HOST-COMMENSAL CROSS TALK AT THE CUTANEOUS INTERFACE

Shruti Naik

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Supervisor of Dissertation

_____

Yasmine Belkaid, Ph.D.

Investigator Chief, Mucosal Immunology Section

Graduate Group Chairperson

_____

Dr. E. John Wherry, Ph.D., Associate Professor of Microbiology

Dissertation Committee

Dr. David Artis, Ph.D., Associate Professor of Microbiology

Dr. Terri Laufer, M.D., Associate Professor of Medicine

Dr. Phillip Scott, Ph.D., Professor of Microbiology and Immunology, Associate Dean for Research

Dr. Christopher Hunter, Ph.D., Professor of Parasitology

Dr. E. John Wherry, Ph.D., Associate Professor of Microbiology

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ABSTRACT

HOST-COMMENSAL CROSS TALK AT THE CUTANEOUS INTERFACE

Shruti Naik

Yasmine Belkaid

Our body's epithelial surfaces are colonized by a diverse array of commensal organisms. While the contribution of intestinal commensals to metabolism, tissue development, and immunity has been extensively examined, the role of flora inhabiting other barrier sites in maintaining host physiology is poorly understood. Moreover, how resident commensals control unique physiological niches and the mechanisms underlying the host-microbial dialogue in distinct tissue sites requires elucidation. In **Chapter 2** we find that murine skin, similar to human skin, houses unique commensal communities in topologically distinct niches. Furthermore, skin sites of both mice and non-human primates enriched with commensal niches contain more inflammatory T cells. Given that the skin forms a critical interface with the terrestrial environment and is an opportunistic site for pathogen entry, this tissue represents a highly relevant barrier site for studying the discrete interactions between resident immune cells and local flora outside the intestine.

The findings presented in **Chapter 3** support a non-redundant role for the resident skin microbiota in setting the cutaneous inflammatory tone. More specifically, germ- free mice have an imbalance of effector and regulatory T cells in the skin that can be reversed upon addition of skin, but not gut, flora.

Additionally, skin commensals act as natural adjuvants, boosting both protective immunity and pathology during *Leishmania major* infection. Thus, signals from the indigenous microbiota are necessary for optimal tissue immune fitness and function in the skin under steady state conditions and during infectious challenge.

In **Chapter 4,** we identify the interleukin-1 receptor (IL-1R1) as an essential regulator of cutaneous T cell function. Accordingly, mice deficient in IL-1R1 and its downstream signaling adaptor, Myd88, recapitulate the homeostatic and immune defects observed in the skin of germ-free mice. Emphasizing the partitioning of innate signals in the skin and gut, IL-1 signaling is dispensable for intestinal T cell function. Importantly, cutaneous commensals locally amplify IL-1/MyD88, signaling to tune the function of effector T cells in the dermis. Taken together, these studies underscore the importance of the microbiota as a distinctive feature of tissue compartmentalization and provide insight into the mechanisms of immune regulation by resident commensal niches in health and disease.

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Chapter 1: Introduction

Microbiota In Health and Disease

Mammals have co-evolved with complex communities of commensal microbes that reside in various epithelial barrier surfaces and outnumber host cells by at least a factor of 10 (Savage, 1977). Far from bystanders, commensals play a functional role in host health that is currently being dissected at the genetic and mechanistic level (Gevers et al., 2012). For instance, microbiota supplement their host's genomes by providing a range of enzymatic functions, integral for maintaining host physiology. As such, the microbiota augment our ability to extract energy from food and promote the development and function of various organ systems, including the immune system (Hooper et al., 2002). In turn, hosts provide the microbiota with nutrient-rich environments that are crucial for microbe survival. The following description is intended to provide a comprehensive view of the evolutionary alliance forged between host and microbe and its implications for host health and disease.

Lessons from germ-free mice: The importance of flora in host development and homeostasis.

In the 1600s, Antonie van Leeuwenhoek developed new microscope lenses that allowed him to identify single-celled organisms he called "animalcules." The father of microbiology had for the first time observed the

microorganisms that were soon implicated as the causative agents of disease. Given the abundance of these tiny creatures associated with every surface of our bodies, French microbiologist Louis Pasteur postulated that rather than just causing maladies, microorganisms may also serve a beneficial role in host homeostasis. In fact, in 1885 Pasteur precociously proposed the study of "young animals from birth [that were]…totally deprived of microorganisms" (Pasteur, 1885). Shortly thereafter, Nuttall and Theirfelder actualized Pasteur's proposed animal model by aseptically acquiring guinea pigs via cesarean section and maintaining them under germ-free conditions for a period of ten days (Heinecke, 1990). A germ-free, or anexic, animal was formally defined as "an animal free of bacteria, yeasts, molds, viruses, protozoa, parasites and all other recognizable life forms." Although several attempts to stably establish long-term germ-free animals using guinea pigs, goats, chicken, and monkeys were made, it was not until the late 1940's that germ-free rodent colonies were established. Fueled by advanced knowledge of nutrition and sterile equipment, Reyniers and Gustafsson (Gustafsson, 1948; Reyniers et al., 1946) both independently established germ-free colonies of rats. These advances in germ-free animal models upended the perception of microbes solely from acting as agents of disease to symbionts that provide vital functions for host heath.

Early studies with germ-free animals revealed a critical role for microbiota in metabolism and nutrient extraction. Morphologically germ-free animals were similar to their conventionally raised counterparts. However, because of an

inability to process complex polysaccharides, germ-free mice had significantly enlarged cecums (Wostmann, 1996). Additionally, in order to maintain similar weights as conventionally raised animals, germ-free animals consumed 30% more calories (Wostmann et al., 1983). Increased caloric intake was necessary to compensate for inefficient energy extraction from food in the absence of flora.

Recent advances in proteomics technology have allowed for a greater appreciation of the central role of the microbiota in nutrient processing and energy extraction. Intestinal microbes have a diverse enzymatic repertoire that functions to break down complex plant polysaccharides such as cellulose, xylan, and pectin that the host is incapable of digesting (Hooper et al., 2002). Specifically, members of the phylum *Bacteriodetes* and *Firmicutes* that reside in the distal gut are endowed with the enzymatic capacity to digest starches into monosaccharaides and short chain fatty acids (SCFA), which are then absorbed and processed by the host (Hooper et al., 2002). To this end, adult germ-free mice conventionalized with normal mouse cecal microbiota exhibited a dramatic increase in body fat, hepatic triglycerides, fasting plasma glucose, and insulin resistance (Caricilli et al., 2011; Nicholson et al., 2012; Tilg and Kaser, 2011). Reinforcing the role of flora in energy homeostasis, a recent report observed that administration of sub-therapeutic doses of antibiotics early in life was associated with perturbations of the intestinal *bacteroidetes/firmicutes* ratio and increased accumulation of fat mass relative to total body weight (Cho et al., 2012). Additionally, both the composition of gut flora and its enzymatic repertoire in

obese mice and people are significantly different than those of healthy individuals, indicating that alterations in the intestinal microbiota adversely impact host energy homeostasis (Turnbaugh et al., 2009; Turnbaugh et al., 2006). Thus, by facilitating efficient processing of caloric intake and nutrient absorption, the intestinal microbiota control host metabolic homeostasis.

In addition to globally controlling host metabolism, the microbiota also regulate the formation and structure of the intestinal barrier. A single layer of columnar epithelia, intended for optimal absorption of nutrient metabolites and segregation of resident microbes from host tissues, lines the wall of the intestine. Importantly, the formation of this physical barrier is critically dependent upon signals from the microbiota (Renz et al., 2012). The permeability of the intestinal barrier is regulated by commensals via modulation of epithelial tight junctions; this process helps limit bacterial penetration into the lamina propria (Ulluwishewa et al., 2011). Furthermore, early studies in hydra performed by Rahat and Dimentman showed that commensal signals were essential for epithelial proliferation (Rahat and Dimentman, 1982). Extending these finds to the mammalian intestine, postnatal epithelial activation and turnover were dependent on exposure to microbial ligands (Abrams et al., 1963; Renz et al., 2012). In addition to controlling steady-state epithelial turnover, commensal interactions are necessary for epithelial migration and wound closure after tissue damage (Swanson et al., 2011). Commensal bacteria also control other aspects of intestinal tissue function, such as angiogenesis (Reinhardt et al., 2012). Thus

sensing of commensals by epithelial cells is critical for optimal barrier formation and function.

The sheer magnitude of commensals residing in the intestine and the permissiveness of the small intestinal epithelia prompts the formation of an immunological barrier that functions to reinforce the physical epithelial barrier and provide protection to the host. As such, the mucosal immune system is tasked with corralling commensals that reside in the gastrointestinal (GI) tract and simultaneously protecting the host from invading pathogens. Studies using germ-free mice, mice treated with oral antibiotics, or mice lacking commensal sensing receptors have demonstrated that microbial stimulation has profound effects on immune cell development and function in each of the three small intestinal compartments: intraepithelial lymphocytes, intestinal lamina propria, and peyer's patches (Hooper et al., 2012). Although microbial interactions impact various aspects of the innate immune system, for brevity, this discussion will be limited to the impact of the flora on the adaptive immune system.

One of the first immunological defects observed in germ-free mice was the loss of secretory Immunoglobulin A (Puel et al.). Moreau and colleagues observed a marked reduction in IgA production in the intestines of axenic mice that was reversible upon microbial conventionalization (Moreau et al., 1978). Numerous reports have now shown that IgA is secreted into the gut lumen, where it coats commensal bacteria and limits their penetration into host tissue, thereby highlighting a reciprocal relationship between host immunity and

intestinal microbes (Macpherson and Uhr, 2004a). Additionally, shifts in the composition of the microbiota are observed in the absence of luminal IgA, suggesting that the host may utilize immune mechanisms to specifically modulate the type and location of its intestinal flora (Fagarasan et al., 2002). A cornerstone study emphasized the "supply and demand" aspect of the intestinal IgA response by associating germ-free mice with transiently colonizing bacteria. This reversible colonization system revealed that IgA production was dynamically controlled by the presence of bacteria and suggested the evolution of a system that could tailor its antibody repertoire to existing commensal content (Hapfelmeier et al., 2010). Dendritic cells (DCs) sample luminal contents and migrate to gut-draining lymphoid tissue, where they promote plasma cell formation and the production of IgA. Indeed, in the absence of commensals, peyer's patches and mesenteric lymph nodes have few or no active germinal centers (Macpherson and Uhr, 2004b). Furthermore, commensal ligation of Toll-Like Receptors (TLRs) on intestinal epithelial cells can lead to localized IgA2 class switching (He et al., 2007).

A considerable number of effector, killer, and regulatory T lymphocytes reside in the intestinal lamina propria and are integrated into the epithelial lining of the small intestine. The numbers and frequencies of $CD4^+$ and $CD8^+$ T cells in the small intestine are dramatically reduced in the absence of commensals (Cebra, 1999). This effect is most prominently observed in intraepithelial lymphocytes (IELs) populations that are reduced tenfold in germ-free mice

(Imaoka et al., 1996). In addition to controlling the number of T lymphocytes, commensals and their by-products dynamically regulate the balance of IL-17A and IFN-γ-producing effectors and $Foxp3^+$ T regulatory cells (T_{regs}) in the intestinal lamina propria (Ivanov et al., 2008). For instance, germ-free mice, mice lacking TLR9 a critical sensor of gut flora DNA, as well as orally antibiotic-treated mice exhibit a dramatic reduction in IL-17A production with a concomitant accumulation of T_{regs} in the small bowel under homeostatic conditions (Hall et al., 2008). This disequilibrium in the composition of intestinal lymphocytes can be reversed upon association of germ-free mice with bacteria or their by products.

Systematic colonization of germ-free mice with defined microbes or communities has highlighted the role of specific microbes or their individual byproducts in immunity. For example, *segmented filamentous bacteria* (SFB) are a class of bacteria that are intimately associated with the intestinal epithelia and induce the maturation of intestinal lymphocytes and production of IL-17A (Ivanov et al., 2009). Similarly, Polysaccharide A (PSA) derived from *Bacteriodeties fragilis* induces IL-10 production from dendritic cells and T cells (Mazmanian and Kasper, 2006). Importantly, metabolic byproducts derived from commensals processing of dietary factors such as ATP and SCFA can also modulate the function of various immune cell populations (Hooper et al., 2012). Collectively, these observations emphasize the benevolent role of intestinal commensals in maintaining intestine tissue fitness.

The role of microbes in disease

The microbial symbionts that inhabit our alimentary track are also central players in inflammation. It is now clear from numerous studies that the microbiota have a “Jekyll and Hyde” relationship with their hosts. Signals from the microflora are critical in adjuvanting immune responses to invading pathogens; however, these same microbial signals have also been implicated in promoting aberrant inflammation (Hooper et al., 2012).

When a pathogen penetrates the intestinal barrier, the mucosal immune system mounts a swift response to control the infection. However, the ability of the immune system to rapidly recognize and control invading pathogens is critically dependent on signals from the microbiota. Indeed, germ-free mice show increased susceptibility to several pathogens including *Reovirus, Citrobacter rodentium*, *Listeria monocytogenes,* and *Salmonella enterica*, which can be reversed by the addition of flora (Cebra, 1999; Round and Mazmanian, 2009). Additionally, certain commensals are known to be aggressive drivers of intestinal inflammation and conventionally raised animals with these microbes in their intestines have an enhanced ability to mount protective immune responses. Supporting this, Singer and Nash observed that C57BL/6 mice bred at Taconic Farms were less susceptible to GI infection with *Giardia lambia* than the same strain of mice acquired from Jackson Laboratories (Singer and Nash, 2000). This disparity in parasite clearance was shown to be dependent upon a component of the microbial flora as cohousing mice transferred protection and treating Taconic

mice with oral antibiotics diminished parasite control. About a decade later, three groups simultaneously discovered that mice from Taconic Farms harbor SFB in their small and large bowel and thus have increased inflammatory milieu in their intestine (Ivanov et al., 2009; Salzman et al., 2010; Wu et al., 2010). Although, these studies have singled out the immunogenic effects of certain species of bacteria, how these individual species interact with the host immune system or other members of the microbial community to promote immunity remains to be understood.

