Title
Filaggrin deficiency in mice alters the early life CD4+ response to skin commensal bacteria

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Publication Date
2022

Peer reviewed|Thesis/dissertation

Filaggrin deficiency in mice alters the early life CD4+ response to skin commensal bacteria

by
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DISSERTATION
Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION
of the
UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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Dedication

To my husband Phillip Sandborn, who has been here for me every step of the PhD journey,

and to my parents, Teofilo and Dorothy Gonzalez, who made it possible for me to embark upon it.

Acknowledgements

First and foremost, I'd like to thank my thesis advisor Tiffany Scharschmidt for all her support during my PhD. I'm so very thankful to have had her as a mentor and to work in her lab. I'd also like to thank my thesis committee members: Jason Cyster, Michael Rosenblum, and Mark Ansel for your helpful advice and guidance over the course of my PhD, and in the case of Mark, for his support even before I started my PhD.

I could not have completed the work outlined in the thesis without the help and support of all the Scharschmidt lab members past and present. Thank you all for your help on so many early morning harvests, for taking care of mice or experimental timepoints when I was out of lab, and for all your helpful feedback in and out of lab meeting. Furthermore, I'd like to thank all the labs on HSW12 for helping build such a good scientific environment to spend the past six years in both socially and scientifically. Getting feedback from everyone on the floor during our weekly meetings and bioinformatic journal club has been invaluable. Thank you to Ari Molofsky, Michael Rosenblum, Cliff Lowell, and Abul Abbas for your advice in and out of floor meeting.

Thank you also to my fellow grad students in my BMS cohort who made the transition into grad school so much more manageable with support during classes, feedback on presentations, and practice quals. I'd especially like to thank Didi Zhu and Camille Simoneau for their scientific and moral support.

I’d like to thank also all the friends and family who have supported me along the way during grad school and given me something else to think about besides my thesis project. Special thanks to my friend Jasmine Verret who has been a great support since our first year at UC Berkeley. Thank you to all my extended family, especially to my Bay Area family who have celebrated so many special occasions with me since I moved here over ten years ago: Aunt Maddie, Aunt Gina, Greg, Jenn, Jackie, Xavier, Velma, Joy, Alvin, and Yurany. Thank you to my in-laws Peter, Tammy, Avery, and Hilary for being a second family to me.

Thank you to my siblings Alexis, Julia, Teo, and Paolo Gonzalez for all their support throughout the years. Thank you as well to my parents Dorothy and Teofilo Gonzalez who have been there every step of the way and who supported my wish to attend UC Berkeley, as I may not have stumbled across the field of immunology otherwise. Finally, thank you to my husband Phil Sandborn, who has been so supportive of my whole grad school journey.

Contributions

The majority of Ch. 2-3 is adapted from: Filaggrin deficiency in mice alters the early life $CD4^+$ response to skin commensal bacteria. Jeanmarie R. Gonzalez, Anna Celli, Antonin Weckel, Miqdad O. Dhariwala, Geil Merana, Laura R. Dwyer, Joy Okoro, Oluwasunmisola T. Ojewumi, Jason M. Meyer, Theodora M. Mauro, Tiffany C. Scharschmidt, *In revision*.

JRG performed the vast majority of the experimental work. AC, AW, MOD, GM, LRD, JO, OTO contributed to execution of the experimental work and related analyses. TMM and JMM contributed materials and helped in design and interpretation of key experiments. Michael Rosenblum, Marlys Fassett and Roberto Ricardo-Gonzalez provided critical review of the manuscript form, Marlys Fassett advised on the HDM studies, Sepideh Nozzari and Yongmei Hu helped with animal husbandry and genotyping. Dr. Masa Amagai provided the $Flg^{-/-}$ mice and Dr. Jim Moon shared his 2W-loaded Class II tetramer.

“Well, yes; she had stuck to her work—and that in the face of what might have seemed overwhelming reasons for abandoning it and doing something different. Indeed, though she had shown cause that evening for this particular loyalty, she had never felt it necessary to show cause to herself. She had written what she felt herself called upon to write; and, though she was beginning to feel that she might perhaps do this thing better, she had no doubt that the thing itself was the right thing for her. It had overmastered her without her knowledge or notice, and that was the proof of its mastery.”

-Dorothy L. Sayers, Gaudy Night

Filaggrin deficiency in mice alters the early life $CD4^+$ response to skin commensal bacteria

Jeanmarie Gonzalez

Abstract

Filaggrin mutations underlie ichthyosis vulgaris and increased risk of atopic dermatitis, conditions typified by disruption of the skin microbiome and cutaneous immune response. Yet, it remains unclear if neonatal skin barrier compromise due to filaggrin deficiency alters the quality of commensal-specific T cells and the functional impact of such responses. To address these questions, we profiled changes in the skin barrier and early cutaneous immune response of neonatal C57BL/6 *Flg*$^{-/-}$ and WT mice using scRNAseq, flow cytometry and other modalities. *Flg*$^{-/-}$ neonates showed little alteration in transepidermal water loss or lipid or corneocyte related gene expression. However, they demonstrated increases in barrier disruption genes, epidermal dye penetration and numbers of skin $CD4^+$ T cells. Using an engineered strain of *Staphylococcus epidermidis* (*S. epi*-2W) to study the response to neonatal skin colonization, we found that commensal-specific $CD4^+$ T cells were skewed in *Flg*$^{-/-}$ pups towards effector rather than regulatory T cells. This altered response persisted into adulthood, where it was typified by Th17 cells and associated with increased susceptibility to imiquimod-induced skin inflammation. Thus, subtle but impactful differences in neonatal barrier function in *Flg*$^{-/-}$ mice are accompanied by a skewed commensal-specific $CD4^+$ response, with enduring consequences for skin immune homeostasis.

Table of Contents

Chapter 1: Introduction ---------- 1

1.1 *Atopic dermatitis and Ichthyosis Vulgaris: Two Distinct, but Prototypical Diseases of the Skin Barrier* *2*

1.2 *An overview of the skin as a barrier organ* *4*

1.3 *Filaggrin is a protein with multifunctional roles in skin barrier function* *5*

1.4 *An overview of the cutaneous immune response and its relationship to barrier function* *8*

1.5 *The early life window facilitates neonatal tolerance to commensal antigens* *10*

1.6 *Aims of study* *12*

Chapter 2: Filaggrin deficiency in mice alters the early life $CD4^+$ response to skin commensal bacteria ---------- 14

2.1 *Introduction* *15*

2.2 *Results* *17*

2.3 *Discussion* *27*

2.4 *Figures* *31*

Chapter 3: Materials and Methods ---------- 43

Chapter 4: Future Directions ---------- 53

References ---------- 57

List of Figures

2.1: Characterization of *Flg*$^{-/-}$ mice 32

2.2: Single cell RNA sequencing of WT and *Flg*$^{-/-}$ neonates and increased permeability to exogenous substances in *Flg*$^{-/-}$ neonates 33

2.3: Select increases in cytokines and CD4^{+} T cells in skin of *Flg*$^{-/-}$ neonates 34

2.4: Neonatal immune profiling 35

2.5: Profiling of dendritic cell antigen uptake reveals altered activation and frequency of select cell populations 36

2.6: *Flg*$^{-/-}$ neonates demonstrate a shift in the commensal-specific CD4^{+} towards a Teff rather than Treg phenotype 37

2.7: Immune cell profiles of adult *Flg*$^{-/-}$ mice at baseline and after secondary challenge 38

2.8: Teff-skewing of the commensal-specific CD4^{+} response in *Flg*$^{-/-}$ mice can be recalled in adulthood and is characterized by a Th17 phenotype 39

2.9: House dust mite model in *Flg*$^{-/-}$ mice and profiling of ear inflammation 40

2.10: *Flg*$^{-/-}$ mice have a stronger neutrophil, but not eosinophil response to MC903 treatment 41

2.11: Increased sensitivity to imiquimod-induced type 17 ear inflammation in *Flg*$^{-/-}$ mice 42

Chapter 1: Introduction

1.1 Atopic dermatitis and Ichthyosis Vulgaris: Two Distinct, but Prototypical Diseases of the Skin Barrier

Skin diseases are incredibly common worldwide and have a profound range of negative health impacts (Hay et al. 2014). While skin disease encompasses many broad categories, including malignancy, infection or dyspigmentation, disorders of the skin barrier and the cutaneous immune system account for some of the most chronic and debilitating conditions. Disease of the skin barrier generally originate from inherited alterations in key structural aspects of the epidermis, the skin's outermost layer. Where there is a strong genetic penetrance and known mutation, these conditions are generally grouped under the clinical term "Ichthyosis". Many other inflammatory skin diseases can have disruption of skin barrier function. In some of these instances this is thought to be secondary to immune dysfunction, but in other instances there is evidence for a primary barrier problem playing a key role in pathogenesis. Here, we will discuss in more detail, Ichthyosis vulgaris (IV) and Atopic Dermatitis (AD), two key prototypes for these two categories of genetic and inflammatory skin barrier disorders, which are particularly prevalent and uniquely intertwined.

Hereditary icthyoses encompass a broad umbrella of skin diseases that can be characterized by a compromised skin epidermis, defective keratinization, and altered transepidermal water loss, with a negative effect on patient quality of life (Malik et al. 2019). Some Ichthyoses so profoundly disrupt skin barrier function that they can result in neonatal lethality (Craiglow 2012). Ichthyosis Vulgaris is the most common form, a skin disease characterized by scaly skin and palmar hyperlinearity, and caused by loss

of function mutations in filaggrin, a key protein in the skin barrier (Smith et al. 2006). Homozygotes have more severe disease than heterozygotes, with more severe scaling and greater palmar linearity (Smith et al. 2006). Mutations in the skin barrier gene filaggrin have nearly full penetrance for icthyosis vulgaris (Thyssen et al. 2013). Filaggrin mutations, and therefore icthyosis vulgaris, can be quite common, particularly in European populations where they have a prevalence of ~7% (Thyssen et al. 2013).

Atopic Dermatitis (AD), more commonly known as eczema, is an inflammatory disease distinguished by dry, itchy skin lesions (Al-Shobaili et al. 2016). Patients with AD often go on to develop other allergic diseases as a part of the atopic march (Dainichi et al. 2018). AD pathogenesis is complex and multifactorial, but it is agreed that disruption of skin barrier function and alteration of the skin immune responses, especially among T cells, play central roles (Dainichi et al. 2018). A wide range of genetic and environmental factors contribute to AD (Leung and Guttman-Yassky 2014). Genetic factors include mutations in filaggrin, which is the most robust genetic risk factor (with an increased risk odds ratio of 3.12 (Rodríguez et al. 2009)) followed by mutations affecting the immune response (Bin and Leung 2016). AD usually starts in infancy but can begin later in life (Dainichi et al. 2018). AD is also often accompanied by changes in skin microbiome composition (Paller et al. 2019a). The abundance of both *Staphylococcus aureus*, a pathobiont, and *Staphylococcus epidermidis*, a commensal, are known to be increased in the skin of patients during AD flares (Byrd et al. 2017; Kong et al. 2012). Additionally, certain studies have identified changes in the skin microbiome preceding disease onset in infants who go on to develop AD (Kennedy et

al. 2017; Nakamura et al. 2020a). Ongoing discussion remains as to whether AD is an outside-in phenomenon initiated by barrier defects which elicit inflammation, or an inside-out phenomenon driven by an initial overreactive type 2 or allergic immune response (Leung et al. 2020). Both factors likely perpetuate active disease, with certain patients having a primarily inside-out or outside-in endotype. Barrier and immune function are very intertwined and hard to totally disentangle. For example, immune cytokines can downregulate filaggrin expression (Howell et al. 2007), suggesting that even in patients without filaggrin mutations, lower filaggrin production may still play a key role in the disease process.

1.2 An overview of the skin as a barrier organ

The skin barrier has multiple functions, including limiting water loss out of the skin and protecting from external agents such as chemicals or disease-causing microbes (Yang et al. 2020). Several components contribute to skin barrier function. This is most commonly described in the context of the brick (corneocytes) and mortar (intracellular lipids) model (Nemes and Steinert). The cornified envelope of corneocytes is composed of involucrin and loricrin surrounded by a lipid envelope and the interior is linked to keratin bundles (Madison 2003) that are aggregated by filaggrin. Another critical component are tight junctions (Madison 2003). Additionally, natural moisturizing factors (NMFs) are also found in the stratum corneum. NMFs are comprised of hygroscopic amino acids and other components from proteolyzed filaggrin (Madison 2003). Additionally, the skin microbiome can also contribute to the barrier by acting as a microbial barrier by directly antagonizing more pathogenic bacteria, inducing production

of host antimicrobial peptides or directly augmenting expression of key structural skin components (Harris-Tryon and Grice 2022). These commensal microbes such as *Staphylococcus epidermidis* reside on the surface of the skin and in appendages such as hair follicles (Kabashima et al. 2018).

The skin is comprised of two main layers: the dermis and the epidermis, the latter of which forms both the hair follicle and interfollicular regions (Pasparakis et al. 2014). The dermis is heterogeneous, containing both stromal cells and extracellular matrix (Pasparakis et al. 2014). Keratinocytes undergo a differentiation program to form the stratified epidermis, beginning with the basal layer, then the stratum spinosum, stratum granulosum, and finally the stratum corneum (Goleva et al. 2019). These cell layers are distinguished by the types of keratins they express (Matsui and Amagai 2015). In the stratum granulosum, keratohyalin granules form, containing protein complexes of keratin and keratin associated proteins like filaggrin (Matsui and Amagai 2015). The uppermost layer, the stratum corneum, is formed by dying stratum granulosum cells in a process known as cornification (Matsui and Amagai 2015).

1.3 Filaggrin is a protein with multifunctional roles in skin barrier function

Filaggrin is one of the best recognized molecules in promoting epidermal barrier function. For example, both AD and IV have filaggrin mutations as a core aspect of their pathogenesis. Filaggrin takes on many forms in the skin. Profilaggrin, the multimeric unprocessed form of filaggrin, is first expressed in keratohyalin granules in the skin's stratum granulosum (McLean 2016). It is further processed into 10-12 individual filaggrin

monomers, which help aggregate keratin filaments and give rigidity to the cell (Al-Shobaili et al. 2016). As keratinocytes start to terminally differentiate in the stratum corneum layer, filaggrin is further broken up into its amino acid components called natural moisturizing factors (NMF) (Al-Shobaili et al. 2016). The hygroscopic qualities of NMF are thought to contribute to several important functions such as UV protection and facilitating proper moisturization and pH of the skin. (McLean 2016). Prior work has suggested that loss of filaggrin and its associated barrier-promoting functions leads to an intracellular defect in the stratum corneum, allowing passage of exogenous substances between corneocytes (Scharschmidt et al. 2009a). However, many earlier studies of filaggrin loss in mice have been confounded by secondary mutations in genes later found to also influence skin barrier function (Bonefeld et al. 2016a; Fallon et al. 2009a; Oyoshi et al. 2009). Thus, further work is needed to more precisely define the functional contribution of filaggrin to skin barrier function.

