-naïve and without skin inflammation, the decreased capacity to clear Sendai virus infection found in manuscript II may be a result of the changes in T cell composition arising from the thymus. In line, the increased risk of human *FLG* mutation carriers to develop asthma and allergic rhinitis may be partly caused by increased allergen-penetration via the skin and partly via altered T cell development. On the other hand, previous studies have shown that defects in the development of DETC in the thymus can cause skin inflammation with infiltrating αβ T cells[214,215]. To establish the exact role of filaggrin and mattrin in the thymus without having to take into account their roles in epidermis, we would probably need a model where filaggrin and mattrin are mutated/knocked out only in TECs. Studies with TEC specific knockout of proteins make use of the Cre-Lox recombinase system in cells expressing *Foxn1*[216,217], but *Foxn1* is also widely expressed in skin[218].

The thymus does not only contain developing T cells, but also a small number of recirculating peripheral B and T cells[219]. In one study, the authors find the number of recirculated peripheral T cells in a B6 thymus to be around 100,000 cells irrespective of thymus size[220]. Another study determines that the vast majority of recirculated T cells are regulatory T cells that regulate the development of their precursors[221]. Importantly, the number of recirculating lymphocytes in the thymus increase during inflammatory conditions caused by different types of infections[222]. Therefore, we cannot, from our data, exclude the possibility that recirculation to the *ft/ft* (manuscript I) and *Matt*$^{ma/ma}$ thymus (manuscript II) is increased due to widespread skin inflammation. This is also a speculation in manuscript III, where we apply a contact allergen that causes mild local inflammation. However, treatment with FTY720 during sensitization sequesters lymphocytes in thymus and lymph nodes[223,224], which should in theory keep lymphocytes from recirculating to the thymus.

As described earlier, γδ T cells exit the fetal thymus in distinct waves started by the Vγ3$^+$ DETC and followed by Vγ4$^+$ γδ T cells. Subsequently, the exit of Vγ2$^+$ and Vγ1.1$^+$ γδ T cells starts, which are also the subsets that develop throughout life[160]. In manuscript I we found that Vγ2$^+$ IL-17-producing γδ T cells develop in adult thymus of *ft/ft* mice, but in manuscript II the IL-17A-producing γδ T cells are not found within the Vγ2$^+$ subset in *Matt*$^{ma/ma}$ mice. Instead, we find it likely that the subset that is increased in the epidermis of *Matt*$^{ma/ma}$ mice is the Vγ4$^+$ subset. A change in the thymic environment during fetal development possibly accounts for the different Vγ-chain incorporation in fetal derived γδ T cells. If the increased number of IL-17A-producing

γδ T cells in *Matt*$^{ma/ma}$ epidermis arise as a result of thymic lack of mattrin, one could speculate that the *ma* mutation affects the selecting ligands important for development of the Vγ4^{+} γδ T cells in that temporal window. As this subset only develops during fetal life, it would explain why we do not find more Vγ4^{+} γδ T cells in the adult thymus of *Matt*$^{ma/ma}$ mice (manuscript II, Fig. 4E).

Conclusion

The studies included in this PhD investigated skin and thymus connections. To this end, the first two manuscripts focused on the role of two skin proteins, filaggrin and mattrin, in the thymus and how the lack of these affect the T cell composition in the thymus and the periphery, whereas the third manuscript focused on how skin inflammation induced by contact allergens affects the T cell composition in the thymus.

In **manuscript I** we determined that filaggrin is expressed in the thymus medulla of mice. Furthermore, that this expression is decreased in flaky tail mice, which is associated with more $V\gamma2^+$ IL-17A-producing γδ T cells developing. Finally, these thymic changes are mirrored in the periphery with more IL-17A-producing γδ T cells in spleen and epidermis.

In **manuscript II** we backcrossed the double mutant flaky tail mice, giving rise to two new strains termed *Flg*$^{ft/ft}$ and *Matt*$^{ma/ma}$. In this study, we determined that the mutation in the mattrin gene, and not in the filaggrin gene, caused the IL-17A-skewed T cell profile in thymus, spleen and epidermis, whereas the mutation in the filaggrin gene caused an IFNγ-skewed T cell profile in the thymus and spleen. Notably, these alterations seem to affect the capacity to clear Sendai virus from the respiratory tract and affect the immune response to epidermal contact allergen.

In **manuscript III** we determined that local inflammation in the skin was able to affect development of T cells in the thymus with an increased number of 'innate-like' IL-17A-producing $CD8^+$ αβ T cells.

Overall, the thymus environment and thereby T cell development is affected by mutations in genes so far only associated with roles in the skin. Furthermore, a mild immune response in the skin can affect the thymus and T cell development. Taken together, the data of this thesis suggest that diseases clinically manifested in the skin may have a systemic component with changes in T cell development and function. Furthermore, skin inflammation, which may be seen as non-severe disease, may have serious consequences if able to change our immune system in general.

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