To ascertain the mechanisms underlying host commensal dialogue, Hall and colleagues the examined the importance of TLR9 signaling, which is necessary for sensing gut flora DNA, and discovered that TLR9-deficient animals had impaired protective responses to *Encephalitozoon cuniculi (Hall et al., 2008)*. Several other receptors, including various TLRs, NODS, and NLRs, have been implicated in the host commensal cross talk (Hooper et al., 2012). More recently, a role of commensal microbes in controlling immunity systemically and supplying signals to immune cells at distal sites has been highlighted. Orally antibiotic-treated animals succumb to lung infections with *Influenza Virus* and *Lymphocytic Choriomeningitis Virus*, in part because of insufficient signals derived from the gut flora (Abt et al., 2012; Ichinohe et al., 2011). This gut-centric perspective largely ignores microbial communities residing in other epithelial niches such as the skin, the respiratory tract, and the urogenital tract. In **Chapter 3**, using the skin as model tissue, we example the role of commensals residing in niches

outside the gut in host immunity.

Commensal microbes have also been implicated in protecting their hosts from allergic diseases. Microbial exposure early in life has been shown to have protective effects against allergic inflammation, and reduced microbial diversity in infancy is associated with a predisposition to develop allergic disease (Bisgaard et al., 2011). Indeed, colonization of neonatal (but not adult) mice was able to protect these mice from iNKT-mediated pathology in murine models of asthma. This finding indicates that immune developments are based on temporal set points during which exposure to flora is essential (Olszak et al., 2012). Furthermore, mechanistic studies using antibiotic-treated animals have demonstrated that commensal signals via MyD88 in B cells negatively regulate serum IgE, which drives pathology in allergic disease (Hill et al., 2012). Mice lacking microbial-sensing receptors such as TLR4 are also more susceptible to allergen challenge (Berin et al., 2006). These findings indicate that microbiota are instrumental in setting the appropriate immunological tone, without which the immune system reverts to potentially pathological states.

While microbial interactions enormously benefit the host, these symbionts, under certain conditions, can themselves trigger disease. Certain species of bacteria, which have been dubbed “pathobionts”, peacefully co-exist with the host under steady-state conditions, but have significant pathogenic potential in the presence of environmental or genetic stress (Chow et al., 2011). These pathobionts have been implicated in a host of inflammatory disorders including

inflammatory bowel disease (IBD) and GI cancers. One example of an intestinal pathobiont is *Helicobacter hepaticus* that contributed to the induction of colitis in IL-10 deficient animals (Kullberg et al., 1998). These commensal-mediated pathologies are mirrored in humans with polymorphisms in IL-10, IL-10R, and IL-23R that have a predisposition to intestinal inflammation (Franke et al., 2010). The presence of intestinal SFB is associated with increased systemic inflammation in diabetic, arthritogenic, and encephalitogenic models of autoimmunity (Chow et al., 2011). In fact, infections that cause breaches of the intestinal barrier also result in the systemic dissemination of pathobionts such as *Escherichia coli.* Hand and colleagues found that the immune system is unable to discriminate between commensal bacteria and the pathogens resulting from the activation of effector T cells specific to commensal antigens (Hand et al., 2012). Inappropriate anti-commensal responses could be the underlying cause of disorders such as IBD and psoriasis. Given the dual role of the microbiota in promoting protective responses and driving aberrant inflammation, this precarious relationship must be carefully navigated to sustain the heath of both the host and its commensal microbiota.

Inside out - How host factors impact the microbiota.

The intestinal immune system is organized in a manner that allows for compartmentalization of commensal bacteria in the lumen. This is achieved in part by the presence of mucus barrier, antimicrobial peptides, and secretory IgA.

Importantly, these factors act in concert to not only to shape the composition of microbial communities but also to minimize bacterial exposure to the host immune system (Hooper et al., 2012). For instance, forced expression of α-defensin does not impact total bacterial quantity in the intestine, but it does alter the composition of colonizing bacteria (Salzman et al., 2010). Defects in host immune pathways result in a breakdown of intestinal stratification such as those observed in Myd88-deficient animals that have reduced expression of regenerating islet-derived protein 3-gamma (Reg3γ) and increased commensal penetration of host tissues (Vaishnava et al., 2011). Additionally, bacteria specifically modulate host immune pathways to allow for their persistence. One example of such an interaction occurs in the gut, where gram-negative bacteria induce the production of antimicrobial peptide Reg3γ to limit colonization by gram-positive species (Brandl et al., 2008). Thus, several mechanisms exist to fine-tune the reciprocal relationship between the host and microbiota.

The microbiota are highly sensitive to changes in the intestinal microenvironment. Diet, antibiotic treatment, and inflammation all lead to shifts in the intestinal microbiota that predispose the host to disease (Hooper et al., 2012). Host deficiencies in immune pathways result in similar microbial dysbiosis leading to uncontrolled inflammation and induction of disease. The first example of such an immune-related dysbiosis was provided by Garrett and colleagues, who observed that mice doubly deficient in T-bet and Recombinase-activating gene 2 (Rag) develop spontaneous shifts in flora and colitis that can progress to

colorectal cancer (Garrett et al., 2007). Accentuating the role of a dysbiotic flora in driving disease, wild-type mice given flora from TRUC mice also developed intestinal pathologies. A similar outgrowth of pathogenic flora has been observed in NRLP6, ASC, and IL-18-deficient mice (Elinav et al., 2011). These animals have an outgrowth of *TM7* and *Prevotellaceae* that is associated with increased susceptibility to colitis. Deficiencies in immune pathways can also lead to floral shifts that cause metabolic disease. $TLR5^{-/-}$ mice have an alteration in the composition of their intestinal flora that results in alterations in metabolism and nutrient absorption, and is associated with increased hyperlipidemia, insulin resistance, and fat deposition (Vijay-Kumar et al., 2010). Therefore, commensal fitness is critically dependent upon the intestinal microenvironment, which is determined in a large part by host factors such as immunity.

Who is out there? Microbial communities across the body's epithelial surfaces.

The human body is a scaffold upon which a plethora of organisms flourish, creating diverse ecosystems along physiologically distinct niches. Recent advances in sequencing technologies have opened up the black box of microbiota residing in our barrier surfaces such as the intestine, skin, respiratory tract, and reproductive tract (Costello et al., 2009). The composition of microbial communities residing in these epithelial niches can be operationally defined by the sequencing of polymorphisms in bacterial 16S ribosomal RNA genes (Weinstock, 2012). These genomic analyses are superior to previously used

culture-based techniques, which were able to recover only a small portion of commensal organisms. Costello and colleagues made use of novel sequencing approaches to survey the flora of 27 different sites, including the gut, skin, nostril, hair, and oral cavity, to understand the relationships among the microbes residing in these habitats. This study revealed that commensal communities were controlled primarily by the specific ecology of each body site. Importantly, bacterial communities were largely stable within an individual over time but varied from one individual to another (Costello et al., 2009). A more extensive analysis of the composition and functional capacity of the normal microbiota using 454 pyrosequencing technology from 15(male) to 18(female) body sites from 242 individuals were conducted by of the human microbiome project (HMP) research consortium (2012a; 2012b). In line with the existing paradigm, these confirmed that factors such as oxygen levels, nutrient availably, moisture, pH, microbial interactions and immunological status within a given host environment dictate the colonization of that tissue site (2012a; 2012b). These studies were instrumental in establishing the fitness landscape of normal human microbiota against which changes associated with disease states can be compared. The HMP has also uncovered the presence of “core” pathways that are conserved across body habitants, including ribosome and translational machinery, nucleotide charging and ATP synthesis, and glycolysis, that are essential for microbial survival in any environment. Finally, because of its large sampling size, the HMP studies unveiled that ethnicity and age impact the microbiota and their functional

metabolic capabilities (2012a; 2012b).

Commensal colonization begins only after the newborn is exposed to the external environment and the factors controlling this process have recently been elucidated. A comparative analysis of the intestinal flora of children born either vaginally or via cesarean section revealed that birth through the vagina results in colonization by vaginal communities of *lactobacillus* species, while those born by cesarean section acquire skin-associated *Staphylococcus, Corynebacterium*, and *Propionibacterium* species (Dominguez-Bello et al., 2010). How early colonization of the intestine by certain species sets the immunological tone is not known. After birth, it is thought that microbial communities fluctuate until 2 to 3 years of age, after which stable residents are established. These colonies are controlled by the above-mentioned environmental factors, which are unique to each tissue site (Parfrey and Knight, 2012). For example, vaginal microbial composition has been shown to correlate with pH (2012b). In the intestines, diet appears to be a driving factor in the acquisition of bacteria and their enzymatic capabilities (Ley et al., 2008). Thus sequencing approaches have allowed for a comprehensive understanding of baseline microbial communities as well as shifts associated with disease state. These studies have also opened the door to understanding how we acquire and maintain microbes in and on our body.

The Cutaneous Microbiota

The skin is the body's largest organ with a surface area of

approximately 1.8 square meters (Elsner, 2006). This topographically heterogeneous organ has an abundance of folds and invaginations capable of sustaining microbes (Grice and Segre, 2011). Initially, traditional culture-based methods were employed to isolate bacteria from the surface of the skin and deep in invaginations such as sebaceous glands, sweat glands, and hair follicles (Grice and Segre, 2011). The skin is also home to fungi, viruses, and mites, which reside in distinct niches in and on the skin (Foulongne et al., 2012) (**Figure 1**). In a milestone study, Grice and colleagues used molecular approaches to characterize the flora from 20 different human skin sites and uncovered an unprecedented amount of diversity in the skin microflora (Grice et al., 2009). Additionally, this study also verified existing paradigms that the specific ecology of a particular microenvironment dictates the composition of its microbial inhabitants. For instance, sites with more sebaceous glands housed more *Propinobactium,* which can metabolize lipids in sebum, while dry sites were inhabited by surface-associated *Corynebacterium*, which can tolerate the skin's acidic pH (Elsner, 2006; Grice et al., 2009; Leeming et al., 1984). Consistent with this hypothesis, environmental control of skin flora was also reported by Blaser and colleagues, who observed specific associations of cutaneous microbial communities sampled from U.S. residents and Venezuelan Amerindians (Blaser et al., 2012). The importance of ecology in determining the community composition is also highlighted by comparative studies of the skin microbiota with microbes from other sites, which reveal that the skin houses flora distinct from

other body sites (Costello et al., 2009). Additionally, these studies have found that the skin houses the most diverse communities of bacteria in the body (Costello et al., 2009). Accordingly, sites with the most colonizing variation, such as popliteal fossa, volar forearm, and buttock, are predisposed to dermatological disorders (Grice et al., 2009). These findings support the notion that the microbiota occupy various niches within the skin to prevent colonization of by pathogenic species. Thus areas of the skin lacking stable microbial inhabitants are more susceptible to diseases that may result from colonization of pathogenic species. In support of such a symbiotic relationship, the presence of commensal *Staphylococcus epidermidis* was sufficient to limit colonization and biofilm formation by pathobiont *Staphylococcus aureus* (Iwase et al., 2010).

The skin microbiota have also been linked to various cutaneous inflammatory disorders. Indeed, shifts in the microbiota have been associated with various conditions including psoriasis, atopic dermatitis, rosacea, and acne vulgaris (Gallo and Nakatsuji, 2011). In a mouse model of diabetic wounds, impairments in wound closure were associated with an outgrowth in *Staphylococcus* species and a dramatic increase in the total density of bacteria-wound-associated bacteria (Grice et al., 2010). A similar decrease in bacterial diversity and outgrowth of *Staphylococcus* species is associated with inflammatory flares in children with eczema (Kong et al., 2012). In addition to bacterial commensals, the skin is also home to fungi, viruses, and mites. A recent study found increased colonization of the mite *Demodes*

folliculorum on the skin of patients with Rosacea (Jarmuda et al., 2012). As these mites die, their associated commensal *Bacillus oleronius* is released, which is associated with a localized inflammatory reaction resulting in tissue damage (Jarmuda et al., 2012). Although, fluctuations in the quality and quantity of flora are associated with disease states, little is understood about how the microbiota contribute to the pathophysiology of these cutaneous disorders.

While the interaction of commensals with host immune system in the intestine is well documented, the contribution of microbes residing in other niches (such as the skin) to host immunity has received little attention. The cutaneous immune system is capable of sensing and responding to commensal-derived ligands. For instance, lipoteichoic acid (LTA) purified from skin commensal *Staphylococcus epidermidis* can mitigate inflammation associated with tissue damage (Lai et al., 2009). Additionally, studies co-culturing keratinocytes with commensal isolates have shown that these cells are able to mount tailored responses to each commensal isolate (Wanke et al., 2011). These studies highlight the interaction of individual species or their byproducts with specific components of the immune system. However, the commensal microbiota is comprised of diverse communities of microbes each of which posses a myriad of both activating and inhibitory ligands. Ascertaining the role of microbiota in skin immune fitness will require the systematic analysis of host and commensal pathways such as those employed in dissect intestinal immune commensal cross talk. **In Chapter 3, using germ-free and monoassociated mice, we**

identify a non-redundant role for the skin microbiota in controlling the inflammatory tone in the underlying tissue at steady state and during inflammation.

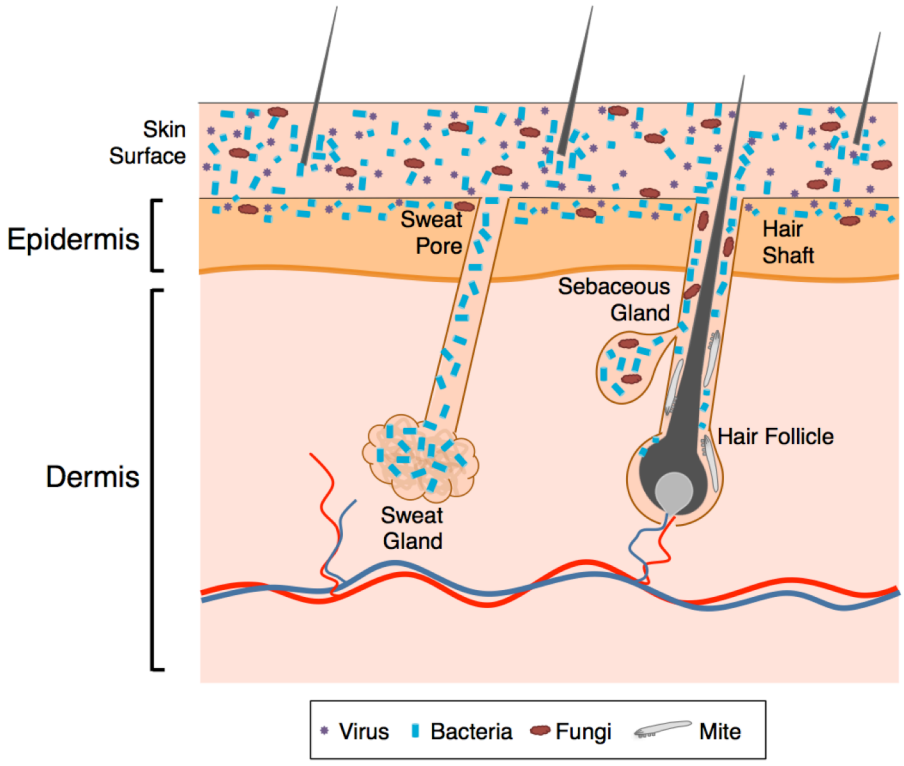

Figure 1: Schematic of commensal localization within the skin.