Other hints toward the function of filaggrin can be gleaned by studying its genetic association with skin diseases, such as IV and AD as noted above. Filaggrin mutations are in fact associated with increased risk for all conditions in the atopic march, e.g. food allergies, eczema associated asthma, and allergic rhinitis (Brown and McLean 2012). While attempts to directly (Brown et al. 2011; Venkataraman et al. 2014) link filaggrin mutations to food allergy have been mixed, its connection to food sensitization is clear (Flohr et al. 2014; Tan et al. 2012). While some patients carry complete loss of function of mutations, a range of mutation types exist that are associated with intermediate or partial function, depending on their location, type, and size. There is also frequent allelic

variation in the number of filaggrin repeats (Sandilands et al. 2007) that can partially modify filaggrin function. Obviously, patients carrying two copies of loss of function mutations will have the more severe phenotypes related to filaggrin gene loss, but even heterozygous individuals or those carrying a single allele that confers partial loss of filaggrin function can have associated disease risk (McLean 2016).

Another important caveat of note is that while filaggrin mutations are well documented in populations of Asian and European descent, there is less evidence for a role of these mutations in populations of African descent, although the latter are still susceptible to developing AD (Brunner and Guttman-Yassky 2019). Additionally, filaggrin genetic variants are highly population specific, so specific mutations generally differ between populations in addition to difference in mutation frequency (Brown and McLean 2012). Because filaggrin mutations are so common, particularly in European populations, several theories have been posited to explain this phenomenon. The first is that filaggrin mutations conferred an evolutionary advantage by allowing natural “vaccination” against various pathogens (Irvine and McLean 2006). Another theory suggests that filaggrin loss of function mutations could have allowed for greater Vitamin D production in northern latitudes (Thyssen et al. 2014). These above findings have led to extensive interest in studying the role of filaggrin in disease.

Mouse models have both contributed to and clouded our understanding of filaggrin deficiency. For many years a commonly used model was the flaky tail (ft/ma). These mice have a spontaneous frameshift mutation in filaggrin (Lane 1972), but for many

years were bred on a background with a Tmem79/Matt gene mutation. One study found an increase in Th17s in adult but not 2-week old mice (Bonefeld et al. 2016b). Adult mice had spontaneous eczematous lesions and increased Th1, Th2, and Th17 cell cytokine responses to OVA (Fallon et al. 2009b; Oyoshi et al. 2009) Separating out the Tmem79 mutation resulted in partial loss of phenotype (Saunders et al. 2013), with sensitivity to OVA retained, but no overt baseline inflammation (Nakatsuji et al. 2016). Several groups have generated new filaggrin knockout lines which have complete loss of filaggrin protein (Kawasaki et al. 2012; Muhandes et al. 2021). One knockout line generated on a B6 background demonstrated dry, flaky skin with increased sensitivity to mechanical disruption with no spontaneous inflammation (Kawasaki et al. 2012), which I use in this thesis. Another knockout line generated on a BALB/c background showed a similar phenotype with no spontaneous atopy (Muhandes et al. 2021). Together, these studies suggest that while filaggrin deficiency has an impact on the host, it may take multiple layered factors to elicit stronger negative consequences.

1.4 An overview of the cutaneous immune response and its relationship to barrier function

As illustrated by the inflammatory phenotypes arising in mice with genetic barrier defects and the key role of the immune system in AD, there is a close relationship between the skin barrier and the cutaneous immune response. This is in part because keratinocytes themselves participate in the skin immune response by secreting cytokines that activate, recruit and coordinate behavior of hematopoetic immune cells (Pasparakis et al. 2014). Certain immune cells, such as CD8+ T cells and antigen-

presenting Langerhans cells reside (Kabashima et al. 2018), reside directly in the epidermis. A special population of dendritic epidermal Gamma delta T cells is also present in the mouse, but not human, epidermis (Kabashima et al. 2018). The dermis contains other immune cell types such as CD4+ T cells, antigen presenting dermal DCs and macrophages, and other innate subsets such as ILCs and mast cells, though the latter may only be found in murine skin (Pasparakis et al. 2014).

T cells comprise an important arm of the adaptive immune response in the skin and are implicated in most forms of chronic inflammatory skin disease. T cells are in fact more abundantly present in skin than in blood (Pasparakis et al. 2014). CD8+ T cells are important in killing other cells and controlling viral infections and cancerous cells CD4+ helper T cells orchestrate immune responses. Naïve CD4+ T cell subsets differentiate into distinct CD4+ helper T cell subsets (Th1, Th2 Th17, Tfh, Treg), defined by lineage-specific transcription factors and production of specific cytokines (O'Shea and Paul 2010). Th1s combat intracellular pathogens, while Th17s target extracellular pathogens. Th2s respond to extracellular parasites, but are also involved in allergic/atopic responses. Tregs help mediate immune tolerance and keep immune responses in check (Zhu and Paul 2008).

Keratinocytes are another key mediator of cutaneous immune responses. They can respond in two phases: in the first to external stimuli, and in the second to inflammatory cytokines produced by immune cells (Dainichi et al. 2018). This interplay creates an inflammatory loop between immune cells and epidermal cells, although the exact

initiating factors in AD have yet to be elucidated (Dainichi et al. 2018). A variety of stimuli can drive keratinocyte cytokine production: allergens, proteases, infection, and barrier disruption (Dainichi et al. 2018). They release cytokines such as IL-33, IL-25, IL-1β and TSLP (Dainichi et al. 2018; Yang et al. 2020). Keratinocytes are known to produce TSLP specifically in AD skin (Yang et al. 2020). Other cells described above also play a role in AD pathogenesis. Patients with AD have dendritic cell and T cell infiltrates, particularly CD4+ T cells, and many patients also have elevated levels of serum IgE (Furue et al. 2017). Another factor in the interconnected immune-epithelial network is the ability of type 2 cytokines such as IL-4 and IL-13 to downregulate expression of filaggrin in keratinocytes (Furue et al. 2017).

AD is typically though of as a type 2 mediated like other atopic diseases, with a Th2 response dominating during acute phases of disease. However, there is evidence to support a role for Th17 cells in Asian and pediatric AD patients (Brunner et al. 2017). While these lineages are thought to be distinct, there is also a substantial amount of evidence for plasticity between different helper T cell subsets (O'Shea and Paul 2010). Most relevant to this study is work showing that Th17 cells exposed to type 2 stimuli have the potential to produce Th2 cytokines (Harrison et al. 2019).

1.5 The early life window facilitates neonatal tolerance to commensal antigens

The neonatal window is a critical time for setting up immune responses. For the past 30 years, the proponents of the hygiene hypothesis have postulated that exposure to appropriate levels of microbes early in life is crucial for appropriate immune tolerogenic

development (Pfefferle et al. 2021). There is mounting evidence to support a unique functionality for neonatal T cells and a predilection for regulatory responses (Rudd 2020). There is also evidence the early life microbiome is correlated with risk of developing asthma (Arrieta et al. 2015). For example, a clinical trial has shown that early life exposure to peanut allergens helps prevent development of food allergy (du Toit et al. 2015).

Our lab has previously shown that tolerance to skin commensals is established in early life (Scharschmidt et al. 2015). In brief, the lab studied this process using a model system of commensal *Staphylococcus epidermidis* expressing the antigen 2W (*S. epi-2W*). Commensal 2W antigen specific CD4+ T cells can be detected using a MHCII tetramer system via flow cytometry. Mice neonatally colonized with *S. epi* develop a high frequency of Tregs (as opposed to effector T cells) among the CD4+ 2W+ T cell compartment, while mice that are not colonized until adulthood do not have this level of antigen-specific tolerance. Mechanisms that contribute to this early life propensity to develop skin commensal-specific Tregs remain an active area of investigation. However, early work showed that a high abundance of polyclonal Tregs in neonatal skin (Scharschmidt et al. 2015), driven in part by hair follicle development, the skin microbiome, and skin chemokines (Scharschmidt et al. 2017), plays an important role. More recent studies have also elucidated that CD301b+ type 2 dendritic cells are important in capturing commensal antigens and facilitating generation of commensal-specific Tregs in the draining lymph nodes (Weckel et al. 2022). Together, the findings

from these studies highlight the critical importance of the neonatal window in establishing tolerance to skin commensals.

However, an open area of interest in the field is understanding what factors disrupt the establishment of tolerance to antigens encountered in early life. Another study from our lab has shown that the immune response is able to distinguish between commensals and pathogens to only establish tolerance to the former (Leech et al. 2019). This is mediated by alpha-toxin production by *S. aureus* and IL-1β production by myeloid cells. This opens the question of what *host* factors may potentially impede the process of establishing tolerance to neonatal antigens.

1.6 Aims of study

There are many open questions regarding the role of filaggrin deficiency in host-commensal interactions in the skin. First, the nature of the barrier defect caused by filaggrin deficiency is not fully characterized especially in mice that do not carry secondary confounding mutations. This has potential implications for how commensals like *S. epidermidis* interact with the host immune system, especially if they differentially locate within the epidermidis in this context. Another important question is to understand the baseline immune defects filaggrin deficiency might confer, particularly in neonates. There are several potential mechanisms by which filaggrin deficiency could alter the baseline immune response: one being increased keratinocyte death or danger signals leading to increased inflammation or another being altered bacteria or antigen penetration into the skin that might further activate cytokines responses from

keratinocyte or other sentinel cell types. Following up on the findings our lab had made regarding the establishment of tolerance to skin commensals, another question is how filaggrin deficiency may alter the host propensity to generation commensal-specific Tregs in the neonatal window. Finally, we want to understand how filaggrin deficiency alters the overall tone of the commensal-specific CD4+ immune response as this might have implications for the nature of T cell inflammation seen in IV or AD.

Chapter 2: Filaggrin deficiency in mice alters the early life $CD4^+$ response to skin commensal bacteria

2.1 Introduction

Early life is a key period for shaping immune responses to cutaneous microbes, with longer-term implications for skin immune homeostasis (Dhariwala and Scharschmidt 2021). We have shown that neonatal colonization preferentially establishes adaptive immune tolerance to skin commensal bacteria via generation of antigen-specific regulatory T cells (Tregs) (Scharschmidt et al. 2015). Studies showing generation of tolerance to orally delivered food antigens when given in the first year of life (Toit et al. 2015) suggest that a similar window of tolerance exists in humans.

In contrast to the Treg-rich response to the commensal *Staphylococcus epidermidis (S. epidermidis)*, *Staphylococcus aureus* (*S. aureus*) colonization of neonatal mice generates a pathogen-specific response enriched for $CD4^+$ effector T cells (Teffs) (Leech et al. 2019). This altered response is driven by bacteria-specific factors, namely toxin production by *S. aureus* that increases skin levels of IL-1β. Whether host-derived factors can undermine the early life Treg-rich $CD4^+$ response to commensal skin bacteria, such as *S. epidermidis*, remains unknown.

Inherited mutations in the gene filaggrin (*FLG*), encoding profilaggrin (FLG), confer the strongest relative risk for Atopic Dermatitis (AD) (Sandilands et al. 2007) and have nearly full penetrance for ichthyosis vulgaris (IV) (Smith et al. 2006). AD has a complex, multifactorial pathogenesis, but altered composition of the skin microbiome during disease flares (Byrd et al. 2017; Kong et al. 2012), as well as before disease onset

(Kennedy et al. 2017; Nakamura et al. 2020b), suggest that a disrupted relationship with skin bacteria plays a central role (Paller et al. 2019b). IV is likewise associated with alterations in skin bacteria or the response to them (Paller et al. 2017; Zeeuwen et al. 2017). FLG serves as a main constituent of keratohyalin granules in stratum granulosum (SG) keratinocytes. FLG monomers support corneocyte integrity by binding intracellular keratins, while their processing into amino acids contributes to stratum corneum (SC) moisturization and acidification (Brown and McLean 2012). Still little is understood about how filaggrin deficiency and its accompanying epidermal changes impact the early life response and longer-term relationship to skin bacteria.

Here we show through studies leveraging systems to track commensal-specific T cells in *Flg*$^{-/-}$ mice that early life filaggrin deficiency alters the quality of the adaptive immune response to *S. epidermidis*. This occurred in the context of increased epidermal penetrability of *Flg*$^{-/-}$ neonates and accompanying subclinical skin immune changes. *Flg*$^{-/-}$ mice demonstrated a shift in the quality of *S. epidermidis*-specific T cells, with relatively fewer Tregs and more T-helper 17 (Th17) cells, which conferred an enduring susceptibility to skin pathology in a model of type-17 inflammation. Collectively these studies reveal new insight into how a clinically-relevant host factor can fundamentally shape the relationship to skin bacteria in the early life window.

2.2 Results

2.2.1 Transcriptional differences in the epidermis of Flg$^{-/-}$ vs. WT neonates

To elucidate the nature of the neonatal barrier dysfunction conferred by filaggrin deficiency and its impact on the response to skin bacteria, we chose to study *Flg*$^{-/-}$ C57BL/6J mice in which expression of Flg protein is eliminated via targeted deletion of putative translation start sites in exons two and three of the *Flg* gene (Kawasaki et al. 2012). Notably, this model is free of secondary gene mutations that confounded interpretations in some earlier studies in flaky tail *Flg*$^{ft/ft}$. Consistent with the original report of this model (Kawasaki et al. 2012), we confirmed reduced *Flg* transcript from the targeted region (Fig. 2.1A), increased skin hyperlinearity (Fig. 2.1B) and relative histologic absence of keratohyalin granules (Fig. 2.1C) in *Flg*$^{-/-}$ neonates as compared to WT mice.