The skin houses bacterial, viral, fungal and arthropodal (mite) organisms. These commensals reside on the surface of the skin, in the outer cornified layers of keratinocytes and in appendages such as sebaceous glands, sweat glands and hair follicles

The Physiology and Ecology of the Skin

The skin barrier is necessary for survival in a terrestrial environment. The development and maintenance of this complex organ is controlled both spatially and temporally and relies on the presence of several self-renewing resident stem cells (Blanpain and Fuchs, 2009). As a result, this barrier is able to repair itself in response to external stress. A variety of structural cell types including keratinocytes, fibroblasts, sebocytes, and melanocytes are organized in a specific manner within the skin to a construct a semipermeable barrier (Wysocki, 1999). Cells of the nervous and immune system are also found in this vast organ, where they contribute to tissue function during homeostasis and in response to environmental assaults. The following discussion is intended to provide a comprehensive overview of the cutaneous microenvironment as it pertains to immune function and barrier maintenance.

The structure of skin

The skin is comprised of three layers: the epidermis, dermis, and hypodermis, each of which serves a specific function in maintaining the integrity of the cutaneous barrier (Wysocki, 1999). The epidermis is responsible for forming a semipermeable layer and the dermis provides tensile strength to fortify this barrier (Egles et al., 2010). Adjacent to the dermis is the hypodermis, which is comprised of fat that insulates the body and aids in shock absorption (Strauss, 2012). Importantly, these three parallel layers are composed of distinct cell types

and connective proteins capable to sensing and responding to tissue stress.

The outermost layer of the skin, the epidermis, is a self-renewing structure that consists of layers of keratinocytes (Fuchs and Nowak, 2008). Epidermal keratinocytes undergo a program of differentiation to lose their nucleus and (Bergboer et al., 2012; Dowlen and Owers, 2011) become corneocytes as they migrate to the skin surface (Fuchs and Nowak, 2008). The epidermis is stratified into the following five layers: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. This stratification limits the passage of harmful substances and microbes (Fuchs and Nowak, 2008). Defects in the formation of these layers result in dramatic barrier defects and increased susceptibility to an array of disorders. For instance, common loss of function variants in the stratum corneum protein filaggrin, carried by approximately 9% of people of European origin, are a major predisposing factor to atopic dermatitis (Palmer et al., 2006). Moreover, dramatic shifts in the composition of the cutaneous microbiota have been observed in filaggrin-deficient animals, suggesting that barrier integrity is crucial for appropriate commensal colonization in the skin (Scharschmidt et al., 2009).

The complex role of the barrier in driving cutaneous inflammation is also supported by a phenomenon termed the “Koebner response” (Sagi and Trau, 2011). First described by Heinrich Koebner in the late 1800s, the Koebner response refers to physical damage that compromises the skin barrier and consequently results in the formation and/or exacerbation of inflammatory lesions

(Sagi and Trau, 2011). Indeed, such an injury response is linked to the acquisition of various cutaneous inflammatory disorders. For instance, damage to the skin barrier triggers psoriatic lesions and results in the onset of uncontrolled inflammation and tissue destruction (Kalayciyan et al., 2007).

The primary constituents of the dermal skin layer, fibroblasts, secrete collagen and elastin to establish and maintain the dermal architecture (Egles et al., 2010). In contrast to the epidermis, which maintains a hypoxic environment, the dermis is highly vascularized and contains a large number of circulating immune cells (Braverman, 1997). Pathology associated with skin disorders such as psoriasis and atopic dermatitis also extends to the dermal layer of the skin underlying the epidermis (Guttman-Yassky et al., 2011a). Additionally, invading pathogens and pathobionts such as *S. aureus* or *Malassezia* species often penetrate the epidermal barrier to establish infection in the dermal tissue (Miller and Cho, 2011). It is therefore crucial that this layer of the skin maintain an alert status to deal with these invading threats and prevent their dissemination systemically.

Mammalian skin controls thermal regulation and retention of normal hydration (Charkoudian, 2003). Specialized appendages within the skin, such as hair follicles, sebaceous glands, and eccrine and apocrine sweat glands, have evolved to serve these specific functions (Blanpain and Fuchs, 2009; Groscurth, 2002; Thiboutot, 2004). The unique microenvironments of cutaneous appendages select for the presence of distinct microbial communities. For

example, *Propionibacterium* are anaerobic bacteria that contain lipases and can metabolize sebum; these are prevalent in sebaceous glands (Leeming et al., 1984). An additional layer of selective pressure for the composition of flora in these niches is applied by the production of antimicrobial peptides, which may control the amount and type of colonizing organisms (Gallo and Hooper, 2012). Bacteria that reside in and metabolize the viscous secretions of apocrine gland are thought to be responsible for body odor (Hofer, 2006). It remains to be understood how host factors such as diet or hygiene impact commensals in these complex cutaneous niches.

The stromal cells responsible for determining the three-dimensional structure of the skin may be essential liaisons of the immune-commensal dialogue. For instance, keratinocytes are in close apposition to various commensal niches and possess the capacity to sense and respond to commensal ligands (Elsner, 2006; Miller and Modlin, 2007). These cells can also interact with immune cells by producing a variety of cytokine mediators. Indeed, the sensing of inflammatory and potentially commensally driven factors by keratinocytes is known to promote skin carcinogenesis. Cataisson and colleagues recently demonstrated that mice with specific deletion of keratinocyte of a signaling molecule downstream of TLRs and IL-1, Myd88 were resistant to transformation and tumor formation (Cataisson et al., 2012). Together, these studies suggest that the structural cells of the skin are not inert, but in fact actively contribute to tissue responsiveness.

Cutaneous topography

The skin is a physiologically heterogeneous organ with regional differences in the thickness of the epidermal and dermal layer, numerical density of appendages, oxygen, temperature, pH, and moisture (Huzaira et al., 2001). These site-specific differences in morphology are controlled developmentally and regulated in part by epidermal mesenchymal interactions. A landmark study of gene expression profiles of fibroblasts from distinct anatomical locations revealed the role of HOX family homeodomain transcription factors in specifying site-specific transcription programs that may dictate the anatomical arrangement of each of these cutaneous microenvironments (Rinn et al., 2007). Importantly, many skin disorders do not manifest uniformly but have a characteristic pattern of expression. For instance, the face and flexors often exhibit symptoms of atopic dermatitis while the scalp and extensors (knees and elbows) are affected in psoriatic patients (Guttman-Yassky et al., 2011a). How structural variations at distinct skin sites relate to these differential disease manifestations requires elucidation.

Tissue responses to environmental assaults also vary by skin site. For example, immune response and pathology during infection with dermal pathogen *Leishmania major* is highly dependent upon the site of inoculation (Nabors et al., 1995). Intradermal infection in the footpads of mice leads to the formation of large necrotic lesions, whereas infections in the flank manifest no macroscopic dermal pathology (Nabors and Farrell, 1994). Additionally, certain skin sites are more

susceptible to tumor formation (Silvers and Mintz, 1998). Thus, this topologically heterogeneous organ is composed of a wide array of physiologically distinct microenvironments with unique levels of responsiveness to environmental and host factors. Given that immune-mediated diseases represent a large portion of disorders afflicting the skin, and that this barrier is a major portal of entry for pathogens, it will be important to understand the composition and function of immune cells in these distinct tissue sites. **In Chapter 2, we define the immune composition of sites with high and low frequencies of appendages in both mice and nonhuman primates. This survey reveals that sites enriched in commensal niches such as sebaceous glands or hair follicles have a higher concentration of inflammatory cells.**

Differences in human and mouse skin

The architecture of murine and human skin varies dramatically. The epithelial barrier in mice is three layers thick and has a faster turnover rate than human epithelia, which is approximately six to ten layers thick (Menon, 2002). The human epithelial layer contains more intrafollicular space, while murine skin has greater density of hair follicles closer together. The dermal tissue compartment in these species also differs significantly. Human dermis is thicker with fewer follicles, and lacks the cutaneous muscle layer that is found in murine skin (Donahue et al., 1999). Additionally, the presence or localization of certain cellular constituents also differs between humans and mice. $\gamma\delta$ T cells that express canonical T cell receptors and aid in wound healing dominantly

populate murine epidermis but are lacking in human skin (Barbee et al., 2011). Melanocytes in mice are associated primarily with hair follicles and with intrafolliclar spaces in human epithelia (Khavari, 2006). **Although skin tissue shows species specific differences, in Chapter 2, using mouse and non-human primate skin (which more closely resembles human skin) we find that normal skin is home to a large number of effector T cells with the potential to produce inflammatory cytokines.**

The immune surveillance system of the skin

A vast immunological arsenal consisting of small molecules and innate and adaptive lymphocytes reinforces the skin's physical barrier (Gallo and Hooper, 2012; Miller and Cho, 2011). This immunologically active site is tasked with protecting the host from invading pathogens while sustaining a myriad of resident organisms (Miller and Cho, 2011). Therefore, a dynamic network of immune cells constantly patrols the skin, surveying for perturbations caused by pathogens and other physiochemical stress resulting from toxic agents or UV irradiation (Kupper and Fuhlbrigge, 2004). Mounting a swift immune response is critical for host protection and relies on the careful orchestration of various immune cells. The skin immune surveillance system is organized into sites where an immune response is initiated, or "induction sites;" and sites where immune cells execute their function, or "effector sites" (Kupper and Fuhlbrigge, 2004). The components of the cutaneous immune system are described below with a particular focus on the formation and function of T lymphocytes. This discussion is intended to provide a framework for considering the complex and dynamic regulation of immunity in the skin. This tissue site is of primary focus in the research to be described herein.

Lymphocyte Activation in Inductive Sites

The lymphoid structures responsible for surveying cutaneous tissues have been creatively named "skin-associated lymphoid tissue" or SALT (Streilein, 1989). The SALT serves to respond to inflammatory tissue signals and

antigens with responsive T lymphocytes to ensure an efficient immune response. To achieve this feat, cutaneous lymphoid structures are spatially organized into B and T cell zones. T-dependent areas contain a mixture of naïve and recirculating $CD44^{hi}$ effector memory cells (Butcher and Picker, 1996). Naïve T cells constitutively enter the lymph node cortex from the blood via high endothelial venules (HEVs), while effector T cells, dendritic cells, and antigens exiting the skin tissue enter via the afferent lymphatics (Mackay, 1993). The importance of lymphoid structures in orchestrating immune responses is evidenced by delayed priming and compromised skin immunity in Lymphotoxin-α deficient mice that have arrested development of skin lymph nodes (Rennert et al., 2001). More recently, Kastenmuller and colleagues proposed that an additional function for lymphoid structures is to prevent the systemic dissemination of invading pathogens (Kastenmuller et al., 2012). Thus lymphoid structures have dual role in organizing an immune response and acting as a "fire wall" to restrict the passage of pathogens into internal organs.

Under steady-state conditions, cutaneous lymph nodes house T cells with both effector and regulatory potential. CD4 and CD8 memory T cells with the potential to produce inflammatory cytokines recirculate through the lymph nodes (Kambayashi et al., 2003; Tomura et al., 2010). Lymph nodes also contain $\gamma\delta^{low}$ T cells with the potential to produce IL-17A, which is derived from both the dermis and the blood (Michel et al., 2012). Additionally, using mice expressing a photo-convertible protein, Tomura and colleagues have shown that T_{regs} from the skin

can migrate to the draining lymph node (Tomura et al., 2010). A recently described population of lymph-node-resident innate-like lymphoid cells also has the capacity of produce IL-17A, which could provide early protection against barrier disruption (Gray et al., 2012). Similarly, an IL-18-responsive population of resident CD8 T cells is also proposed to act as a first responder to inflammation in a TCR independent manner (Kastenmuller et al., 2012).

Because the lymphoid structures serve as a rapid response centers during adverse inflammatory conditions, they are tasked with constant surveillance of the cutaneous environment. This is achieved by two mechanisms that occur simultaneously: One is the constitutive migration of dendritic cells carrying antigens from the skin to the lymph nodes, and the other is draining of interstitial fluid carrying tissue signals to the node (Villadangos and Heath, 2005). Examination of skin migratory dendritic cells in the cutaneous lymph nodes of germ-free animals reveals no differences in the frequency and status of activation from conventionally raised animals, indicating that commensal signals are not necessary for this evolutionally conserved process (Wilson et al., 2008). Furthermore, a dermal-derived subset of migratory dendritic cells has recently been implicated in the production of retinoic acid (RA) and consequently in the de novo generation of T_{regs} in cutaneous lymph nodes. Commensal-derived signals were also found to be dispensable for RA production and T_{reg} conversion in skin-draining nodes (Guilliams et al., 2010).

In addition to migratory dendritic cells, antigen- and tissue-derived signals

drain to the lymph node and are sampled by the resident macrophages and dendritic cells (Manolova et al., 2008). The importance of these lymphoid-resident dendritic cell populations in presenting antigens to T lymphocytes has been appreciated because of an elegant study conducted by Allenspach and colleagues (Allenspach et al., 2008). Restriction of the antigen presentation capacity of either lymphoid resident or migratory dendritic cells with the use of sophisticated genetic and chimeric animal models revealed for the first time that both these subsets of dendritic cells are necessary for antigen presentation and proper induction of an adaptive immune response in the cutaneous lymph nodes. Upon activation, T lymphocytes acquire context-dependent effector functions, exit the lymph node, and migrate into skin tissue, where they are able to play their roles in immune responses (Kunkel and Butcher, 2002).