We first employed single-cell RNA sequencing (scRNAseq) to gain in depth understanding of epidermal composition and function in filaggrin-deficient neonatal skin. To magnify any differences elicited by early life bacterial exposure, WT and *Flg*$^{-/-}$ pups were colonized with *S. epidermidis Tu3298*, a strain we have worked with extensively and have shown to elicit immune tolerance in WT neonates (Scharschmidt et al. 2015). Bacteria was applied on day 7 (D7) and D10 of life, and on D12 the epidermis was isolated via trypsinization and processed into single cell suspensions. Epidermal cells from three mice of each genotype were pooled and submitted for scRNAseq using the 10X platform. Analysis of the integrated datasets using Seurat (Hao et al. 2021) revealed sixteen distinct cell clusters (Fig. 2.2A). Cluster identities were assigned based

on examination of the top differentially expressed genes in each cluster and cross referencing with prior epidermal single-cell datasets (Cheng et al. 2018; Joost et al. 2016; Rezza et al. 2016). These clusters included hair follicle associated keratinocytes (1-9), interfollicular keratinocytes (10 & 11), contaminating red blood cells (12), melanocytes (13), dermal papilla/fibroblasts (14), Langerhans cells (15), and T cells (16), all of which were present in both genotypes (Fig. 2.2B). The relative frequency of certain clusters differed subtly in *Flg*$^{-/-}$ versus WT mice, with small increases for the epidermal T cells and hair-follicle associated clusters and a decrease in interfollicular keratinocytes (Fig. 2.2C).

As Flg is produced in the SG and proposed to have its primary function in the interfollicular epidermis, we further probed the transcriptional profile of basal layer (10) and stratum spinosum (SS)/SG (11) keratinocytes by genotype. In both, only a handful of genes showed greater than 2-fold expression change (Fig. 2.1D). As classical barrier function-related genes did not appear on this short list, we specifically examined relative expression of genes involved in epidermal lipid processing and corneocyte formation. Expression of these genes was overall higher in SS/SG than basal keratinocytes, but differences by genotype were overall subtle (Fig. 2.2D-E, 2.1E-F). Of note, several genes in each functional category were detected in only a minority of cells and at low levels (Fig. 2.1E-F), consistent with known limitations of single cell versus bulk RNA sequencing. This included *Flg* transcript, which was detected at low levels in both genotypes (Fig. 2.1F) consistent with engineering of *Flg*$^{-/-}$ mice via gene modification rather than deletion. Thus, to understand the functional significance of the very subtle

transcriptional differences we detected in epidermal lipid and corneocyte genes, we measured transepidermal water loss (TEWL) on back skin of 1 week old *Flg*$^{-/-}$ and WT mice. In agreement with prior work (Kawasaki et al. 2012), we did not observe genotype-associated differences in TEWL (Fig. 2.2G).

2.2.2 The epidermis of Flg$^{-/-}$ *neonates demonstrates increased permeability to exogenous substances*

Other studies of Flg-deficient mice have detected heightened skin fragility and exogenous penetration of substances, at least in adulthood (Kawasaki et al. 2012; Muhandes et al. 2021; Scharschmidt et al. 2009a). We thus examined our scRNAseq data for interfollicular expression of genes implicated in desmosomal disruption (Sumigray et al. 2014), tight junctions (Morita et al. 1998; Yuki et al. 2016), calcium signaling following physical skin injury (Celli et al. 2021), and barrier repair (Uberoi et al. 2021) (Fig. 2.2F & 2.1G). This showed a mixed picture with modest increases in *Ahr, Cldn1, Cyp1a1* and *Ocln* among *Flg*$^{-/-}$ keratinocytes, while expression of *Cldn12, Hsp90aa1, Smpd1, Slc9a1* and *Tjp1* was somewhat enriched in WT keratinocytes. Next, to functionally test epidermal permeability to exogenous substances, we exposed skin taken from D6 WT and *Flg*$^{-/-}$ mice to media containing calcium green dye in a Franz chamber (Fig. 2.1H). After overnight incubation at 37°C, the surface of the skin was washed to remove excess dye and confocal imaging was performed, revealing deeper dye penetration into *Flg*$^{-/-}$ skin (Fig. 2.2H white arrows). Calculation of dye penetration volume throughout the surveyed tissue confirmed more extensive penetration into the epidermis of *Flg*$^{-/-}$ versus WT mice (Fig. 2.2I-J). Collectively, these studies support and

add to earlier work suggesting that neonatal filaggrin deficiency is associated with a subtle barrier defect, specifically marked by increased permeability to exogenous substances (Kawasaki et al. 2012; Muhandes et al. 2021; Scharschmidt et al. 2009b).

2.2.3 The skin of Flg$^{-/-}$ neonates demonstrates select increases in cytokines, CD4^{+} T cells, and myeloid cells

Epidermal infiltration can often elicit cutaneous immune activation. Although histology did not reveal overt inflammation in neonatal *Flg*$^{-/-}$ skin (Fig. 2.1C), our scRNAseq revealed a subtle relative increase in the proportion of epidermal T cells (Fig. 2.2C). To further investigate the impact of increased epidermal permeability in *Flg*$^{-/-}$ neonates on immune activation, we performed flow cytometry on whole skin and skin-draining lymph nodes (SDLN) of specific pathogen free (SPF) 14 day-old *Flg*$^{-/-}$ and WT mice. This revealed an increased percentage and number of CD4^{+} T cells in *Flg*$^{-/-}$ neonatal skin (Fig. 2.3A-B), within which the percentage of Tregs was comparable (Fig. 2.3C). Frequencies but not numbers of skin CD8^{+} T cells were decreased in *Flg*$^{-/-}$ neonates, and no consistent differences were noted among dermal $\gamma\delta$ T cells (Fig. 2.3D-F). In the SDLN, no consistent trends in these populations were seen except for a slight increase in the percentage of Tregs in *Flg*$^{-/-}$ mice only seen in one experiment (Fig. 2.4A-F).

To determine whether antigen-presenting cells such as dendritic cells were altered in the skin of *Flg*$^{-/-}$ neonates, we used a previously described antigen uptake model (Leech et al. 2019). Mice were colonized on day 9 of life with *S. epi* expressing the stable fluorophore zsgreen. Flow cytometric analysis of the skin on D10 allows for detection of

cells that take up bacteria by gating on zsgreen^{+} cells. While we saw no difference in the frequency or number of total zsgreen^{+} cells in WT vs *Flg*$^{-/-}$ mice (data not shown), we were able to note that CD11c^{+}MHCII^{+} DCs that took up commensal antigen had higher expression of CD86, indicating perhaps increased ability to stimulate T cells (Fig. 2.5A-B). We also characterized the frequency and number of dendritic cell populations in the skin. Our panel allowed for detection of CD103^{+} DC1s, CD11b^{+}Epcam^{lo} Langerhans cells, CD11b^{-}Epcam^{+} DCs, CD301b^{+}CD11b^{hi} DCs, and CD301b^{+}CD11b^{lo} DCs (Fig. 2.5C). While examining the frequencies and numbers of these cells across experiments, the only consistent difference was an increase in CD11b^{-}Epcam^{+} DCs in *Flg*$^{-/-}$ mice (Fig. 2.5D-E). These cells, however, are not a well-described population in the skin and represent an overall very small proportion of skin APCs. Thus, the significance of this difference in unclear.

Examination of our scRNAseq data for interfollicular keratinocyte expression of innate immune and alarmin-associated genes (Lessard et al. 2013) showed a mixed picture with small differences in gene expression in either direction (Fig. 2.3G, Fig. 2.4G). As cytokine transcripts were largely undetectable by scRNAseq (Fig. 2.4G), we performed a qPCR array for 38 cytokines on whole skin from D12 *Flg*$^{-/-}$ and WT mice following *S. epi* colonization. This revealed non-significant trends towards elevated expression in *Flg*$^{-/-}$ neonatal skin for *Il6, Il25, Il1f9* (Fig. 2.4H-J) and more substantial elevations of *Ccl2, Cxcl5* and *Il10* (Fig. 2.4K-M) that were significant by individual unpaired t-test but not when corrected for multiple comparisons. We also performed a high-sensitivity Luminex cytokine array on skin homogenates from D12 *S. epidermidis*-colonized skin.

This revealed slight elevations in IL-1β, IL-7 and CXCL2 that were individually significant but not following Bonferroni correction (Fig. 2.3H). Notably there were no differences in production of the type 2 cytokines IL-4, IL-5 or IL-13 (Fig. 2.4O-Q). Collectively, these data suggest that neonatal filaggrin deficiency is accompanied by subtle immunological alterations.

2.2.4 $Flg^{-/-}$ neonates demonstrate a shift towards Teffs rather than Tregs in the commensal-specific $CD4^+$ response

As prior studies have shown that spontaneous or induced inflammation in the skin of adult filaggrin-deficiency mice is influenced by the presence of skin bacteria (Archer et al. 2018), we next examined if the neonatal immune response to commensal skin bacteria is altered in *$Flg^{-/-}$* mice. To do so, we employed our established model in which colonization with a strain of *S. epidermidis* Tu3298 expressing the model antigen 2W (*S. epi*-2W) enables tracking of *S. epi*-specific $CD4^+$ T cells using 2W-loaded major histocompatibility complex (MHC) class II tetramers (Scharschmidt et al. 2015). Litters of *$Flg^{-/-}$* pups were cross-fostered with age-matched WT C57BL/6J mice starting on postnatal D7 to promote microbial sharing and mitigate potential genotype-associated differences in microbial flora composition. Repeated skin colonization with *S. epi*-2W was performed on postnatal D7, D10 and D13, before harvest at weaning age to examine the primary immune response to *S. epi* (Fig. 2.6A). Importantly, we confirmed that *S. epi*-2W application resulted in comparable levels on *$Flg^{-/-}$* and WT skin up to 10 days later (Fig. 2.6B). Examination of the SDLN by flow cytometry at postnatal D21-24 revealed a non-significant trend towards increased total numbers of 2W-specific $CD4^+$ T

cells in *Flg*$^{-/-}$ pups (Fig. 2.6C). More notably, among these commensal-specific CD4^{+} in *Flg*$^{-/-}$ as compared to WT mice, there was a shift towards *Foxp3*^{neg} effector CD4^{+} cells (Teffs) and away from Tregs, as reflected by increased percentages of *S. epi*-specific Teffs, reduced percentages of *S. epi*-specific Tregs and an elevated ratio of *S. epi*-specific Teffs to Tregs (Fig. 2.6D-G). Consistent with our previous studies (Leech et al. 2019), the absolute number of tetramer positive cells recovered from the SDLN of each neonate was quite variable and no statistically significant differences were observed in total numbers of *S. epi*-specific Tregs or Teff (Fig. 2.6H). No substantial genotype differences were noted among polyclonal SDLN CD4^{+} cell populations (Fig. 2.6I). Thus, neonatal filaggrin deficiency leads to an altered early life immune response to commensal skin bacteria, demarcated by relative skewing towards *S. epi*-specific Teffs.

2.2.5 Teff-skewing of the commensal-specific CD4^{+} response in Flg$^{-/-}$ mice can be recalled in adulthood and is characterized by a Th17 phenotype

Examination of adult skin by flow cytometry demonstrated that early life elevations in skin CD4^{+} T cells among *Flg*$^{-/-}$ mice resolved with age, suggesting general immune activation had abated (Fig. 2.7A-B). To understand if Teff-skewing of commensal-specific CD4^{+} T cells persisted and could be recalled in adulthood, we performed 2W antigen re-exposure in neonatally colonized mice via intradermal injection of 2W peptide and incomplete Freund's adjuvant (IFA) (Fig. 2.8A). This allowed re-expansion of *S. epi*-specific memory cells in skin while minimizing the potential impact of genotype-associated differences in adult epidermal barrier function (Fig. 2.8A). Flow cytometry of skin revealed a greater percentage of *S. epi*-specific Teffs in *Flg*$^{-/-}$ as compared to WT

adult mice (Fig. 2.8B), a pattern not replicated in polyclonal skin $CD4^+$ T cells (Fig. 2.8C).

We next sought to define the phenotype of *S. epi*-specific Teffs in *Flg*$^{-/-}$ mice, something precluded in neonatal mice due to limited cell numbers. For this, we used our previously established (Leech et al. 2019; Scharschmidt et al. 2015) *S. epi*-2W re-challenge in which 4-6 week old WT and *Flg*$^{-/-}$ mice, previously colonized as neonates with *S. epi*-2W, were re-exposed to the bacteria three times in conjunction with light tape stripping to disrupt the skin barrier and augment the magnitude of 2W-specific response (Fig. 2.8D). Harvest of SDLN following re-challenge revealed persistently elevated percentages of *S. epi*-specific Teffs in *Flg*$^{-/-}$ versus WT mice (Fig. 2.8E). This occurred in the setting of comparable numbers of $2W^+$ $CD4^+$ T cells and equivalent percentages of polyclonal Teffs (Fig. 2.7C and 2.8F). The absolute number of *S. epi*-specific Tregs was also somewhat reduced in *Flg*$^{-/-}$ mice (Fig. 2.7D-E). *Ex vivo* re-stimulation of SDLN with PMA and ionomycin revealed increased percentages of IL-17A-producing cells among *S. epi*-specific versus polyclonal Teffs across both genotypes (Fig. 2.8G-I). There was very little production of either IFNγ or IL-13, with even less IFNγ in *S. epi*-specific Teff, and no differences by genotype (Fig. 2.7F-K). Thus, early life differences in the response to commensal bacteria can be recalled in adult *Flg*$^{-/-}$ mice, revealing persistent skewing towards Teffs that demonstrate a Th17 phenotype.

2.2.6 Increased sensitivity to imiquimod-induced type 17 ear inflammation in Flg$^{-/-}$ *mice*

FLG mutations are associated with increased risk of atopy, and *S. epi*-specific T cells have been reported to demonstrate plasticity between a Th17 and Th2 phenotype (Harrison et al. 2019). We therefore tested whether the *S. epi* response in *Flg*$^{-/-}$ mice might alter susceptibility to Th2-type inflammation in an established house dust mite (HDM) dermatitis model (Kanemaru et al. 2019). Mice were neonatally colonized with *S. epi*-2W then 3.5 weeks later underwent five bi-weekly treatments with HDM paste to the back skin, preceded by sodium dodecyl sulfate (SDS) to optimize penetration. The final three treatments were accompanied by *S. epi*-2W re-colonization. No differences in Th2 cytokines were observed by genotype or among commensal-specific CD4^{+} Teffs (Fig. 2.9A-C).