Cutaneous Lymphocyte Recirculation

During priming and activation in the SALT, T lymphocytes are imprinted with chemokine receptors and adhesion molecules that act as “zip codes” to promote their migration into skin tissue (Sigmundsdottir and Butcher, 2008). A combination of cytokines and tissue-specific external clues, such as the presence of nutrient metabolite Vitamin D, regulate the expression of skin-specific trafficking programs on T cells. Indeed, Vitamin D signaling, via Vitamin D receptors, programs the expression of the skin-homing chemokine receptor CCR10 on T lymphocytes (Sigmundsdottir et al., 2007). CCR4 has also been described as a skin-specific trafficking receptor on T lymphocytes (Campbell et

al., 1999). The ligands for CCR4 and CCR10, CCL17 and CCL27 respectively, are constitutively expressed by various cell types in the skin, and therefore may be susceptible to modulation by commensal signals, but haven't been measured in germ-free mice. The importance of generating skin tropic cells is evidenced by the eruption of spontaneous inflammation in mice in which T_{regs} lack these receptors (Dudda et al., 2008; Sather et al., 2007). In addition to skin-specific trafficking, effector lymphocytes also migrate into tissue in response to inflammatory chemokines, many of which are expressed during skin inflammation.

Entering extra-lymphoid tissues requires circulating T effectors to sense and bind to activated vascular endothelium and then extravasate into the surrounding skin tissue (Springer, 1995). This is achieved by the adhesion of molecules such as E and P selectins in mice, and by the adhesion of cutaneous lymphoid antigen (CLA) expressed on T cells to their corresponding ligands expressed by vascular endothelium (Steeber and Tedder, 2000). Importantly, the expression of these markers is used to identify skin-homing T cells in the blood. Isolation of circulating skin-homing T cells from blood revealed that these T cells are enriched for cytokine production and pathogen recognition (Duhen et al., 2009; Zielinski et al., 2012). Upon entry into the skin, both transmigration and retention in tissue is mediated by integrin CD103 (Schlickum et al., 2008). The capacity of skin T_{regs} to be retained in the skin is critically dependent upon the presence of CD103 (Suffia et al., 2005).

T lymphocyte accumulation in both normal and inflamed skin is orchestrated by a series of chemokine and adhesion molecules that control entry into and exit from the skin. The expression of inflammatory chemokines and their receptors are increased when skin disorders are present. Indeed, expression of both CCR6 and its ligand CCL20 is dramatically increased in psoriatic lesions in comparison to non-lesional skin (Mabuchi et al., 2012), and affected skin from AD patients releases more CCL17 and CCL27 (Gros et al., 2009). Moreover, T cells in cutaneous T cell lymphomas express high levels of CCR4, and blocking antibodies to this receptor are being tested as a therapeutic modality (Han et al., 2012).

A portion of T effector and regulatory lymphocytes that enter tissues also leave to the draining lymph nodes via the afferent lymphatic and return to circulation. Early studies observed this phenomenon by assessing the phenotype of T cells leaving normal skin via the afferent lymph. Indeed, CD4 T cells with effector phenotype (high CD44 expression) were enriched in the afferent lymph when compared to blood and efferent lymph, which had more naïve T cells (Mackay et al., 1990). T lymphocytes egress from skin tissue into the afferent lymph is facilitated by ligation of chemokine receptor CCR7 with CCL21 expressed in the lymphatic endothelium (Debes et al., 2005). Importantly, the skin's migratory network may be modulated by commensals as a means to control the composition of T cells in the skin. The link between commensals and tissue-derived chemokines in the skin is largely unexplored. **In Chapter 3, we**

examine the role of commensals in controlling the frequency of skin tropic T lymphocytes in circulation.

Lymphocyte Function in Effector Sites

Although the epidermal and dermal layers are located adjacent to each other, these two skin compartments house distinct populations of immune cells that have functionally adapted to their local microenvironment. Approximately 70% of the hematopoietic compartment of murine epidermis is comprised of dendritic epidermal γδ T cells (DETCs) and Langerhans cells represent the other 30% of this compartment (Jameson and Havran, 2007; Merad et al., 2002). DETCs developed early in ontogeny express high levels of canonical receptor Vγ3/Vδ1. Importantly, in both mouse and man, production of insulin-like growth factor-1 by epidermal γδ T cells is essential for mediating epidermal homeostasis and repair (Havran and Jameson, 2010; Jameson and Havran, 2007; Jameson et al., 2002). Additionally, it has been proposed that these cells also regulate the proliferation of Langerhans cells (LCs) within the epidermis by producing GM-CSF (Kadow et al., 2011). Thus, DETCs sense environmental stress and consequently engage in dialogue with both structural and immune cells in their microenvironment, which is essential for tissue fitness. Additionally, cutaneous infection results in the formation non-migratory effector CD8 cells that localize to the epidermis and are sustained there independently of antigens even after resolution of infection (Gebhardt et al., 2011; Jiang et al., 2012). These cells

continually survey the epidermis and provide enhanced protection from any pathogens they re-encounter.

The dermis is home to both γδ and αβ T cells, which play a central role in maintaining the skin's responsiveness. The importance of skin-resident T_{regs} is evidenced by the spontaneous development of dermal pathologies in mice and humans that lack the essential lineage specific T_{reg} transcription factor Foxp3 (Wildin et al., 2001). Additionally, an elegant study by Campbell and colleagues, in which T_{regs} were restricted specifically from the skin, phenocopied the dermal pathology of scurfy mice and indicated that T_{regs} constitutively limit dermal effector T cells and thus restrict unwanted inflammation (Dudda et al., 2008; Sather et al., 2007). Conversely, the presence of T_{regs} in the skin can potentially be harmful in the context of an infection, where ensuring proper protective immune responses relies on limiting excessive regulation. Indeed, a milestone study by Belkaid and colleagues illustrated that T_{reg}-derived IL-10 restricts the formation of protective immunity to *Leishmania major (Belkaid et al., 2002)*. Although T_{regs} represent a dominant dermal cell population, little is known about their regulation in the skin. *In vitro* co-culture studies with autologous LCs and T cells from human skin revealed an MHCII dependent increased in T_{regs} proliferation (Seneschal et al., 2012). Nevertheless, factors that control dermal T_{reg} homeostasis and function *in vivo* remain undefined.

A population of highly motile $\gamma\delta^{low}$ T cells distinct from the epidermal DETC resides in the dermis. These cells have a heterologous Vγ4 and Vγ3 expression

and produce IL-17A and IL-22 in response to IL-1 and IL-23 (Gray et al., 2011; Sumaria et al., 2011). A role for γδ T cell cytokine production has been implicated in early responsiveness to breach of the skin barrier. This early function of γδ T cells in barrier protection is crucial, as the formation of a protective conventional T cell response requires approximately seven days. Accordingly, these cells play an essential role in neutrophil recruitment after intradermal BCG inoculation (Sumaria et al., 2011). Early production of γδ T cell-derived IL-17A also determines long-term susceptibility to pathogens because γδ T cells are necessary to set the appropriate immunological tone. In response to *Staphylococcus aureus* infection, mice deficient in γδ T cells exhibit a defect in neutrophil recruitment, have larger skin lesions, and increased bacterial count (Cho et al., 2010). Remarkably just one dose of recombinant IL-17A provided early, after inoculating with *S. aureus*, can reconstitute protective immunity in γδ T cell-deficient animals *(Cho et al., 2010)*. In addition to γδ T cells, innate lymphoid cells have also been implicated in the production of IL-17A and IL-22 in the skin during various disease states (Pantelyushin et al., 2012).

Accentuating the importance of IL-17A in barrier function, individuals with defects in this pathway are highly susceptible to cutaneous infections with *Candida albicans* and *Staphylococcus aureus (Puel et al., 2011).* However, how the cutaneous immune system regulates the composition and localization of resident commensals and the interaction between commensals, pathogens, and host immunity requires elucidation. These lymphocyte-derived inflammatory

cytokines play a central role in mediating protection to cutaneous pathogens but are also important for regulating the tissue parenchyma. IL-17A signaling via IL-17 receptor in keratinocytes is known to promote their proliferation during a cutaneous wound healing response. While these inflammatory cytokines are indispensible for sustaining normal barrier function, they must be tightly regulated to avoid disease states. Indeed, dysregulation of IL-17A results in immune pathology and tissue destruction in psoriasis (Nakajima, 2012). The blockading of IL-17A or IL-17 receptor in psoriasis patients has shown to be an effective and fast-acting therapy (Leonardi et al., 2012; Papp et al., 2012). Similarly, intradermal injection with recombinant IL-23, a major driver of IL-17 and IL-22, results in dermal inflammation and acanthosis (Zheng et al., 2007). A shift in the microbiota is associated with excessive IL-17A inflammation and psoriasis, yet the role of commensals in promoting these T cell phenotypes is unknown (Gao et al., 2008). Although it is widely recognized that T lymphocytes are an important component of the cutaneous immune surveillance system, their steady state composition, function, and regulation in the skin is poorly understood. **In Chapters 2-4, we identify the resident lymphocytes in various skin sites and study their regulation by skin commensals.**

Innate Immunity in the Skin

A dense network of innate immune sentinels populates the skin; these can sense and rapidly respond to tissue stress (Merad et al., 2008). Epidermal LCs can extend their dendrites through the keratinocyte barrier and sample

antigens from the surface of the skin (Kubo et al., 2009). These cells also have a specialized function in the induction of IL-17A responses to trans-epidermal *C. albicans* infection (Igyarto et al., 2011). It is tempting to speculate that these cells may be involved in the sampling and transport of commensal antigens from the skin to the lymph node. In contrast, dermal CD103 DCs are essential for eliciting antigen-specific IFNγ responses and for cross-presenting antigens to CD8 T cells in the lymph nodes (Bedoui et al., 2009). Tissue stress associated with inflammation or wounds results in recruitment of special subsets of dendritic cells that curb tissue damage. pDCs enter the dermis early after wounding and facilitate wound closure by secreting type 1 interferons (Gregorio et al., 2010). Other innate cells, including mast cells and eosinophils, also reside in the skin. These cells contain granules of histamines and innate cytokines that are secreted in response to physical or chemical stress in the skin barrier (Macfarlane et al., 2000). In addition to immune cells, the cutaneous stroma also contributes to immunity in the skin. For example, dermal fibroblasts can augment proliferation of T_{regs} *in vitro* in the presence of IL-15 and IL-2, suggesting that they play an essential role in shaping the immune repertoire of the skin (Clark and Kupper, 2007).

Keratinocytes contain stores of inflammatory cytokines, including IL-1, that are secreted upon junction disruption (Kennedy-Crispin et al., 2012). The over-expression of these factors in the epidermis under keratinocyte-specific promoter (Keratin 14 promoter) results in altered immunity (Tian et al., 2009).

Importantly, IL-1 receptor antagonist immune deficiency results in spontaneous and uncontrolled dermal inflammation (Aksentijevich et al., 2009). Keratinocytes express pathogen recognition receptors and are therefore endowed with the capacity to recognize both commensals and pathogens (Miller and Modlin, 2007). Keratinocytes also have anti-microbial functions; they produce an array of anti-microbial peptides including a-defensins, RNase 7, psoriasis, and cathelicidin. AMPs also play a central role in cutaneous disorders (Gallo and Hooper, 2012). Outgrowth of certain bacteria in AD is associated with diminished AMP expression, conversely in psoriasis aberrant inflammation results in overexpression of AMPs that coat the surface of the skin (Guttman-Yassky et al., 2011b). Given the reciprocal regulation of these small molecules by intestinal bacteria, it is likely that a parallel control of the inflammatory and anti-microbial function of the cutaneous epithelial is linked to cutaneous microbiota.

Model of Skin Infection – Leishmania major

To gain a functional and mechanistic understanding of the role of commensals in promoting immune responses and inflammation in the skin, we have employed *Leishmania major* as a physiological model of skin infection *(Belkaid et al., 2000)*. *Leishmania major* is a natural non-disseminating mouse pathogen and a model for human cutaneous *Leishmaniasis (Kaye and Scott, 2011).* A range of factors, including host immunity and parasite virulence, controls the manifestation of this spectral disease. Intradermal inoculation with this parasite results in the slow development of necrotic dermal lesions

in a dose-dependent manner (Belkaid et al., 1998). Both pathology and protective immunity are dependent upon CD4 T cell-derived IFNγ *(Scharton-Kersten and Scott, 1995)*. Indeed, Balb/c mice that are predisposed to a Th2 polarization and IL-4 production are highly susceptible to *L. major (Jones et al., 1998).* These anaerobic parasites are housed in vacuoles with dermal macrophages, where they multiply stealthily (Naderer and McConville, 2011). Activation of macrophages with bacterial lipopolysaccharide (LPS) and IFNγ results in production of reactive oxygen species and parasite killing (Mauel et al., 1991). Importantly, mice deficient in the MyD88 signaling complex, essential for signaling downstream toll-like receptors (TLRs), are unable to form protective responses to *L. major* (Muraille et al., 2003). Because a parasite-derived TLR ligand capable of *in vivo* recognition of *Leishmania* has yet to be identified, it is highly probably that commensal-derived signals or tissue damage associated with infection are required for a development of immunity to *L. major*. **In Chapter 3, we examine the contribution of skin commensals to the acquisition of protective immunity to *L. major.***

Chapter 2: Topographically distinct skin sites have unique immunological and commensal signatures

Abstract

The skin is a critical interface that separates the body from the external environment. This physical barrier is reinforced by an immunological barrier, which protects from pathogenic invasion while maintaining a plethora of resident organisms. Yet little is understood about the composition and regulation of immune cells in normal skin tissue. In order to evaluate the immune cell compartment of the skin, we established novel cell isolation techniques that allowed us to reach the following conclusions:

1. **Physiologically distinct murine skin sites house unique compositions of innate and adaptive immune cells.**
2. **Murine skin is an ecologically heterogeneous organ with commensal communities that are characteristic of specific niches.**
3. **Inflammatory T cells preferentially reside in skin sites that are rich in hair follicles and sebaceous glands in non-human primates (NHPs).**

Rationale

A dense network of innate and adaptive immune cells patrol the skin and respond to environmental assaults. Using mice deficient in either immune cells or their activating receptors, previous studies have demonstrated the importance of these resident cells in maintenance of the cutaneous barrier (Kupper and Fuhlbrigge, 2004). However, our understanding of skin-resident immune cells and the factors that regulate their function has been limited by lack of efficient cell isolation techniques. Moreover, the skin is a large heterogeneous organ with variations in tissue morphology and immune responsiveness (Braverman, 1997; Huzaira et al., 2001). For instance, pathogen challenge has dramatically different outcomes in physiologically distinct skin sites (Nabors and Farrell, 1994; Nabors et al., 1995). Furthermore, cutaneous inflammatory disorders such as atopic dermatitis, psoriasis, and acne characteristically affect certain skin sites but not others (Guttman-Yassky et al., 2011a). Thus, mapping the cutaneous immune landscape will be essential to gain a proper understanding of barrier function and tissue disorders in the skin.

Results

Novel cell isolation technique allows for characterization of innate and adaptive lymphocytes from the skin.