We then sought to determine whether *Flg*$^{-/-}$ mice might be more susceptible to MC903, the application of which is commonly used to model an atopic dermatitis-like dermatitis in mice. MC903 is a vitamin D analogue that drives TSLP-mediated type 2 inflammation, with recruitment of eosinophils and production of type 2 cytokines (Li et al. 2009). However, neutrophils are also known to be associated with MC903 treatment, indicated some component of concurrent type 17 inflammation (King et al. 2019). In WT and *Flg*$^{-/-}$ mice that were colonized neonatally with *S. epi*, we treated their ears as adults with MC903 daily for 7 days, in conjunction with three rounds of *S. epi* re-colonization every 3 days (Fig. 2.10A). Ear thickness was measured daily during treatment and three days after the final treatment, ear skin was harvested for flow cytometry. *Flg*$^{-/-}$ mice demonstrated greater ear thickness in the later stages of MC903 treatment compared to WT mice (Fig. 2.10B). Flow cytometry analysis revealed that while *Flg*$^{-/-}$ mice showed

no difference in eosinophil frequencies or numbers, neutrophil numbers were increased in the ears of *Flg*$^{-/-}$ mice (Fig. 2.10C-D).

Due to this neutrophil phenotype and observations that a Th17 phenotype among *S. epi*-specific Teff and type 17 inflammation is detectable in pediatric AD (Brunner et al. 2018; Esaki et al. 2016) and forms of ichthyosis (Malik et al. 2019; Paller et al. 2017), we next opted to test if the altered commensal-specific response in *Flg*$^{-/-}$ mice impacted their susceptibility to a model of type 17 inflammation. Imiquimod-induced ear swelling has previously been shown to be reduced in adult mice when oral antibiotics are used to broadly reduce commensal flora (Zákostelská et al. 2016). To test if imiquimod-induced skin inflammation relies more specifically on the presence of commensal skin bacteria, we performed imiquimod experiments in WT and *Flg*$^{-/-}$ mice with topical application of Neosporin (Neo) or Aquaphor (Ctl) ointment and real vs. sham cage changes (Uberoi et al. 2021) (Fig. 2.11A). This revealed significant reductions in ear swelling as measured by daily caliper readings among Neo-treated *Flg*$^{-/-}$ mice (Fig. 2.11B), with a similar but non-significant trend among WT mice (Fig. 2.11C).

Next, we compared the effects of imiquimod in *Flg*$^{-/-}$ vs. WT mice neonatally colonized with *S. epi*-2W. Here, adult mice were re-colonized three times with *S. epi*-2W in tandem with five days of daily imiquimod application to the ears (Fig. 2.11D). As previously reported in adult Balb/c *Flg*$^{-/-}$ mice (Muhandes et al. 2021), we noted a slight baseline increase in the ear thickness of adult C57BL/6 *Flg*$^{-/-}$ mice (Fig. 2.9D). Blinded daily measurements during imiquimod treatment further revealed a greater percentage

(Fig. 2.11E) and absolute increase (Fig. 2.9E) in ear thickness among *Flg*$^{-/-}$ mice. In the SDLN, this was accompanied by increased IL-17 production among *S. epi*-specific (Fig. 2.11F-G) but not polyclonal (Fig. 2.11H) CD4^{+} Teffs. Collectively these data suggest that persistent differences in the early life response to commensal bacteria in adult *Flg*$^{-/-}$ mice are associated with increased sensitivity to a microbiome-dependent model of type-17 skin inflammation.

2.3 Discussion

Our studies reveal notable contributions of filaggrin to epithelial barrier integrity and shaping of the adaptive immune response to commensal skin bacteria. Whereas neonatal filaggrin deficiency minimally affects “inside-out” barrier function, i.e. TEWL and genes involved in stratum corneum lipid processing and corneocyte formation, it confers molecular and functional metrics of “outside-in” barrier dysfunction as well as transient neonatal accumulation of skin CD4^{+} T cells and subtle elevations in certain cutaneous innate cytokines. Our results confirm and extend upon prior studies in murine models of isolated filaggrin deficiency (Kawasaki et al. 2012; Muhandes et al. 2021), reinforcing that large spontaneous TEWL differences (Leisten et al. 2013) reported in *Flg*$^{ft/ft}$ mice may have been attributable to secondary confounding mutations, e.g. in Tmem79 (Muhandes et al. 2021). Together these results highlight an important concept in skin research: distinct, separable molecular mechanisms maintain “outside-in” vs. “inside-out” barrier function.

Amongst our most striking results was the altered immune response to *S. epi* in *Flg*$^{-/-}$ neonates, denoted by a shift towards commensal-specific Teffs (as opposed to Tregs), which could be recalled as a memory response in the skin of adult mice. The etiology of this shift may be multifactorial, but the subtle increase in IL-1β levels in neonatal *Flg*$^{-/-}$ skin is notable considering that topical IL-1β application prompts a similar shift toward *S. epi*-specific Teffs in WT neonates (Leech et al. 2019), that IL-1 family cytokines have been implicated in dermatitis in *Flg*$^{ft/ft}$ mice (Archer et al. 2018; Schwartz et al. 2019), and that these cytokines are often elevated in AD patients with *FLG* mutations (Kezic et al. 2012). Likely sources of this IL-1β include skin myeloid populations responding to greater epidermal infiltration by skin bacteria as a consequence of *FLG*-dependent barrier dysfunction.

S. epi-specific CD4^{+} Teffs showed enriched production of IL-17A but not IFNγ or IL-13, consistent with other work examining CD4^{+} responses to *S. epidermidis* (Harrison et al. 2019). We used two mixed models of type 2/type 17 inflammation: HDM dermatitis and MC903, which did not reveal any type 2 mediated genotype-dependent differences. However, the latter did show *Flg*$^{-/-}$ mice had increased numbers of neutrophils, and imiquimod treatment of *S. epi*-2W-colonized *Flg*$^{-/-}$ mice yielded increased ear swelling and heightened IL-17A production by *S. epi*-specific Teffs. This is in line with other studies showing type 17 inflammation in Flg-deficient mice (Archer et al. 2018; Bonefeld et al. 2016c; Hoff et al. 2015). The lack of spontaneous dermatitis in adult *Flg*$^{-/-}$ mice is consistent with other studies (Kawasaki et al. 2012; Muhandes et al. 2021) and our own work showing that cutaneous sequela of altered early life responses to skin bacteria are

manifested only upon secondary bacterial challenge in an inflammatory context (Leech et al. 2019; Scharschmidt et al. 2015). Our ability to reduce imiquimod-induced ear swelling via topical Neosporin further reinforces that the immune response to skin bacteria plays a critical role in this model and more generally in these type 17 responses.

Collectively, these results have intriguing implications for how we conceptualize the immune relationship to skin bacteria in FLG-deficient humans. The evidence for FLG-dependent shifts in composition of the AD skin microbiome remain mixed (Clausen et al. 2018; Mierlo et al. 2022; Nath et al. 2020; Zeeuwen et al. 2017). We specifically sought to minimize the impact of any such compositional differences by cross-fostering and co-housing *Flg*$^{-/-}$ and WT mice and, where applicable, repeatedly applying *S. epi*-2W to ensure comparable bacterial burden before examining *S. epi*-specific CD4^{+} T cells. The Teff-skewed response to *S. epi* in this context suggests that, irrespective of any effects on microbiome composition, filaggrin-deficiency leads to a fundamental shift in the adaptive immune response to a given commensal bacterium.

Although we did not seek to model human disease in any of these studies, it is intriguing to consider potential parallels to IV and AD patients with *FLG* mutations. The skin immune signature of IV has not been deeply characterized, but IL-17 features prominently in other ichthyoses (Malik et al. 2019; Paller et al. 2017) and a small cohort study found elevated blood Th17 cells in subjects carrying *FLG* mutations (Bonefeld et al. 2016c). AD is classically considered a type 2 inflammatory condition, but AD

subtypes, e.g. pediatric (Brunner et al. 2018), intrinsic (Suárez-Fariñas et al. 2013) and Asian (Noda et al. 2015) AD patients, can display a concurrent type 17 signature. Targeting type 17 immune responses in established AD has not proven efficacious (Ungar et al. 2021). However, murine studies suggest an earlier role for IL-17 in potentiating subsequent Th2 responses (Nakajima et al. 2014). Our work supports a model in which *FLG* mutations confer early differences in the host response to commensal microbes, which could facilitate early immune 'kindling' on the path to atopy and or lead to heightened immune responses to commensal bacteria in the setting of AD flares (Byrd et al. 2017), even in the absence of additional factors such as *S. aureus* colonization (van Drongelen et al. 2014).

We are currently in an explosive phase of therapeutic development for AD. It will be fascinating to learn how *FLG* mutation status influences the efficacy of different targeted immune pathways (Wu and Guttman-Yassky 2020) or microbially-directed (Nakatsuji et al. 2021) therapies. Knowledge gained at the intersection of these therapeutic advances and mouse dermatitis models will significantly advance our understanding of the important, nuanced relationship between filaggrin, skin barrier function, and cutaneous atopy.

2.4 Figures

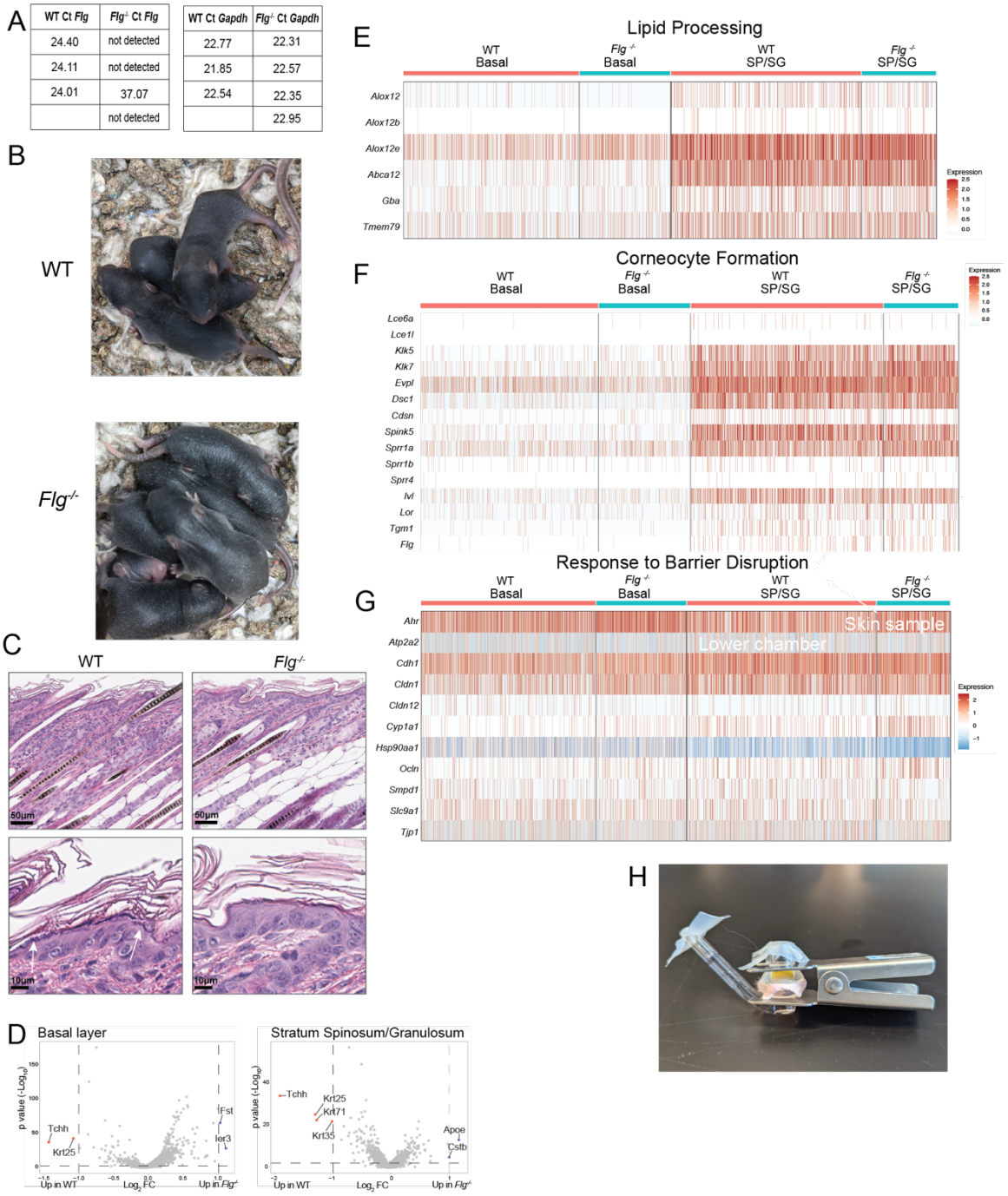

Figure 2.1: Characterization of *Flg*$^{-/-}$ mice (A) qRT-PCR of *Flg* expression and housekeeping gene GAPDH in the skin of neonatal *Flg*$^{-/-}$ and WT mice. (B) Image of D7 neonatal *Flg*$^{-/-}$ and WT mice. (C) Histology of D10 skin from WT and *Flg*$^{-/-}$ mice, with arrows indicating keratohyalin granules in WT mice in bottom left magnified image. (D) From scRNAseq described in Fig. 1, volcano plots of -Log10 p values and (Log2FC) of gene expression in WT and *Flg*$^{-/-}$ mice for Basal and SP/SG keratinocytes, with Log2FC >1 colored red or blue by genotype. (E) Heatmaps showing individual cell expression of genes involved in lipid processing, (F) corneocyte formation and (G) response to barrier disruption. (H) Example of Franz chamber set up to measure epidermal penetration of calcium green dye.