Previous studies have employed histological analysis or gene deficient animals to identify the function of immune cells in the skin. A comprehensive single-cell analysis has been limited by the availability of proper reagents such as purified enzymes that minimize nonspecific cleavage of identifying surface markers and the ability to discern live and dead cells. Therefore, we optimized a novel cell isolation technique with the use of newly available reagents including ultrapure enzymes, fixable live/dead markers, and machines that mechanically disrupt tissues resulting in uniform cell extractions.

In order to perform a single-cell analysis of T lymphocytes in the skin, we first isolated cells directly from the skin using a cocktail of enzymes that disrupt the tissue by cleaving the extracellular matrix and allowing for any associated cells to be extracted. We then analyzed these isolated cells for their expression of surface markers and functional potential using flow cytometry (**Figure 2A**). This technique allowed for isolation of both $\gamma\delta$ and $\alpha\beta$ T cells from whole skin. To determine the localization of $\gamma\delta$ and $\alpha\beta$ T cells within the skin, the dermal and epidermal compartment were separated by cleaving the basement membrane. In line with previous reports, cells expressing high levels of the TCR $\gamma\delta$ known as Dendritic Epidermal $\gamma\delta$ T Cells (DETCs) localized to the epidermis (Jameson and

Havran, 2007), while TCR$\gamma\delta^{low}$ and TCRβ^{+} cells were found to be primarily dermal resident cells (**Figure 2B**). Dermal resident TCR$\gamma\delta^{low}$ cells are a recently described population of cells that maintain heterogeneous TCR$\gamma\delta$ expression and have the potential to produce substantial amounts of IL-17A (**Figure 2B and 3A**). Work from our lab and others have shown that Foxp3^{+} regulatory T cells (T_{regs}) are critical regulators of skin immunity, without which dermal pathologies have been observed in both mouse and man (Belkaid et al., 2002; Wildin et al., 2001). Indeed, we found that normal skin housed a large number of T_{regs} (**Figure 3B**). In fact a comparative analysis of extralymphoid tissues including the skin, small intestine, colon, liver, and lungs revealed that the skin houses the greatest frequencies of T_{regs}, indicating that these cells are likely a dominant form of regulation in this tissue (**data not shown**). In addition to regulatory cells, the TCRβ^{+} compartment also contained cells with the potential to produce considerable amounts of inflammatory cytokines IFNγ and IL-17A (**Figure 3B**). Both CD4^{+} and CD8^{+} T cells possessed the ability to make IFNγ (**data not shown**), while IL-17A was produced by CD4^{+} T cells and a previously uncharacterized population of CD4^{-} CD8^{-} double negative TCRβ^{+} T cells in the dermis (**Figure 3C**).

Our newly optimized cell isolation technique also enabled us to characterize the innate cell populations of the skin. While several groups have identified the resident dendritic cells of the ear skin (Helft and Merad, 2010; Ortner et al., 1996), these isolation techniques often utilize crude enzymes

that do not allow for efficient extraction of innate cells from all skin sites, especially those with higher densities of hair follicles or extracellular matrix materials (S. Naik unpublished observation). Additionally, existing techniques for isolation of dendritic cells required the separation of the dermis and the epidermis and prolonged incubation periods that result in either activation or cell death of these highly sensitive immune cells. We were indeed able to recover a considerably larger number of viable dendritic cells with our protocol than with existing protocols (**Figure 4A and data not shown**). In agreement with previous studies, there were four distinct subsets of cutaneous dendritic cells, including Dermal Dendritic Cells (DDC), Langerhans cells (LC), Langerin^{+} Dermal DC (CD103DC) and Double Negative Dendritic Cell (DNDC) (**Figure 4A**). This cell isolation strategy also allowed us to retrieve eosinophils, mast cells, and several uncharacterized populations of CD45^{+} immune cells from the skin (**data not shown**). These findings corroborate existing evidence that the skin is a dynamic immune environment with a host of cells whose function and regulation can be further explored using the above-mentioned techniques.

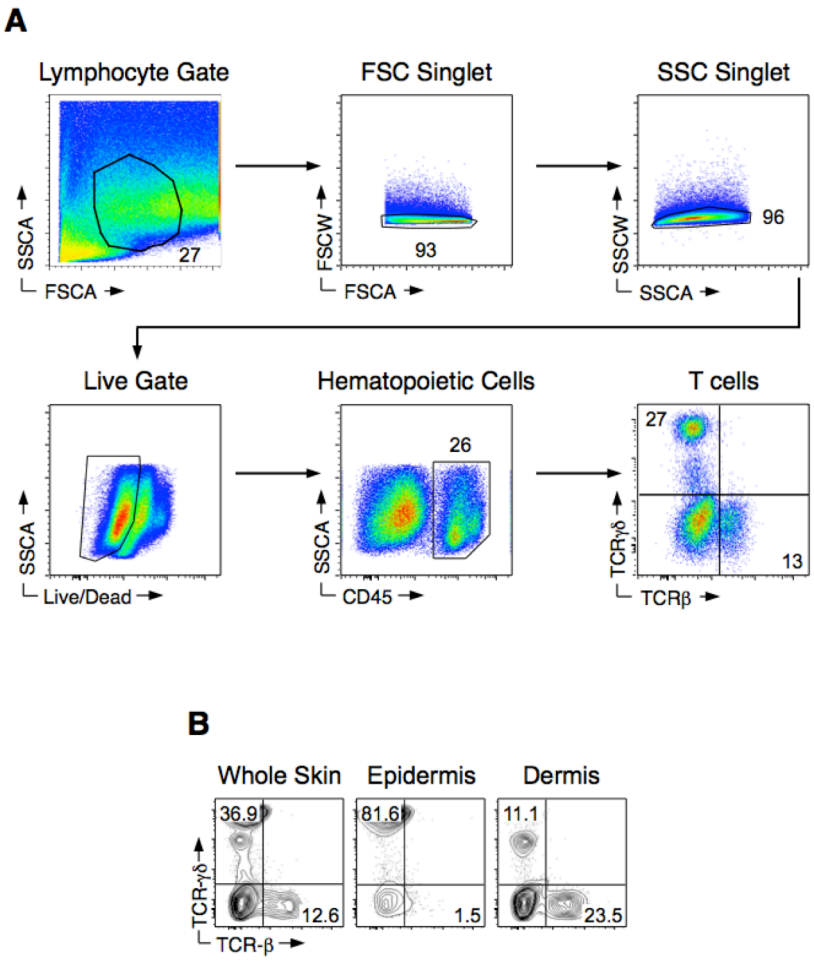

Figure 2: Isolation of T lymphocytes from skin tissue.

(**A**) Gating strategy for γδ and αβ T cells from skin tissue of specific-pathogen-free (SPF) mice. (**B**) Representative flow cytometric plots of γδ and αβ T cells isolated from whole skin, epidermis, and dermis of SPF mice.

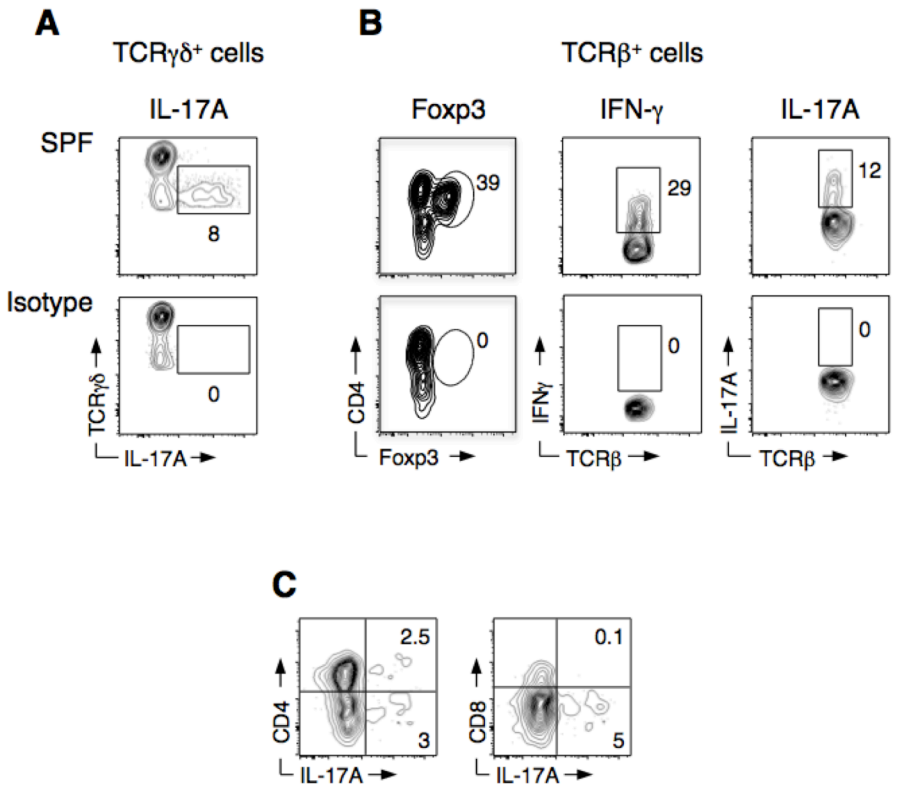

Figure 3: Characterization of effector and regulatory T lymphocyte subsets from the skin.

(**A-B**) Representative plots of IL-17A expression in live CD45$^+$ TCR$\gamma\delta^+$ cells and Foxp3, IFN-γ, and IL-17A expression in live CD45$^+$ TCRβ^+ cells and negative isotope control after phorbol myristate acetate (PMA)/ Ionomycin stimulation from skin tissue of SPF animals. (**C**) Representative plots of IL-17A expression by CD4$^+$ and CD8$^+$ TCRβ^+ single positive cells and TCRβ^+ double negative cells.

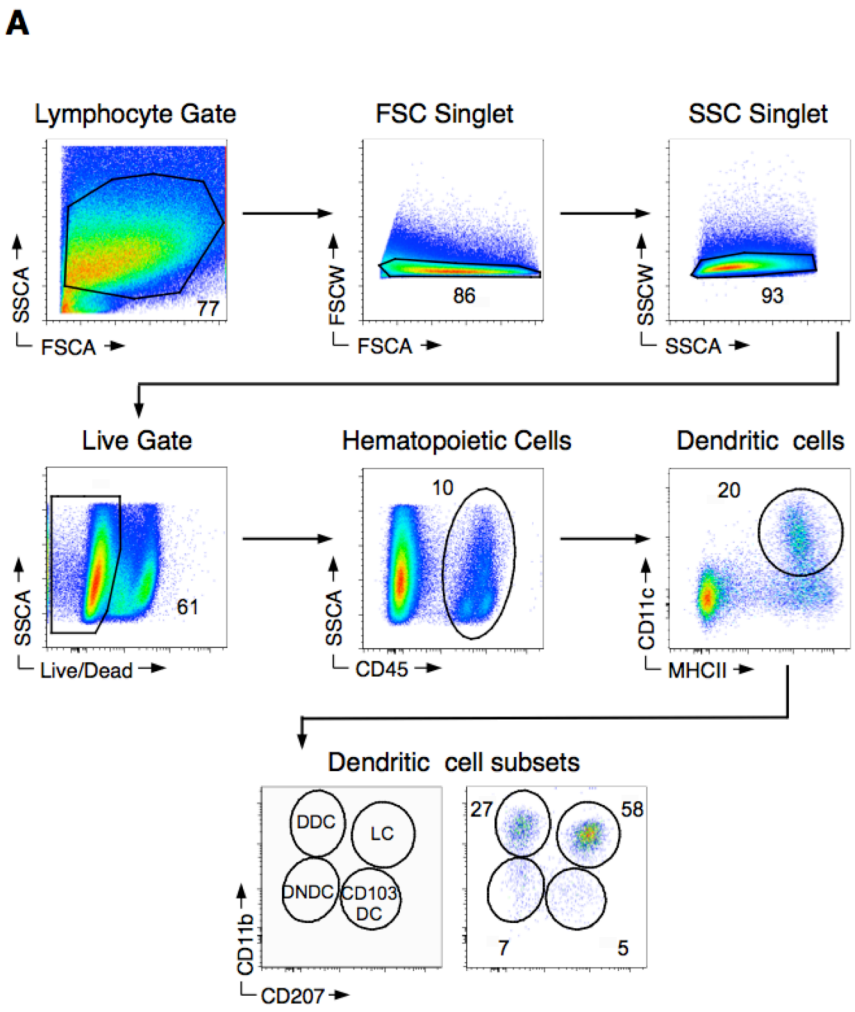

Figure 4: Identification of dendritic cell subsets from skin tissue.

(**A**) Gating strategy for dendritic cell subsets from skin tissue of SPF mice. Cells are gated on live $CD45^+$ $CD11c^+$ and $MHCII^+$. Subsets are defined as follows: Langerin cell (LC) are gated on $CD11b^+$ $CD207^+$ (Langhans) cells, $Langerin^+$ Dermal DC (CD103 DC) are gated on $CD11b^-$ $Langerin^+$ cells, Dermal Dendritic cell (DDC) gated on $Langerin^-$ $CD11b^+$ cells and double negative dendritic cells (DNDC) are gated on $CD11b^-$ $Langerin^-$.

Murine skin is an immunologically heterogeneous organ.

Topographical surveys of the human skin have revealed that this organ is not structurally uniform. The physiology of this heterogeneous organ is defined by invaginations formed by sweat glands, sebaceous glands and hair follicles, variations in epidermal and dermal thickness, varying numbers of blood vessels, and differences in temperature and pH (Huzaira et al., 2001). Additionally, these ecologically distinct niches within the skin house unique communities of commensal organisms (Grice et al., 2009; Grice and Segre, 2011). Some or all of these factors may orchestrate tissue function and control immune fitness locally. Indeed, in the skin, responsiveness to environmental challenges has previously been observed to be site-specific. For instance, variable immunity and pathology to dermal pathogen *Leishmania major* has been observed in the skin as infection in some locations, resulting in the development of palpable necrotic lesions, while other locations do not exhibit any dermal pathology (Nabors and Farrell, 1994; Nabors et al., 1995). However, the immune cell compartments of distinct skin sites have not been studied due to limitations in cell isolation techniques.

In order to construct a representative map of immune cells in the skin, we characterized this cellular compartment in three distinct skin sites using the abovementioned novel skin isolation techniques (see pg. 130). The ear skin, back skin, and belly skin were assessed because of differences in the numerical density of appendages such as hair follicles (Schlake, 2007)(**Figure 5A**). We

enumerated the $CD45^+$ hematopoietic cell compartment from $100mm^2$ of skin at each of these sites. Areas with increased invaginations, such as the back skin or belly skin, housed a greater number of $CD45^+$ immune cells when compared to the ear skin, which has lower densities of hair follicles, a thinner dermal layer, and maintains a lower temperature (**Figure 5B**).

To further probe the composition and functional capacity of these cells, we performed an in-depth analysis of innate and adaptive immune cells at the three defined skin sites. The T lymphocyte compartments varied considerably between the ear, back, and belly skin. More specifically, the ear housed the greatest frequency of epidermal $TCR\gamma\delta^{hi}$ cells (DETCs), while the back and the belly had significantly larger proportions of dermal resident $TCR\gamma\delta^{low}$ cells and $TCR\beta^+$ cells (**Figure 6A**). In addition to the differences in the proportions of T lymphocytes, we also noted site-specific variations in the functional capacity of these T lymphocytes. The ear skin contained the lowest frequency of both T_{regs} and IFN-γ^+ T effectors, while the back and belly skin both held substantially more T_{regs} and IFN-γ^+ T effectors (**Figure 6B and C**). Although the total amount of IL-17A produced in these three areas was comparable (~5-8%), the relative contribution from cellular sources varied from site to site (**Figure 6D**). Consistent with the increase in frequency of $TCR\beta^+$ cells in the back and belly skin, the $TCR\beta^+$ cells within these sites also made a greater contribution to the total amount of IL-17A produced (**Figure 6D**). Conversely, $TCR\gamma\delta^+$ made up a greater proportion of IL-17A producers in the ear skin (**Figure 6D**). Thus, the T lymphocyte composition

in skin tissue varies in a site-specific manner not only in the proportions of T cells that are housed, but also their functional capacities.

The skin is also home to a variety of innate sentinels, such as dendritic cells, mast cells, and eosinophils, that are primary responders during inflammatory challenges (Clark and Kupper, 2005). We sought to characterize these populations in previously defined skin sites (**Figure 5A**). Similar frequencies of $CD11c^+$ $MHCII^+$ dendritic cells were isolated from each of the three skin sites (**Figure 7A**). However, these sites harbored distinct compositions of dendritic cell subsets (**Figure 7B**). Langerhans cells made up a dominant proportion of ear dendritic cells, while the back and belly skin contained more dermal dendritic cells and double negative dendritic cells (**Figure 7B**). Of note, $CD103^+$ dendritic cells uniformly localized to the three areas of the skin (**Figure 7B**). Next, we analyzed the defined cutaneous locations for eosinophils and mast cells, and observed a reciprocal pattern in the localization of these two populations. The ear skin contained a considerable amount of mast cells but few eosinophils (**Figure 7C and D**). On the other hand, back and belly skin, which had few mast cells, was inhabited by a significant amount of eosinophils (**Figure 7C and D**). This survey of innate and adaptive cells revealed that murine skin is not only a structurally heterogeneous organ but also exhibits dramatic differences in the immune cell populations that occupy these distinct tissue sites.

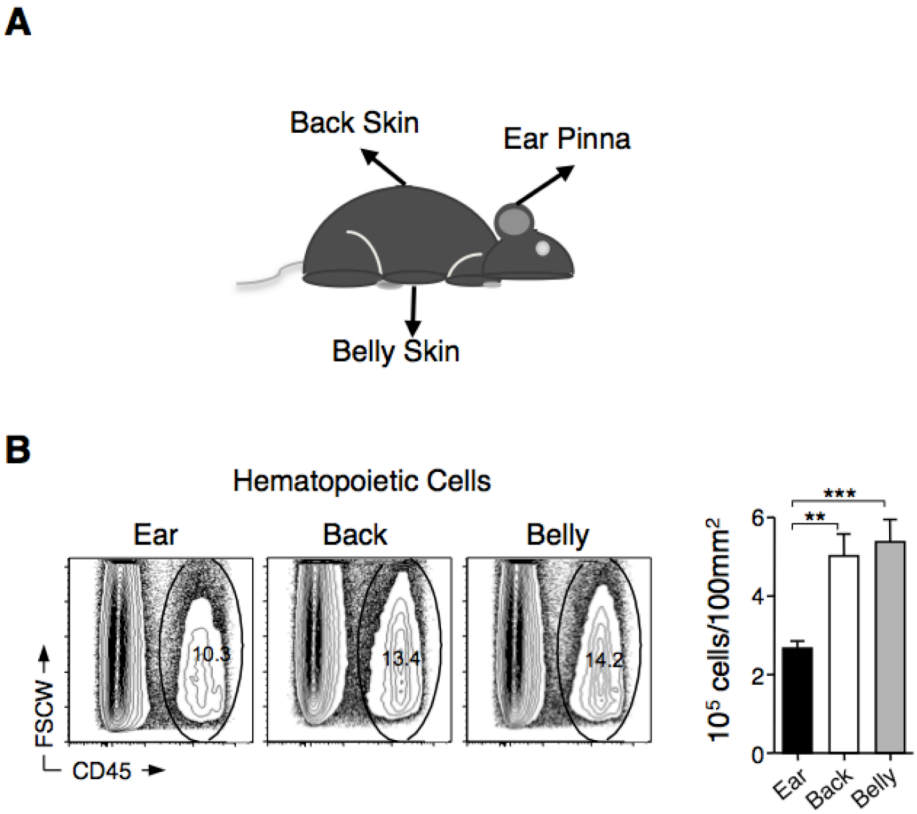

Figure 5: Defining physiologically distinct sites in murine skin.

(**A**) Schematic of three physiologically distinct sites in murine skin: ear pinnae, back skin, and belly skin. (**B**) Representative flow cytometric plots and summarized bar graphs of $CD45^+$ cells per $100mm^2$ of skin tissue from defined skin sites. Graphs are a compilation of 2 experiments and show the mean of 9 mice $\pm$ SEM (** $p< 0.005$, *** $p< 0.0005$).

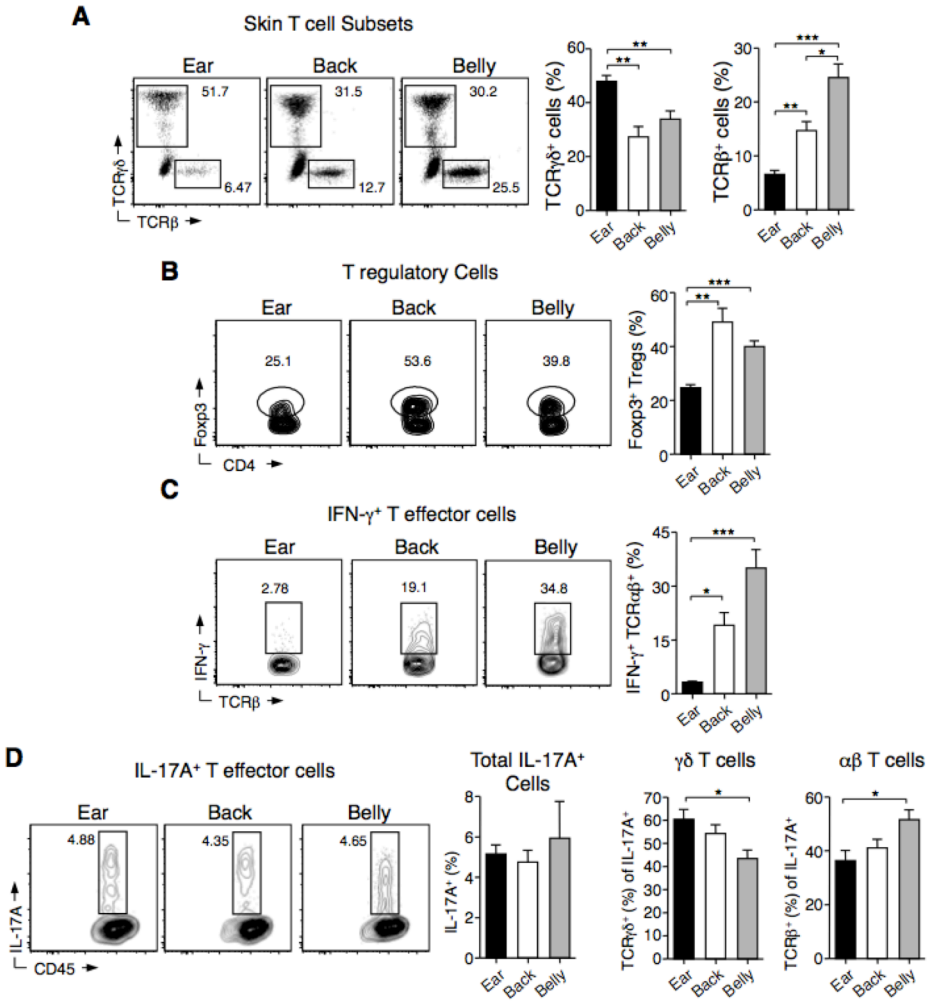

Figure 6: Distinct skin sites house unique compositions of T lymphocytes.

(**A**) Representative flow cytometric plots and summarized bar graphs of live CD45$^+$ TCR$\gamma\delta^+$ and TCRβ^+ cells extracted from ear (■), back (□), and belly (■) skin of SPF mice. Graphs show the mean of 4 mice ± SEM. (**B**) Representative flow cytometric plots and summarized bar graphs of Foxp3 expression in live CD45$^+$ TCRβ^+ CD4$^+$ cells extracted from ear (■), back (□), and belly (■) skin. Graphs show the mean of 4 mice ± SEM. (**C-D**) Representative flow cytometric plots and summarized bar graphs of IFN-γ and IL-17A expression in live CD45$^+$ TCR$\gamma\delta^+$ and TCRβ^+ cells extracted from ear (■), back (□), and belly (■) skin. Graphs show the mean of 4 mice ± SEM (*p< 0.05, ** p< 0.005, *** p< 0.0005).

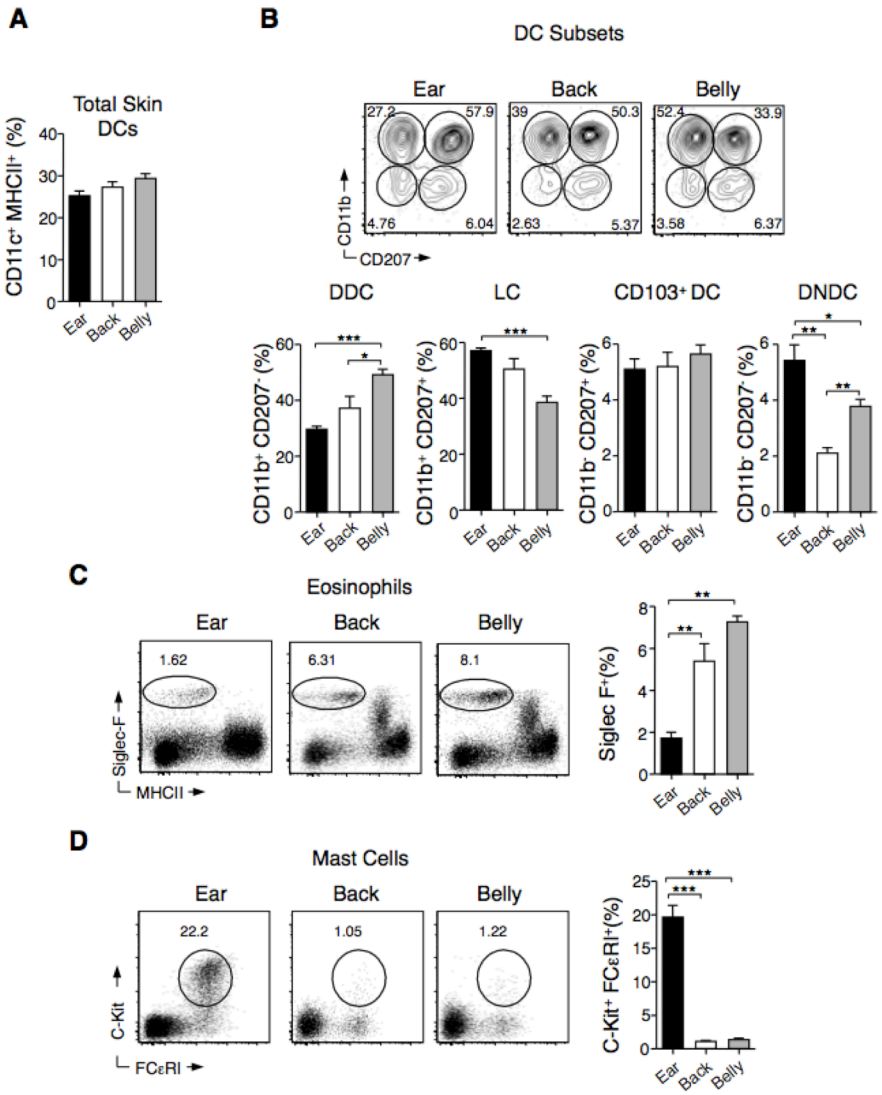

Figure 7: Distinct skin sites house unique compositions of innate leukocytes.

(**A-B**) Representative flow cytometric plots and summarized bar graphs of cutaneous dendritic cells (See Fig. 2 for definition) extracted from ear (■), back (□), and belly (■) skin of SPF mice. Graphs show the mean of 4 mice ± SEM. (**B**) Representative flow cytometric plots and summarized bar graphs of Eosinophils gated on live $CD45^+$ $MHCII^-$ $Siglec\text{-}F^+$ cells extracted from ear (■), back (□), and belly (■) skin. Graphs show the mean of 4 mice ± SEM. (**C**) Representative flow cytometric plots and summarized bar graphs of Mast Cells gated on live $CD45^+$ $Cd11b^-$ $C\text{-}Kit^+$ $FC\varepsilon RI^+$ cells extracted from ear (■), back (□), and belly (■) skin. Graphs show the mean of 4 mice ± SEM. (*p< 0.05, ** p< 0.005, *** p< 0.0005).

Anatomically distinct skin sites in mice house unique commensal communities.