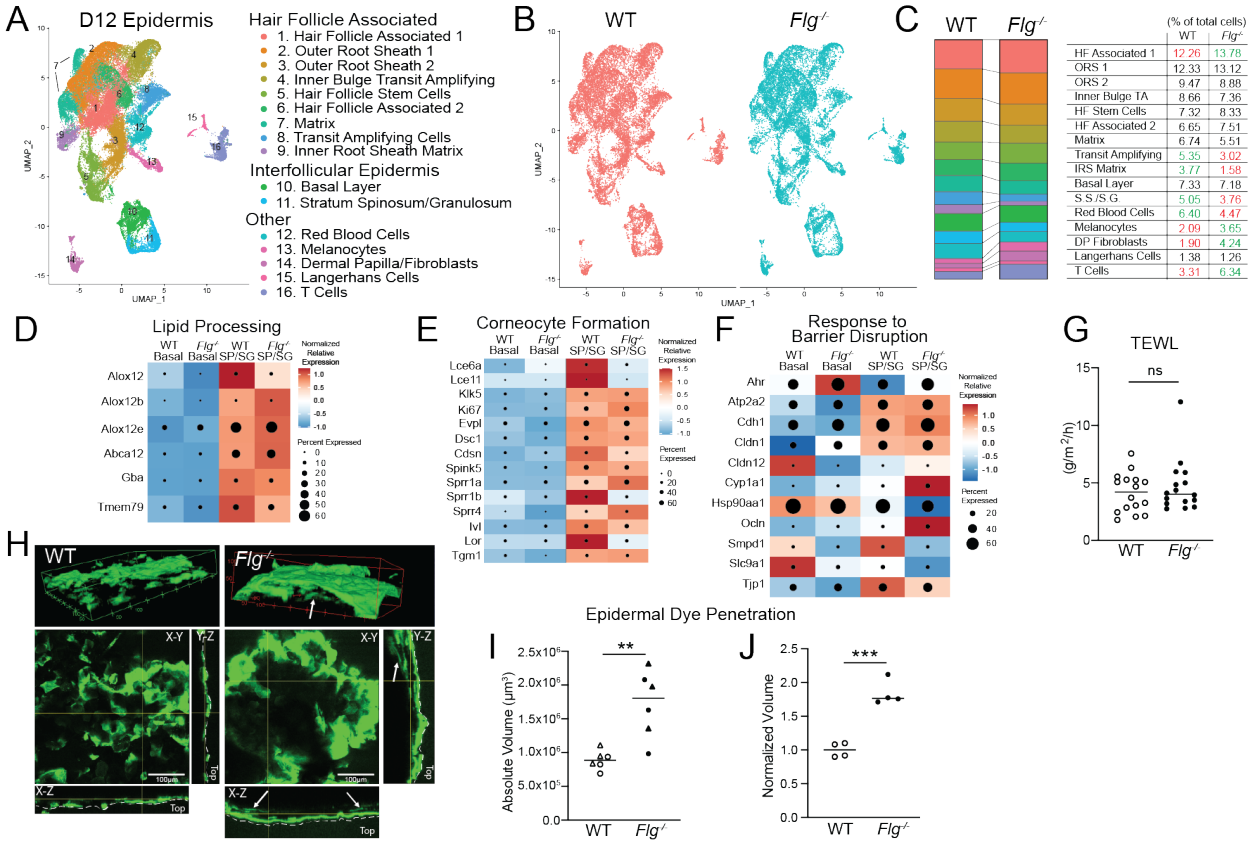

Figure 2.2: Single cell RNA sequencing of WT and *Flg*$^{-/-}$ neonates and increased permeability to exogenous substances in *Flg*$^{-/-}$ neonates. scRNAseq of epidermis of D12 WT and *Flg*$^{-/-}$ pups colonized with *S. epidermidis* on postnatal day 7 (D7) and D10. UMAP plots of (A) combined clusters and (B) split by genotype with (C) relative cell proportions (red= >1.25% lower, green= >1.25% higher). For (D) lipid processing, (E) corneocyte formation, and (F) barrier disruption response genes, normalized relative expression heat maps overlaid with percentage expression in WT and *Flg*$^{-/-}$ Basal and SP/SG clusters. (G) TEWL in week old mice pooled from 3 experiments. (H) Images of calcium green dye on D6 *Flg*$^{-/-}$ and WT skin in Franz chambers with (I) total penetration volume (one of two representative experiments, 3 technical replicates from 2 mice per group shown), and (J) dye penetration volume normalized to WT mice average (each dot represents average of three technical replicates from 1 mouse; data pooled from two experiments). P value symbols: ns = not significant, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$. Basal, Basal layer; SP/SG, Stratum Spinosum/Stratum Granulosum, WT, wildtype, Log2FC, log two-fold change, TEWL, transepidermal water loss

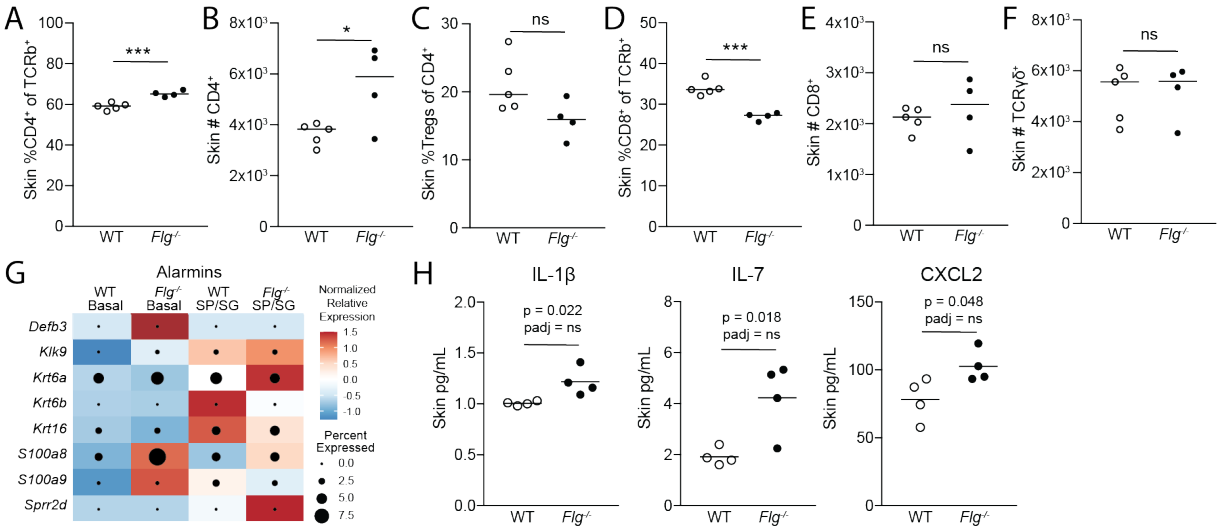

Figure 2.3: Select increases in cytokines and CD4⁺ T cells in skin of *Flg*$^{-/-}$ neonates. Flow cytometry of (A-C) CD4^{+}, (D-E) CD8^{+}, and (F) dermal γδ T cells from whole skin of 2 week old WT and *Flg*$^{-/-}$ mice, data from 1 of 2 representative experiments. (G) Heat map of normalized relative expression and percent cells expressing for innate immune and alarmin-associated genes in the WT and *Flg*$^{-/-}$ Basal layer and Stratum Spinosum/Stratum Granulosum (SP/SG) scRNAseq clusters. (H) Cytokine concentration by Luminex in whole skin homogenates of D12 *S. epidermidis* pre-colonized WT and *Flg*$^{-/-}$ mice. ns = not significant, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, padj = t-test following Bonferroni correction.

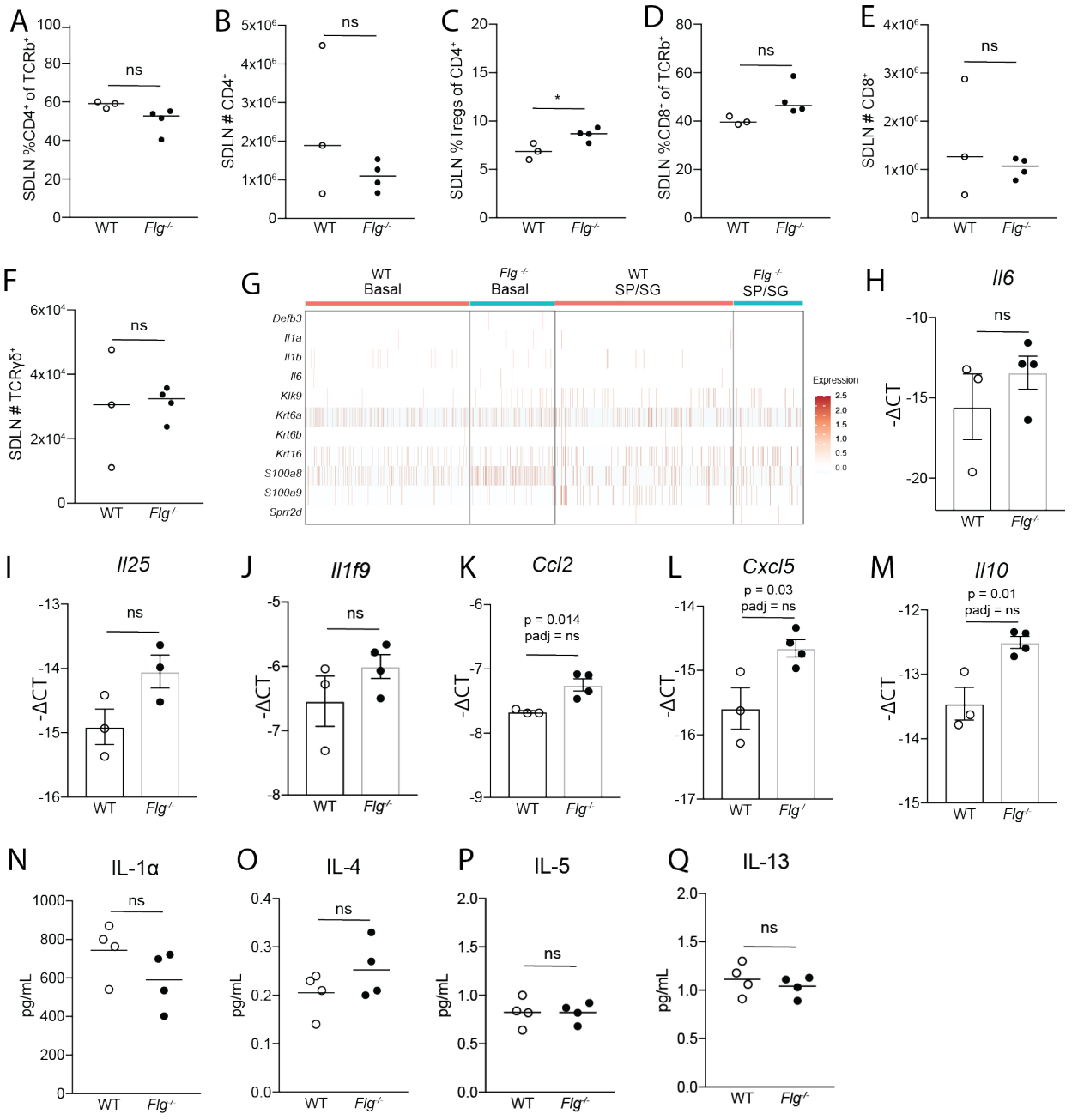

Figure 2.4: Neonatal immune profiling. Flow cytometry of (A-C) $CD4^+$, (D-E) $CD8^+$, and (F) γδ T cells from skin draining lymph nodes (SDLN) of 2 week old WT and *Flg*$^{-/-}$ mice, data from 1 of 2 representative experiments. (G) Heatmap of individual cell expression of genes involved in innate alarmin response from scRNAseq described in Figs.1 & 2, with the addition of *Il1a*, *Il1b*, and *Il6* genes to show negligible expression. (H-M) Selected data from qRT-PCR array for 38 cytokines on whole skin from D12 WT and *Flg*$^{-/-}$ mice following *S. epi* colonization. (N-Q) Further data from a Luminex array from D12 WT and *Flg*$^{-/-}$ skin described in Fig. 2H. P value symbols: ns = not significant, $p > 0.05$, * $p \leq 0.05$, padj reflects Bonferroni correction where appropriate.

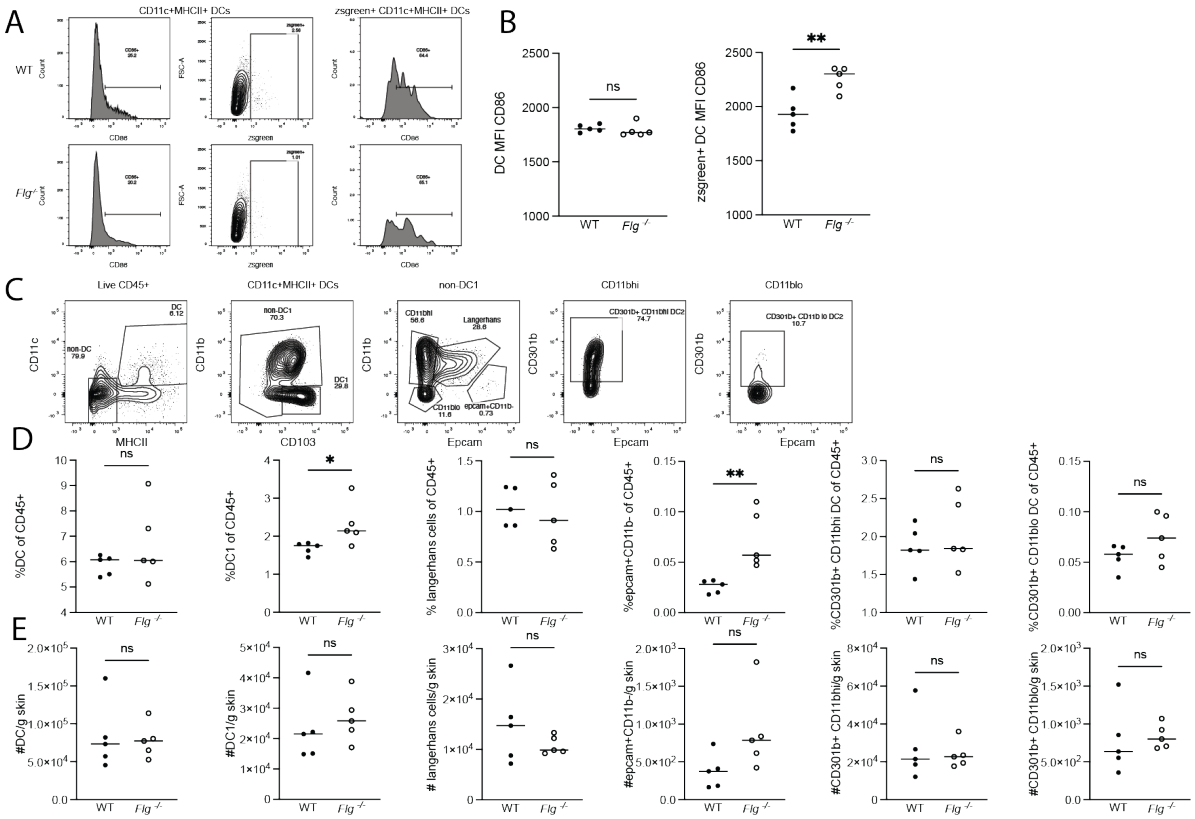

Figure 2.5: **Profiling of dendritic cell antigen uptake reveals altered activation and frequency of select cell populations.** Neonatal mice were colonized on D9 with *S.epi* expressing the stable fluorophore zsgreen (*S. epi-zsgreen*) to measure antigen uptake and characterize neonatal antigen presenting cell populations. (A) Flow cytometry was performed on D10 pups and CD86 MFI was measured in both bulk (left) and zsgreen+ (right) $CD11c^{+}MHCII^{+}$ DCs, quantified in (B) with one of three representative experiments shown. (C) Flow cytometry gating of immune cell populations within CD45+ gate, with (D) frequencies and (E) numbers quantified from one of two representative experiments.