Mammalian skin is a complex ecosystem that harbors diverse populations of microbial communities (Grice et al., 2009). Recent advances in 16S rDNA sequencing technology have dramatically improved our appreciation of commensals that reside in the skin by revealing that this reservoir houses unprecedented diverse communities of bacteria. A survey of human skin microbiota revealed that physiologically and topographically comparable sites house similar bacterial communities (Grice et al., 2009). Additionally, this study also revealed that the skin microbiota exhibit more intrapersonal than interpersonal differences. To date, the microbial communities that occupy distinct skin sites of specific-pathogen-free mice (SPF) mice have not been examined. Because mice are housed in groups of four or five per cage and are in constant contact with each other and their own feces, it important to discern if murine skin, like human skin, houses unique microbial communities associated with characteristic features of distinct skin sites. To address this, we analyzed the 16S rDNA sequences of bacteria communities found in the three defined skin sites (**Figure 5A**) from four individual animals housed in the same cage. Remarkably, we found that l the commensal composition of murine skin, like that of human skin, was associated with the specific ecology of each skin site (**Figure 8A**). *Firmicutes* preferentially reside in ear skin, while back and belly skin were dominantly colonized by *Proteobacteria* (**Figure 8A**).

Additionally, a cluster analysis used to group sequenced communities on the basis of similarity revealed that communities of commensals grouped together by body site rather than by individual animals, indicating a greater degree of intrapersonal rather than interpersonal variability in the bacterial communities living on murine skin (**Figure 8B**). Our data also revealed that distinct microbial communities inhabit the cutaneous and intestinal epithelial surfaces (**Figure 8A and B**). These findings underscore the importance of the ecology of a particular skin site in controlling the composition of resident microbial communities. Importantly, the identification of site-specific differences in skin microbiota of mice further validates murine skin as a relevant model for studying the discrete immune commensal interactions at barrier sites outside the intestine.

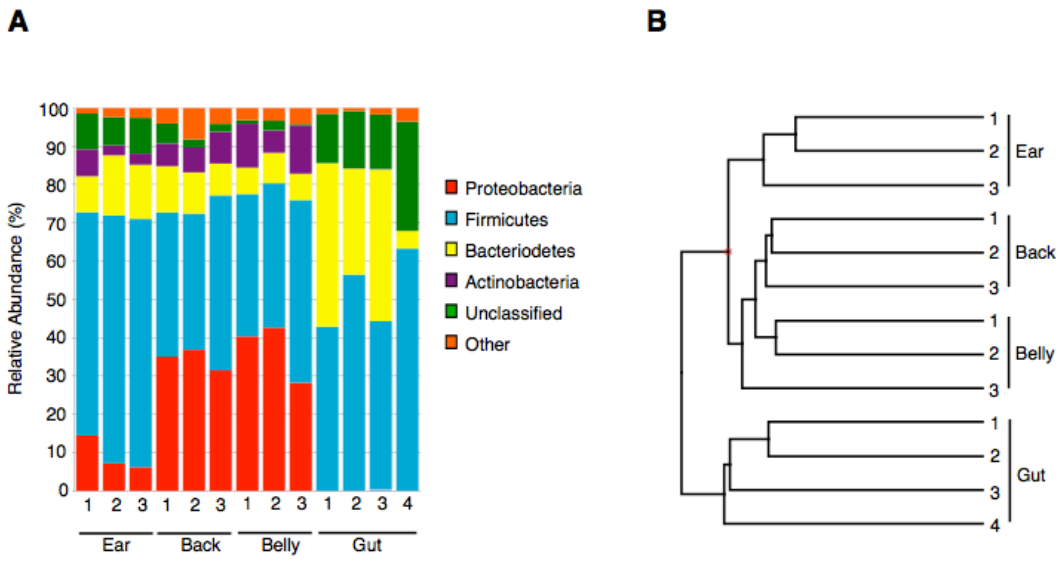

Figure 8: Unique microbial communities reside in topographically distinct murine skin sites.

(**A**) Taxonomic classifications at the phylum level for 16S rDNA gene sequence data clustered at 97% identity from ear, back, and belly skin and fecal pellet of specific-pathogen-free mice. (**B**) Hierarchical cluster analysis of OTUs from ear, back, and belly skin and fecal pellet.

Physiologically distinct skin sites in non-human primates have unique immune landscapes.

To understand the immune surveillance system in the skin of higher-order mammals that have similar skin structure to humans, we evaluated the composition and function of T lymphocytes from normal skin of several non-human primate (NHP) species, including rhesus macaques, pig-tailed macaques, and cynomolgus monkeys (Godin and Touitou, 2007). We re-optimized our cell isolation technique for NHP skin, which is considerably thicker than murine skin with more subcutaneous fat (see pg. 132). Remarkably, a very large number of $CD3^+$ T lymphocytes including $CD4^+$ and $CD8^+$ T cells as well as double negative T cells were recovered from normal skin tissue of NHPs (**Figure 9A**). Of note, a majority of double negative T cells did not express $\gamma\delta$ TCR (**data not shown**). We next assayed these cells for their potential to produce inflammatory cytokines. Considerable portions of cutaneous $CD4^+$ and $CD8^+$ T cells were equipped with the potential to produce inflammatory cytokines either IL-17A or INF-γ and few double producers were observed in normal skin (**Figure 9B**). These findings reiterated the importance of normal skin as an immune effector site.

Our survey of murine skin revealed an immense amount of heterogeneity in the composition and function of immune cells in different areas of the skin. To ascertain if the skin of NHP has similar variations in resident immune cells, we compared the T lymphocyte compartment of skin sites that had a higher density

of sebaceous glands and hair follicles, such as the face and chest, to those with fewer invaginations such as the skin from biceps and quadriceps (**Figure 9C**). Indeed, analogous to our analysis of murine skin, sites with more invaginations, such as the face and chest, contained more $CD3^+$ T cells per gram of tissue. Furthermore, these sites also contained a significantly greater number of cells with the potential to produce inflammatory IL-17A and INF-γ when compared to sites with fewer appendages (**Figure 9D**). Thus, NHP skin is an immunologically active organ with site-specific differences in composition of inflammatory T lymphocytes.

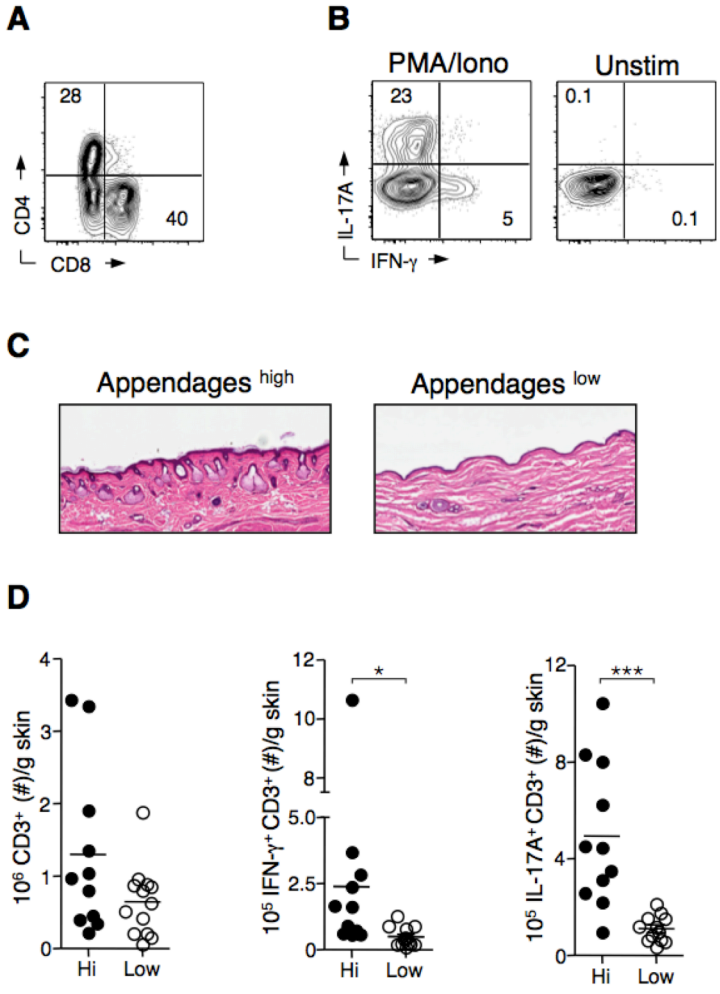

Figure 9: Non-human primate skin sites that have a high density of appendages house more inflammatory T cells.

(A) Representative flow cytometric plots of $CD4^+$ and $CD8^+$ T cells isolated from normal nonhuman primate (NHP) skin. (B) Representative flow cytometric plots of IL-17A and IFN-γ by live $CD3^+$ T cells either stimulated with PMA/Ionomycin or unstimulated control from normal NHP skin. (C) Representative histological image of NHP skin sites with a high density of appendages (appendage ^{high}) and with a low density of appendages (appendage ^{low}). (D) Comparative analysis of the total number of $CD3^+$ T cells and the number of $CD3^+$ T cells with the potential to produce IL-17A or IFN-γ in sites with high or low frequency of appendages (Defined in Figure 9C)

Chapter 3: Compartmentalized control of cutaneous immunity by resident commensals

Abstract

The data presented in **Chapter 2,** along with numerous published reports, have demonstrated that resident immune cells are a major component of skin tissue. However, the exogenous factors that regulate the homeostasis and function of immune cells in the skin require elucidation. The commensal microbiota dominantly control immune cells at other barrier sites, such as the intestines. Although complex communities of commensal organisms densely populate the skin, their role in tissue function and immune fitness has not been evaluated. We thus postulated that indigenous commensal communities, which are in close apposition with immune cells in the skin, critically regulate their function. To address this hypothesis, we evaluated immune cells in the skin of germ-free mice at steady state and during *Leishmania major* infection. These studies led to the following conclusions:

1. **The balance of inflammatory and regulatory T cells in the skin is altered in the absence of commensal microbes.**
2. **Distinct microbial communities regulate immune cells in the skin and intestine.**
3. **Skin microbiota drive immunity and pathology during *Leishmania major* infection.**

Rationale

The body is comprised of various tissue microenvironments with finely tuned local immune-surveillance systems that are intimately associated with distinct communities of commensal organisms. These microbes are not “innocent bystanders” but play an active role in maintaining host physiology. In particular, intestinal microbiota are essential for metabolism, tissue development, and host defense (Hooper et al., 2012; Hooper et al., 2002). More recently, gut-dwelling microbes have been implicated in driving systemic inflammation in murine models of autoimmunity (Hooper et al., 2012). However, the degree to which the gut flora act as the dominant source of commensal signals in the body and contributes to immunity at distal sites remains unclear.

The skin represents the primary interface between the host and the environment and is densely populated by diverse communities of commensals (Grice et al., 2009). The cross talk between resident skin bacteria and underlying dermal compartment is likely to play a complex role in tissue functions. Supporting this idea, defined microbial products from the skin commensal, *Staphylococcus epidermidis*, can mitigate inflammation in the context of tissue injury (Lai et al., 2009). On the other hand, inflammatory skin disorders such as psoriasis, atopic dermatitis, and rosacea have been associated with dysbiosis in the resident skin flora (Gallo and Nakatsuji, 2011; Kong et al., 2012). Owing to its accessibility, this tissue represents an ideal organ system in which to study the unique mechanisms that underlie the dialogue between local flora and resident

immune cells and the consequence of such interactions on tissue immune fitness and pathology. Despite the primary role of the skin as a physical and immunological barrier, the direct contribution of skin commensals to cutaneous immunity and host defense has not been addressed. Understanding the host–commensal dialog in the skin will provide novel insights into the regulation of immune cells at this tissue site and reveal the contribution of commensals residing in sites outside the intestinal mucosa to host immunity.

Results

Commensals control the effector and regulatory T cell equilibrium independently of tissue development in the skin.

In humans, commensal microbes densely cover the surface of the skin and reside in appendages such as hair follicles, sebaceous glands, and sweat glands. To detect the location of microbial byproducts within skin of mice we stained the skin of conventionally raised or specific-pathogen-free (SPF) mice with an anti-*E. coli* antibody. This antibody is capable of detecting major bacterial cell wall and cytoplasmic components (**data not shown**). Consistent with previous analysis of human skin, we found that microbes or microbial byproducts were intimately associated with the cornified layer of interfollicular epidermis and within appendages such as hair follicles and sebaceous glands in the skin of SPF mice (**Figure 10A**). Importantly, these microbial signatures were absent from the skin tissue of germ-free mice born in aseptic conditions and reared in sterile isolators in the absence of any living bacteria, fungi, protozoa, and exogenous virus (Wostmann, 1996)(**Figure 10A**).

Using GF mice, mice treated with oral antibiotics, or mice deficient in commensal sensing receptors, previous work has revealed that the balance between effector and regulatory T lymphocytes in the intestine is tightly controlled by commensally derived signals (Hall et al., 2008; Ivanov et al., 2008). In the absence of commensal stimuli, a reduction in the frequency of T cells with

potential to produce IFN-γ and IL-17A and a concomitant increase in the frequency of T_{regs} is observed in the small intestine lamina propria (Ivanov et al., 2008). Similarly, we observed a significant reduction in the frequency and number of TCRβ$^+$ cells with the potential to produce pro-inflammatory cytokines IFN-γ and IL-17A in skin tissue of GF mice compared to SPF mice (**Figure 11A and B**). The capacity of dermal TCRγδ^{low} cells to make IL-17A was also diminished in the absence of commensals (**Figure 11B**). Simultaneously, an increase in the frequency and number of cutaneous CD4$^+$ Foxp3$^+$ T_{reg} cells was observed in the skin of GF mice when compared to SPF mice (**Figure 11A**).

One possible explanation for the above-mentioned differences in cutaneous T cell subsets is a requirement for the flora in priming cells in the skin-draining lymph nodes. An analysis of the brachial, inguinal, and retroauricular lymph nodes revealed similar frequencies of effector and regulatory T lymphocytes (**Figure 12A**). Additionally, the structure and cellularity of cutaneous lymph nodes were comparable between SPF and GF animals, excluding the possibility that T cells were not appropriately committed to effector fates in lymphoid tissues in the absence of commensals (**Figure 12B and C**). Because alterations in T cell subsets were restricted to the skin tissue and did not extend to cutaneous lymph nodes, we excluded a role for the flora in priming and polarization of these cells in lymphoid organs at steady state. Instead, we postulated that the ability of certain T lymphocytes subsets to migrate to the skin is altered, and/or the development of innate cells required for maintenance of T

cells in the skin was affected, in the absence of commensals.