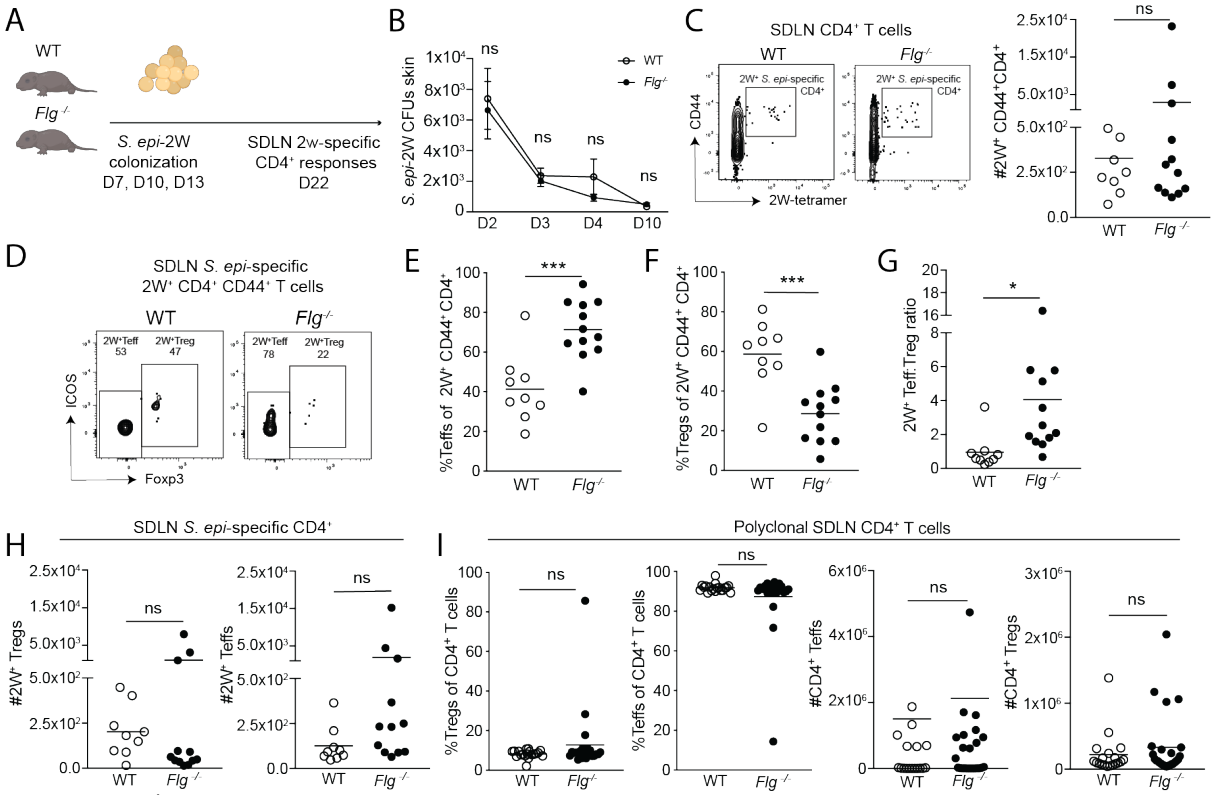

Figure 2.6: *Flg*$^{-/-}$ neonates demonstrate a shift in the commensal-specific CD4^{+} towards a Teff rather than Treg phenotype. (A) Primary antigen-specific response to *S. epidermidis:* WT and *Flg*$^{-/-}$ mice colonized on days 7/10/13 of life. SDLN harvested at weaning age. (B) *S. epi*-2W CFUs from skin swabs 2/3/4 days post colonization and from homogenized whole skin 10 days after colonization (1 of 2 experiments shown). (C) Gating of 2W-MHCII tetramer^{+}CD44^{+} cells (pre-gated on live DUMP^{neg}TCRβ^{+}CD4^{+}); total numbers in WT and *Flg*$^{-/-}$ mice on right. Within SDLN *S. epi*-specific 2W^{+}CD4^{+}CD44^{+} T cells: (D) gating of Tregs and Teffs and quantification of (E) %Teffs (F) %Tregs and (G) ratio of Teffs:Tregs. If fewer than 20 total 2W^{+} cells recovered, or poor tetramer staining, samples excluded for rigor. (H) Quantification of SDLN 2W^{+} Teffs and Tregs. (I) Polyclonal (CD4^{+}) percent and absolute numbers of Teffs and Tregs in SDLN. (C-I) Data pooled from 4 experiments. ns = not significant, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. SDLN, Skin Draining Lymph Nodes;

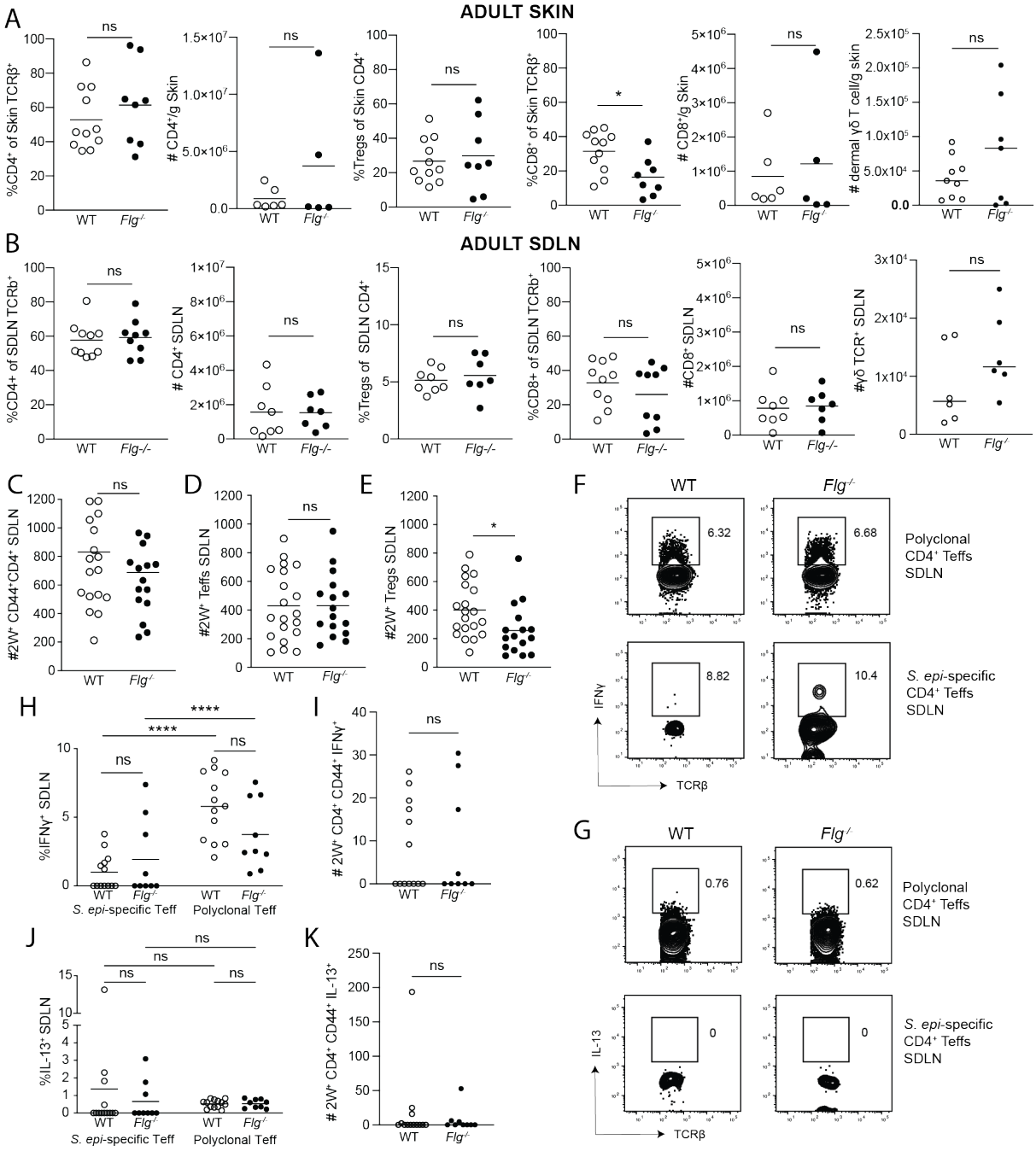

Figure 2.7: Immune cell profiles of adult *Flg*$^{-/-}$ mice at baseline and after secondary challenge. In uncolonized adult WT and *Flg*$^{-/-}$ mice, quantification of CD4$^+$, Tregs, CD8$^+$ and gamma delta T cells in (A) skin and (B) SDLN, data pooled from 2-4 of 4 replicate experiments. From tape stripping experiments shown in Fig.4, quantification of SDLN (C) *S. epi*-specific (2W$^+$CD44$^+$) CD4$^+$ T cells (D) *S. epi*-specific CD4$^+$ Teffs, and (E) *S. epi*-specific Tregs (data pooled from 5 replicate experiments). Representative gating of (F) %IFNγ$^+$ and (G) %IL-13$^+$ *S. epi*-specific (2W$^+$CD44$^+$CD4$^+$) cells and polyclonal (CD44$^+$CD4$^+$) cells in WT and *Flg*$^{-/-}$ mice with quantification of the percentage and numbers of (H-I) IFNγ$^+$ and (J-K) IL-13$^+$ cells (data pooled from 3 replicate experiments). Individual T tests (or 2 way ANOVA with Sidak's multiple comparison test in H and J) were used. P value symbols: ns = not significant, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. SDLN, skin draining lymph nodes

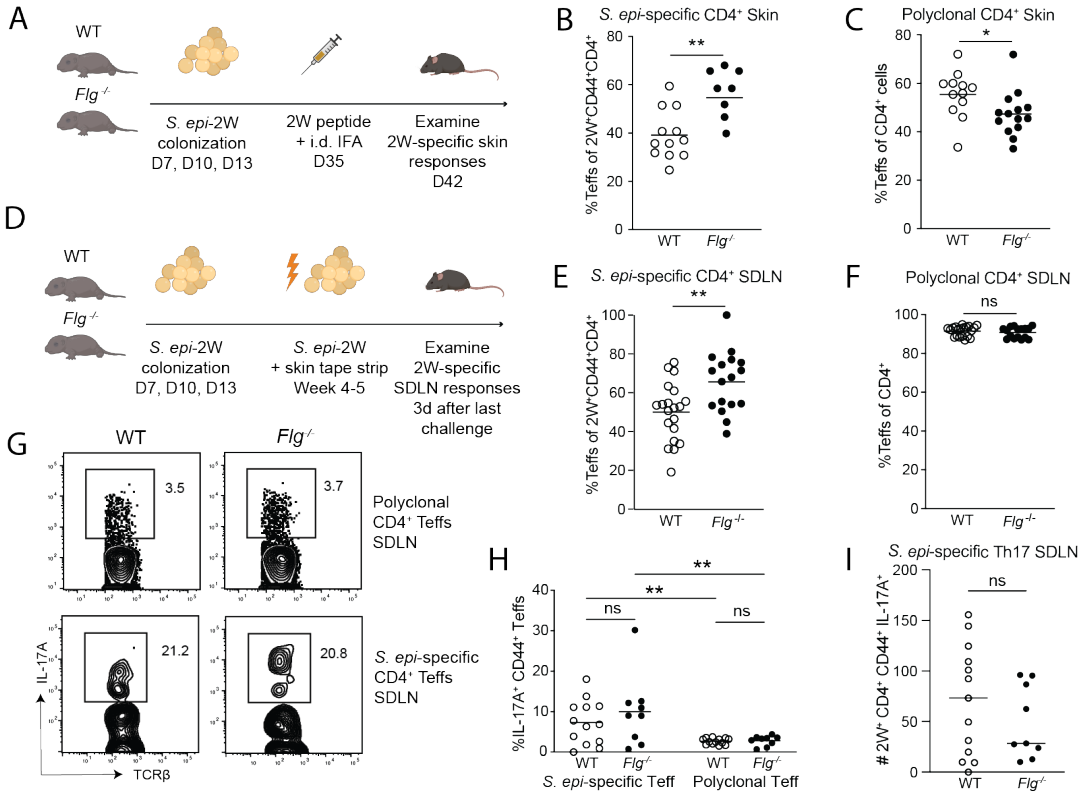

Figure 2.8: Teff-skewing of the commensal-specific CD4⁺ response in *Flg*$^{-/-}$ mice can be recalled in adulthood and is characterized by a Th17 phenotype. (A) Antigen recall model with i.d. injection of 2W peptide and IFA with skin harvest one week later (B-C) % Teffs of *S. epi*-specific (2W⁺CD44⁺CD4⁺) cells and polyclonal (CD4⁺) cells in skin (pooled from 3 experiments) (D) *S. epi*-2W recall model with three colonization plus tape stripping followed 3 days later by SDLN harvest to look at cytokine, (E) % Teffs of *S. epi*-specific and (F) polyclonal CD4+ in SDLN (pooled from 5 experiments). (G) Example gating of % IL-17⁺ in polyclonal (CD44⁺CD4⁺) (top) and *S. epi*-specific (2W⁺CD44⁺CD4⁺) cells (bottom) with (H) quanitifed frequencies and (I) numbers (from combined bound/unbound tetramer fractions) (data pooled from 3 replicate experiments). Individual T tests (or 2 way ANOVA with Sidak's multiple comparison test in H) were used. ns = not significant, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$. i.d., intradermal, IFA, incomplete Freund's adjuvant,

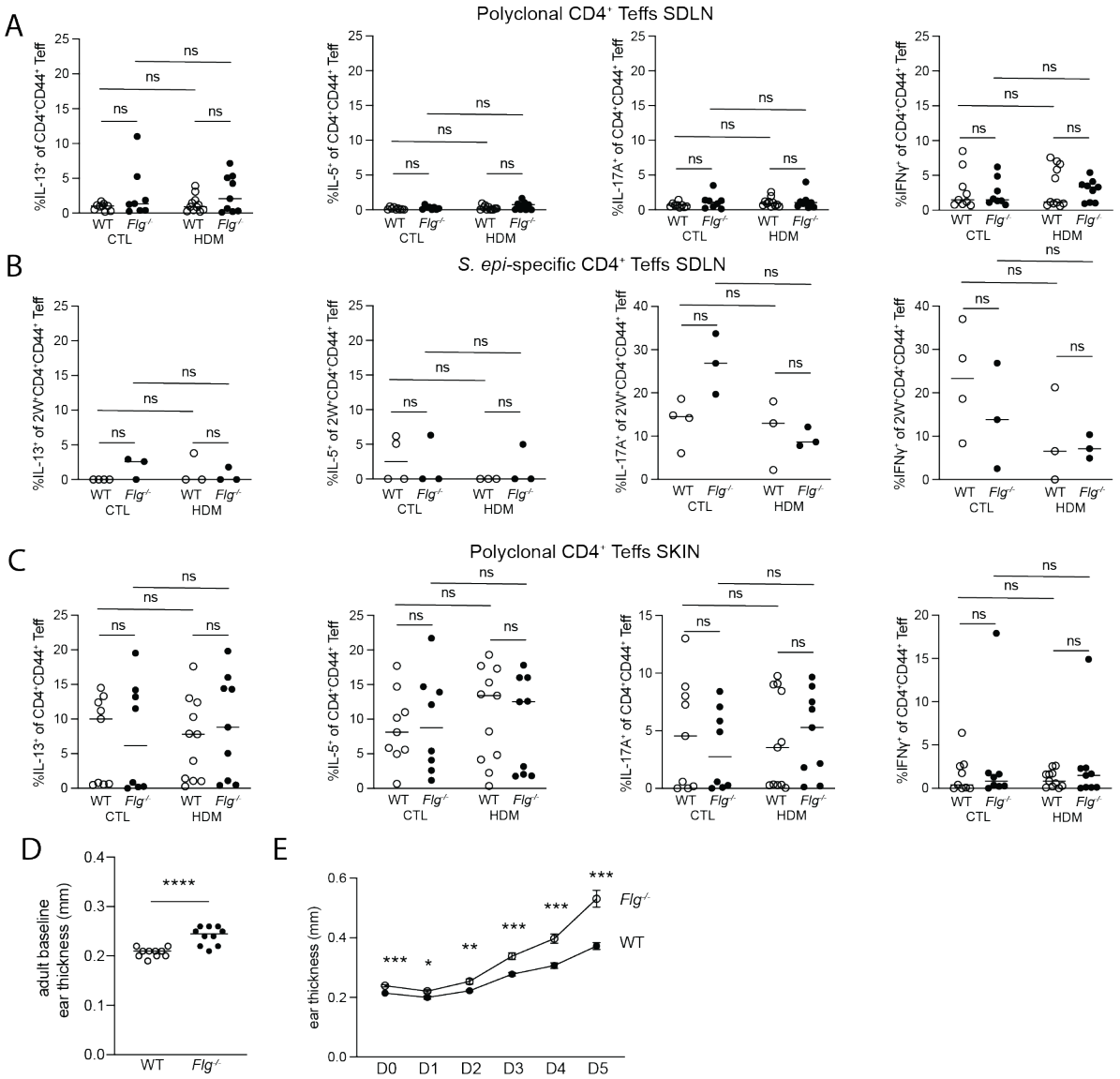

Figure 2.9: House dust mite model in *Flg*$^{-/-}$ mice and profiling of ear inflammation. Mice were neonatally colonized with *S. epi*-2W. Three weeks later, mice were treated with SDS to weaken barrier 2 hours before topically applying HDM paste to back skin twice weekly for 5 weeks and re-colonized with *S. epi*-2W during the final 3 treatments. Cytokine production by (A) SDLN polyclonal ($CD4^+CD44^+$) T cells pooled from 2 experiments, (B) SDLN *S. epi*-specific ($2W^+CD44^+CD4^+$) T cells from 1 experiment, and (C) skin polyclonal ($CD4^+CD44^+$) T cells pooled from 2 experiments quantifying IL-5^+(IL-13^{neg}), IL13^+(IL5^{neg}), IFNγ^+(IL-$17A^{neg}$), and IL-$17A^+$(IFNγ^{neg}) (D) Baseline ear thickness in adult WT and *Flg*$^{-/-}$ mice. From *S. epi* and imiquimod treatment described in Fig. 5D: (E) longitudinal ear thickness measurements, data pooled from 3 experiments. P value symbols: ns = not significant, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. HDM, house dust mite,

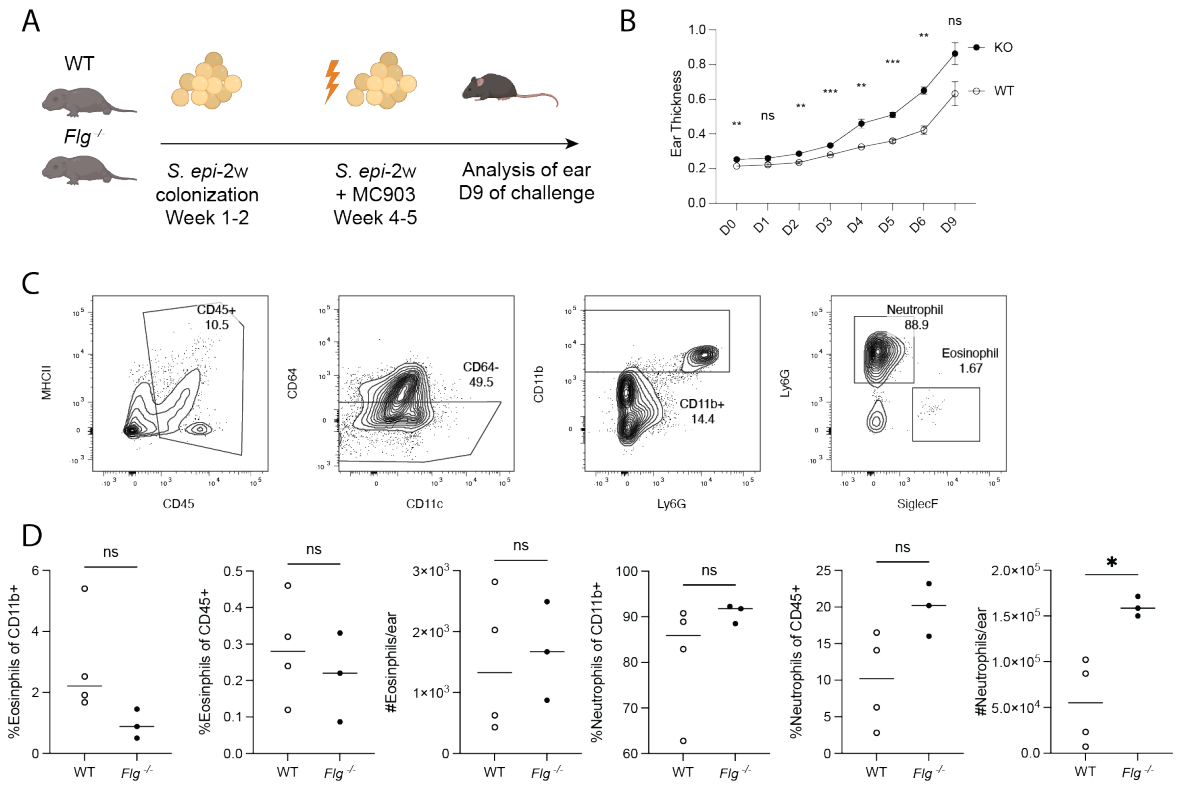

Figure 2.10: *Flg*$^{-/-}$ mice have a stronger neutrophil, but not eosinophil response to MC903 treatment. (A) MC903 challenge model in *S. epi* colonized WT and Flg$^{-/-}$ mice with (B) Mouse ear thickness measured longitudinally shown from one of three representative experiments. (C) Sample flow gating of neutrophils and eosinophils with (D) frequencies and numbers quantified, one of three representative experiments shown.

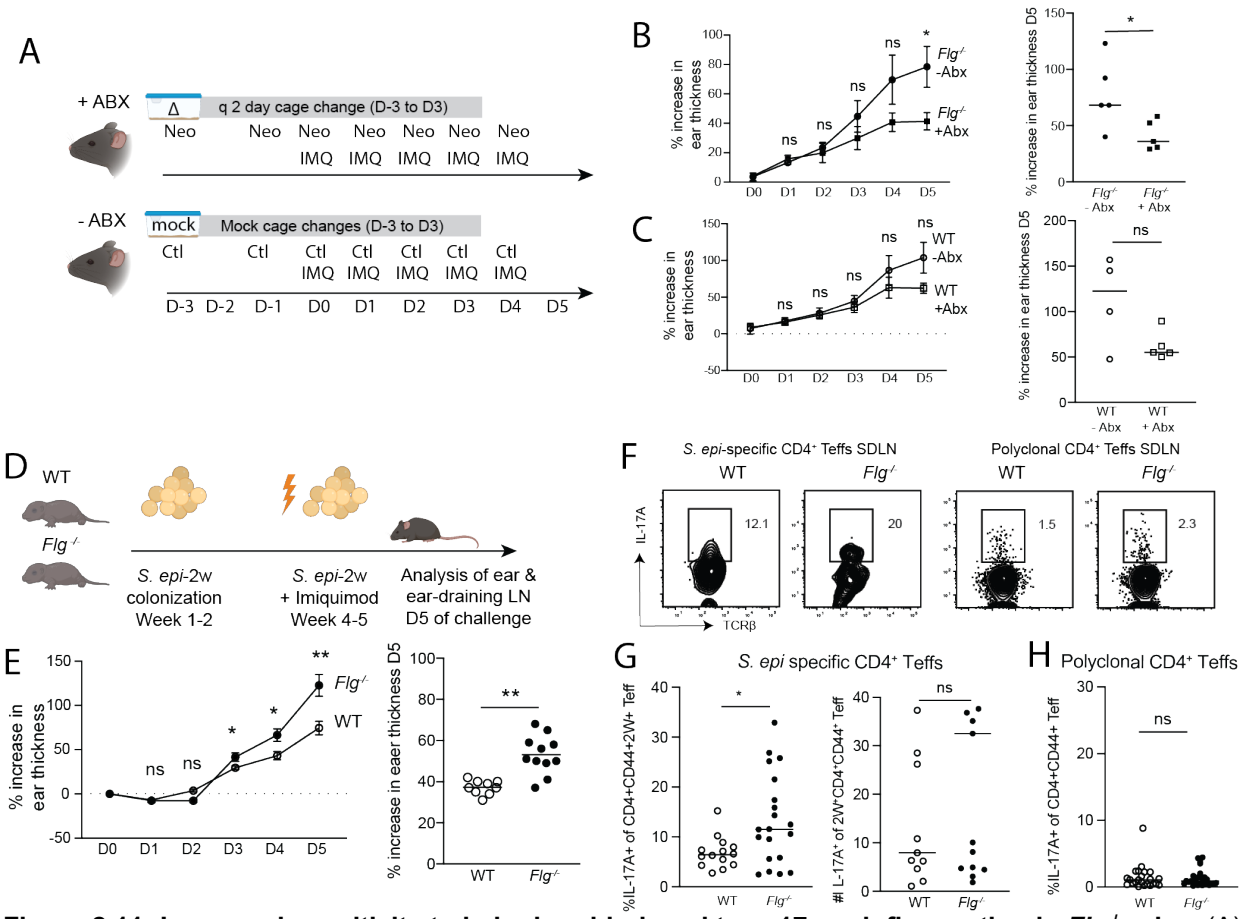

Figure 2.11: Increased sensitivity to imiquimod-induced type 17 ear inflammation in *Flg*$^{-/-}$ mice. (A) Skin bacteria effect on imiquimod response: Adult WT and *Flg*$^{-/-}$ mice were treated with Neosporin (+ABX) or Aquaphor control (-ABX) beginning 3 days before imiquimod, plus real (+ABX) or mock (-ABX) cage changes. In (B) *Flg*$^{-/-}$ mice and (C) WT mice (left) Percent increase in ear thickness in +Abx versus -ABX groups and (right) D5 data for individual mice (1 of 2 representative experiments shown). (D) Impact of flg status on imiquimod response during memory response to *S. epi*-2W: Mice neonatally colonized with *S.epi*-2W were re-exposed in adulthood in tandem with ear imiquimod treatments. (E) (left) Longitudinal and (right) D5 % increase in ear thickness (1 of 3 replicate experiments shown). (F-H) Gating and quantification of IL-17^{+} cells among *S. epi*-specific (2W^{+}CD44^{+}Foxp3^{neg}CD4^{+}) and polyclonal (CD44^{+}Foxp3^{neg}CD4^{+}) Teffs in mice ns = not significant, p > 0.05, * p ≤ 0.05, ** p ≤ 0.01.

Chapter 3: Materials and Methods

Materials and Methods

Bacterial Skin Colonization and Skin Abrasion Models

S. epi-2W was cultured for 48 h to achieve consistent high gpmcherry expression. Cells were washed and re-suspended in PBS, and 10^8-10^{10} colony-forming units (CFUs) were applied to the back skin of mice starting at 1 week of age and repeated every 3 days for a total of three applications. To assess the primary response to *S. epi*-2W, mice were colonized as above and then SDLNs (axillary, brachial, and inguinal) were harvested at weaning age (postnatal D21-24) and processed for tetramer staining. In certain experiments, mice were colonized neonatally and then re-challenged two to three weeks later via intradermal injection into the back skin with 50 mg of 2W1S peptide (EAWGALANWAVDSA; Genscript) in 100μL of Incomplete Freund's Adjuvant (IFA). Skin was harvested 7 days after re-challenge. In other experiments, clippers and Nair™ Hair Remover Body Cream were used to depilate the mice. The upper layers of epidermis were then disrupted via repeated application and removal of adhesive tape (Shurtape HP-500), and *S. epi*-2W was applied as above. This was repeated every 3 days for a total of three times for one round of challenge. SDLNs were harvested 9 days after challenge initiation.

Imiquimod Model

The ears of mice were treated daily with Imiquimod (Taro) for 5 days with ear skin and ear-draining lymph nodes harvested one day after final treatment. In experiments with neonatal colonization and re-challenge, *S.epi*-2W was applied as described above every 3-4 days for a total of three times, with final application three days before harvest.

In some experiments, imiquimod-treated mice received topical ear treatment with Neosporin (Johnson & Johnson) or vehicular control Aquaphor (Beiersdorf AG) three and one day prior to first imiquimod treatment, with daily application thereafter. Mice were also subjected to cage changes or mock cage changes every other day from the first antibiotic treatment.

MC903 model

Mouse ears were treated for daily for 7 days with 20 uL/ear of 200 uM MC903 (Sigma). Three days later, ear draining lymph nodes and ear skin were harvested. In experiments with neonatal colonization and re-challenge, *S.epi*-2W was applied as described above every 3 days for a total of three times, with final application three days before harvest.

Antigen uptake S. epi zsgreen model

Mice were colonized with *S. epi-zsgreen* on day 9 of life, and skin and skin draining lymph nodes were harvested on day 10. To inhibit antigen uptake during processing, digestion solutions were supplemented with 2.5 ug/mL cytochalasin D.

Tissue Processing

Skin and SDLN were isolated and processed into single cell suspensions, stained with antibodies and 2W-loaded MHC-II tetramers and analyzed by flow cytometry as previously described (Leech et al. 2019). Whole skin was processed for qPCR and Luminex assays as per standard protocols. Isolation of lymph node cells was performed by mashing over sterile 100uM filters in 2ml of PBS. For cell isolation from skin, the

back skin or ear skin was harvested, lightly defatted if back skin, or split apart if ear skin, then minced with scissors and re-suspended in a 50ml conical with 1-3ml of digestion media (2mg/mL collagenase XI, 0.5mg/mL hyaluronidase and 0.1mg/mL DNase in RPMI with 1% HEPES, 1% penicillin-streptomycin and 10% fetal calf serum). Following 45 min of shaking incubation at 37°C, an additional 15ml of media was added and the suspension shaken vigorously by hand for 30 s. It was then filtered through sterile cell strainers (100μm cell followed by 40μm), pelleted and re-suspended in PBS for cell counting. For myeloid cell analysis, skin draining lymph nodes were incubated for 15 min at 37°C in digestion media (2mg/mL collagenase I, 2mg/mL collagenase IV, 0.1mg/mL DNase in RPMI with 1% HEPES,1% penicillin-streptomycin and 10% fetal calf serum). After the first 15 min of incubation, cells were pipetted up and down repeatedly, then returned for a second 15 min incubation.

Calcium Green Assay

Freshly excised D6 neonatal skin was placed in a Franz chamber with the SC facing the donor chamber, which was filled with 150μl of 40μM Calcium Green 5N (Life Technologies) in fully supplemented cell culture media (154 CF plus HKGS and 0.07mM Ca2+, Gibco-life technologies). Identical media without dye was added to the acceptor chamber. After sealing ports with parafilm, samples were incubated overnight at 37°C. Sample were rinsed three times in HBSS (Gibco, Life Technologies). For imaging of epidermal dye penetration after Franz chamber experiments, the dermal side of the skin was glued to a 35mm plastic petri dish which was secured to the stage of an upright Zeiss 780 LSM multiphoton microscope with a W-Plan-Apochromat20X/1.0 dipping lens

(Carl Zeiss Microscopy, NY). Z-stacks were acquired tuning the laser source at 900nm and spectral detection window between 506-604nm. Images were acquired every 1μm with 3 z-stacks at different positions for each sample. Parameters (image size, detector gain, laser attenuation) were kept constant. Dye penetration volumes were calculated in Fiji (Schindelin et al. 2012) by thresholding each stack and integrating each image's positive fractional areas.

Measurement of Transepidermal Water Loss

TEWL (g/h/m²) was measured on the backs of 1 week old mice using a Tewameter® TM 300 (Courage + Khazaka electronic GmbH).

Single cell RNA Sequencing of Neonatal Epidermal cells

Mice were colonized on postnatal D7 and 10. Skin was harvested on day 12 and digested in 0.5ml of 0.5% trypsin at 37°C for 30 minutes. The epidermal layer was removed, resuspended and assessed for cell numbers and viability (Nucleocounter, Chemometec). scRNA-seq was performed by the UCSF Genomics core facility using the 10x Chromium Single Cell 3' Gene Expression Kit, according to the manufacturer's instructions (10x Genomics, Pleasanton, CA). Sequencing was completed on a NovaSeq 6000 instrument with standard 10x Gene Expression cycling parameter configurations (R1:28; R2: 91). The Cell Ranger analysis pipelines (10x Genomics) were then used to process the generated sequencing data using Loupe Browser (10x Genomics). The R package Seurat (version 3.0) was used for all gene expression analysis. Filtered gene-barcode matrices were loaded, and quality control steps were

performed (filtering based off of number of features, number of reads, and percent mitochondrial reads). Data was integrated and scaled to visualize clusters with nonlinear dimensional reduction with uniform manifold approximation and projection (UMAP) plots. Clusters were identified based on differentially expressed genes using FindAllMarkers and scaled non-integrated data was used to generate dot plots and heatmaps. Volcano plots were generated using VolcaNoseR (Goedhart and Luijsterburg 2020).

Experimental Animal Use

C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) then bred and maintained in the UCSF specific pathogen-free facility on the Parnassus campus. C57BL/6 *Flg*$^{-/-}$ mice were obtained from Dr. Masayuki Amagai via Riken. All mice used in experiments were socially housed under a 12 h light/dark cycle. Animals were 6 days to 10 weeks old at the time of experiments. When experimental design precluded use of littermates, mice from age-matched litters were used and cross-fostered to minimize cage effects. Both male and female mice were included in experiments, with equal distribution of sexes across groups. Animal work was performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the guidelines of the Laboratory Animal Resource Center and Institutional Animal Care and Use Committee of the University of California, San Francisco,

Bacterial Strains and Culture Conditions

S. epidermidis strain Tu3298 transformed with pJL74-2W-gpmcherry or pJL74-2W-gpmcherry-cat (*S. epi*-2W), previously generated and described (Leech et al. 2019; Scharschmidt et al. 2015), was used experiments in this study. Bacteria were grown in tryptic soy broth at 37°C, with erythromycin or chloramphenicol for plasmid selection where appropriate.

Flow Cytometry

Cells were stained in PBS for 30 min at 4°C with surface antibodies and a live dead marker (Ghost Dye™ Violet 510 or Red 780, Tonbo Biosciences). For intracellular staining, cells were fixed and permeabilized using the Foxp3 Staining kit (eBioscience), or in certain cases to optimize Th2 staining, fixed in 4% PFA at room temperature for 8 min and permeabilized using the Foxp3 Transcription Factor Staining Buffer Set (eBioscience). Fluorophore-conjugated antibodies were purchased from eBioscience, BD Biosciences or BioLegend. Samples were run on a Fortessa (BD Biosciences) in the UCSF Flow Cytometry Core. AccuCheck counting beads (Invitrogen) were used calculate absolute numbers of cells. Flow cytometry data was analyzed using FlowJo software (FlowJo, LLC).

Tetramer Staining and Enrichment

For identification of $2W^+$ cells, skin or SDLN suspensions were stained for 1 hour in the dark at room temperature (15–25°C) with 2W1S:I-Ab–streptavidin-phycoerythrin (PE) at a concentration of 10nM. Skin was then directly stained for other surface and

intracellular markers as above. For SDLN samples, enrichment for the tetramer-bound fraction was performed via an adapted protocol of the EasySep PE selection kit II (StemCell Technologies, Inc.) developed by Marc Jenkins' lab. In brief, 6.25μl of EasySep PE selection cocktail was added to each sample in a total volume of 500μl and the cells were incubated in the dark at room temperature for 15 min. Subsequently, 6.25μl of EasySep magnetic particles were added and the cells were incubated at room temperature for an additional 10 min. Finally, cell suspensions were brought to a total volume of 2.5mL and placed into the EasySep magnet for 5 min. The supernatant (unbound fraction) was poured off and collected in another tube and this process of washing and enriching for magnetically-bound cells was repeated up to three times until the positively-selected cells (bound fraction) and pooled unbound fraction for each sample were taken for cell counting and staining.

Measurement of skin CFUs

After one colonization with *S. epidermidis*, mice were sampled daily with a PBS soaked swab, that was then vortexed in 2ml of PBS and plated to enumerate CFUs.

Tissue Processing for Histopathology

For histopathology, skin tissue was fixed in 10% formalin, followed by 70% ethanol and embedded in paraffin, sectioned, and stained with H&E by HistoWiz. Whole slide scanning (40x) was performed on an Aperio AT2 (Leica Biosystems). Downloaded images were analyzed using QuPath (Bankhead et al. 2017).

qPCR

Square 6mmx6mm pieces of mouse skin were placed in sterile eppendorf tubes in 1ml of RNAlater (ThermoFisher), stored overnight at 4°C and then maintained at -80°C until further processing. Total tissue RNA was isolated using RNAeasy fibrous tissue mini kit (QIAGEN) according to the manufacturer's protocol with the following modification. Tissue was first transferred to 500μL of RLT buffer in MACS M-tubes (Miltenyi) and physically disrupted using a gentleMACS Octo Dissociator (Miltenyi). The handbook protocol was followed thereafter including DNase treatment. For qRT-PCR chemokine array, cDNA was made using RT2 FirstStrand kit (QIAGEN) and then run on RT2 Profiler PCR Array Mouse Cytokines & Chemokines (QIAGEN). Alternatively, for traditional qRT-PCR, appropriate Taqman Gene Expression Assays were used according to manual. All reactions were run on a StepOnePlus instrument (Applied Biosystems).

Luminex Assay

Back skin of D12 mice that had been colonized with *S. epidermidis* on D7 and D10 was flash frozen on dry ice and stored at -80°C. For protein extraction, each skin sample was placed in a gentleMACS M tube with 50ml of modified RIPA Buffer (150mM NaCl, 5mM EDTA, 50mM Tris-HCL pH=8, 1% NP-40, Sodium deoxycholate 0.5%, Glycerol 10%) with one Pierce Protease Inhibitor mini tablet (ThermoFisher Scientific) per 10ml and homogenized with the gentleMACS Protein_01 protocol. Lysates were centrifuged at 4°C for 5 minutes at 4300g, after which the supernatant was isolated and re-centrifuged at 4°C for 15 minutes at 15000g. The supernatant was again collected and

the concentration of protein therein was calculated using a Pierce BSA Protein Assay Kit (Thermo Fisher Scientific). Luminex Assay “Mouse High Sensitivity T-Cell 18-Plex Discovery Assay Array” was completed by Eve Technologies.

Statistical Analyses

Unpaired student’s t -test was used throughout, with Bonferroni correct as appropriate, with the exception of cytokine experiments comparing multiple conditions, where a 2 way ANOVA with Sidak’s multiple comparison test was used.

Chapter 4: Future Directions

One of our key findings was that filaggrin deficiency leads to “outside-in” barrier defects and enhanced antigen penetration. As natural question arising from this is whether penetration of larger bacteria is similarly altered in the setting of filaggrin-deficiency. Are bacteria able to penetrate more deeply into the skin, thus interacting with different immune cells and setting off a cascade of effects? Previous work in the lab suggests the hair follicle may be a primary location in healthy skin for interaction between bacteria and immune cells. Many commensals, such as *S. epi*, can be seen to reside in hair follicles and our commensal-induced colonization of hair follicles in neonatal mice induces cytokines that recruit Tregs to this skin region (Scharschmidt et al. 2017). However, in the context of filaggrin deficiency, perhaps this interaction is supplanted by interactions in other parts of the tissue and that location is key.

Another item of interest is whether the enhanced immune response to *S. epidermidis* in *Flg*$^{-/-}$ mice, extends to other commensals. In experiments not shown, the effect of filaggrin deficiency on the T cell response to the pathobiont *S. aureus* is unclear, in part likely because it is difficult to parse if filaggrin deficiency would confer a reduced frequency of *S. aureus* specific T effectors, when this frequency is already so low in WT mice (Leech et al. 2019). But commensal bacteria more similar to *S. epidermidis* might also display a tolerogenic response in wildtype mice that is lost in *Flg*$^{-/-}$ mice.

We have shown that filaggrin deficiency leads to an altered CD4+ T cell response to the skin commensal *S. epidermidis* and have further demonstrated that neonatal mice have a temporary increase in CD4+ T cells. Additionally, dendritic cells that took up *S.*

epidermidis have increased CD86 expression. A potential mediator of this phenomenon is IL-1β, which in addition to being a general driver of DC function (Koide et al. 1987), is also known to promote CD86 expression on DCs (Pang et al. 2013).

In addition to profiling the cell populations present in *Flg*$^{-/-}$ mice during this critical neonatal window, we have noted subtle changes in cytokine levels in these mice. While we have shown that IL-1 levels are altered in the immune responses in filaggrin deficient mice, this avenue bears further investigation. We would like to determine if loss of IL-1b ameliorates some of the phenotypes seen in these mice. Of further interest is determining which cells produce the IL-1b in *Flg*$^{-/-}$ mice. One candidate would be myeloid cells, who could produce extra IL-1b in response to increased contact with *S. epidermidis*. Another potential candidate would be keratinocytes, as IL-1a release has been shown to be triggered in a model of filaggrin deficiency (Archer et al. 2018). Moreover, we would be interested in determining what other cytokines may be mediating the immune response to *S. epi* in *Flg*$^{-/-}$ mice. This dissertation has also hinted at a potential role for several cytokines including IL-7, which is known to be involved in T cell responses. Future studies could address the role of this and other cytokines in affecting T cell activation.

Using several experimental models of skin inflammation, we have shown that these mice have altered type 17, but not type 2 responses. While this might be due in part to colony specific microbiome effects, this phenotype is interesting and further work to understand why filaggrin deficiency alone does not alter type 2 responses is of definite

interest. This may help further our understanding of why mutations in filaggrin have incomplete penetrance for development of Atopic Dermatitis.

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