T cell infiltration into the skin is controlled in part by the surface expression of specific chemokine receptors such as P-selectin ligand and CD103 that mediate transendothelial migration and tissue retention (Dudda et al., 2008). To determine if the observed differences in skin T cell subsets of germ-free mice were due to altered frequencies of circulating skin tropic T cells, we assess effector and regulatory T cells from the blood, cutaneous lymph nodes, and spleens of SPF and GF animals. The proportion of circulating $CD4^+$ and $CD8^+$ effectors and T_{regs} (P- $selectin^{hi}$ and $CD103^+$ cells) was comparable between GF and SPF animals (**Table 1**). The ability of cells to migrate to peripheral tissues is also dependent upon local chemokine expression. Aberrant expression of CXC16 by lung and colonic epithelia of germ-free mice has been associated with increased migration of iNK T cells to these tissues (Olszak et al., 2012). While our data show that there are no intrinsic defects in the T cells' ability to migrate to the skin in the absence of commensals, we cannot exclude the possibility that changes in chemokine expression in the tissue could be responsible for alterations in the types of cells recruited or retained.

Commensal flora stimulates the development of the intestinal immune system by promoting the maturation of both innate and adaptive lymphocytes (Cebra, 1999). Differences in the composition of intestinal T cells have been partially attributed to developmental deficiencies in the intestine of germ-free mice (Cebra, 1999; Round and Mazmanian, 2009). We therefore assessed if

development defects were similarly responsible for aberrant cutaneous T cell homeostasis in germ-free mice. In contrast to the intestine, the cellularity of the $CD45^+$ immune cell compartment in the skin was not altered in GF mice (**Figure 13A**). Additionally, the composition of both $\gamma\delta TCR^+$ and $\alpha\beta$ TCR^+ cells in GF mice was comparable to that of their SPF counterparts (**Figure 13B**). Innate cells express activating ligands and secrete a variety of cytokines in response to environmental clues that modulate the function of T lymphocytes in tissues. Previous studies have identified differences in the composition and function of specific dendritic cell subsets isolated from the intestine of germ-free mice (Lee et al., 2011; Niess and Adler, 2010). We therefore analyzed the innate cell compartment of the skin and found no changes in the frequency of dendritic cells subsets, mast cells, and eosinophils that reside in the skin (**Figure 13C through E**). Taken together, our findings show that the absence of commensal microbes results in the disequilibrium of effector and regulatory T lymphocytes in the skin. Furthermore, alterations in T cell polarization in the lymph nodes, differential skin homing capacity, or tissue development were not responsible for changes in T cell subset distribution in the skin of GF mice.

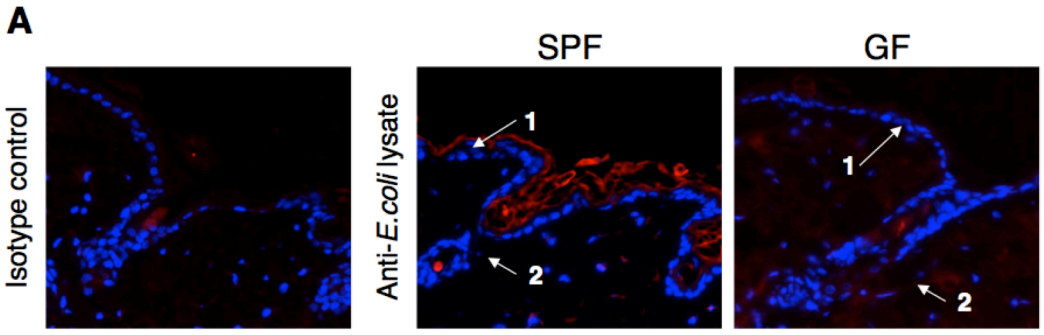

Figure 10: Commensal byproducts are found on the surface of the skin and in appendages.

(**A**) Immunofluorescence labeling of bacterial products in (1) interfollicular keratinocytes and (2) hair follicles from skin tissue of specific pathogen-free (SPF) and germ-free (Bisgaard et al.) mice. Representative images of naïve skin stained with anti- *E. coli* lysate antibody (Red) or isotype control and HOECHST (Wen et al.)

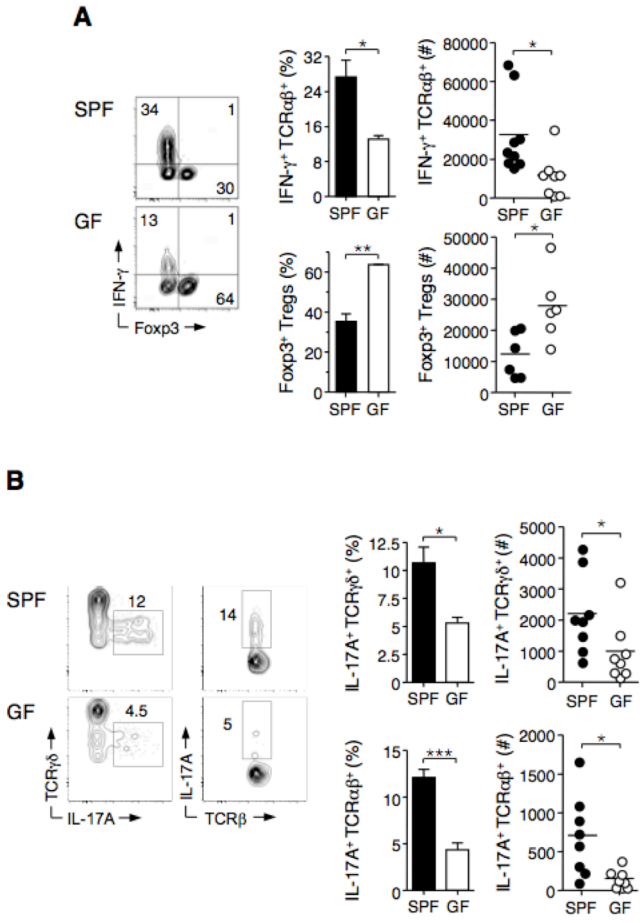

Figure 11: Commensal microbiota control the balance of effector and regulatory T cells in the skin tissue.

(**A**) Representative flow cytometric plots and summarized graphs of frequency and numbers of IFN-γ, and Foxp3 expressing live $CD45^+$ $TCR\beta^+$ cells extracted from skin tissue of SPF (■) and GF (□) mice after (PMA)/ Ionomycin stimulation. Bar graphs show the mean of 3-4 mice ± SEM. Results are representative of 3 experiments. (**B**) Representative flow cytometric plots and summarized graphs of frequency and numbers of IL-17A$^+$ live $CD45^+$ $TCR\gamma\delta^+$ or $CD45^+$ $TCR\beta^+$ cells from skin tissue of SPF (■) and GF (□) mice post PMA/ Ionomycin stimulation. Graphs show a mean of 3-4 mice ± SEM. Results are representative of 3 experiments. (*$p< 0.05$, ** $p< 0.005$, *** $p< 0.0005$).

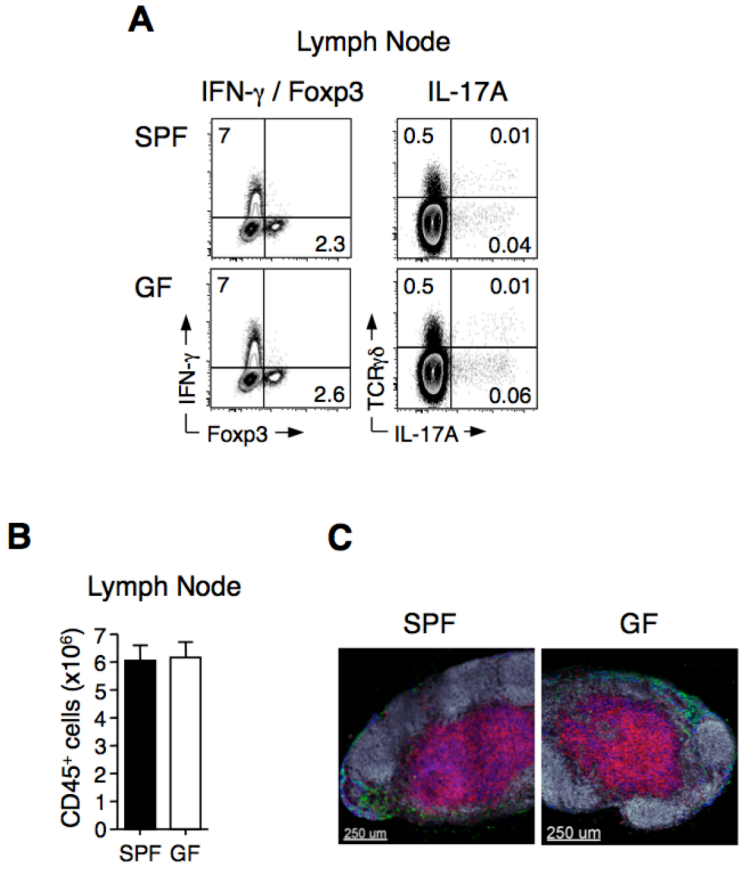

Figure 12: Comparable cellularity and structure of skin draining lymph nodes from SPF and GF mice.

(**A**) Representative plots of IFN-γ, and Foxp3 expression in live $CD45^+$ $TCR\beta^+$ $CD4^+$ cells and IL-17A expression in live $CD45^+$ cells extracted from cutaneous lymph nodes of SPF (■) and GF (□) mice post PMA/ Ionomycin stimulation. Results are representative of 3 experiments. (**B**) Quantification of total $CD45^+$ hematopoietic cells from the skin associated lymph nodes of SPF (■) and GF (□) mice. Error bar is a mean of 4 mice ± SEM. Results are representative of 3 experiments. (**C**) Representative images of retroauricular lymph node sections from naïve SPF and GF mice immunolabeled with anti-B220 (White; labels B cells), CD8 (Red) CD4 (Wen et al.) and Collagen IV (Green). Results are representative of 2 experiments.

Table 1

% Skin Homing T cells

	Blood		cLN		Spleen	
	SPF	GF	SPF	GF	SPF	GF
$CD4^+$ CD44hi T regs	6 ± 3	4 ± 2	19 ± 2	18 ± 1.5	5 ± 1	6 ± 1
$CD4^+$ CD44hi T effectors	5 ± 3	4 ± 0.5	16 ± 1	15 ± 1	11 ± 1	15 ± 2
$CD8^+$ CD44hi T effectors	2 ± 0.5	2 ± 0.5	10 ± 2	7 ± 1	10 ± 1	11 ± 1

Table 1: Circulating T lymphocytes from SPF and GF mice have equivalent surface expression of skin homing markers.

(**A**) Summary of skin-homing markers expressed by T cells from blood, cutaneous lymph node, and spleen of SPF and GF mice. Skin-homing subsets were defined as follows: CD44hi $CD4^+$ T_{regs} and T effectors were evaluated for CD103 and high P-selectin ligand surface expression and CD44hi $CD8^+$ T effectors were analyzed for high P-selectin ligand surface expression. Results are representative of 2 experiments.

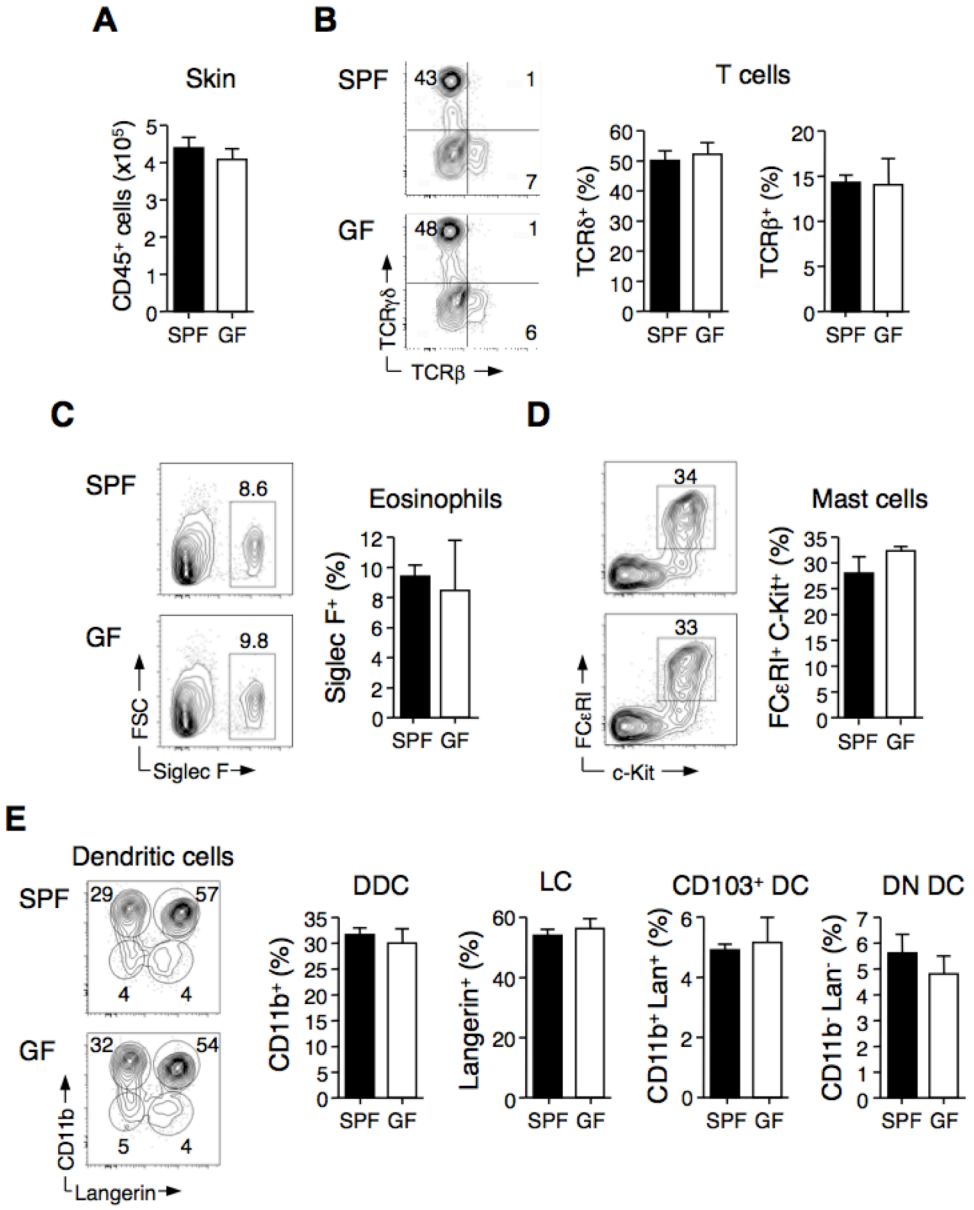

Figure 13: Composition of skin immune cells is maintained in the absence of commensals.

(**A**) Quantification of total CD45$^+$ hematopoietic cells from the skin of SPF (■) and GF (□) mice. Error bar is a mean of 4 mice ± SEM. Results are representative of 3 experiments. (**B**) Frequency of TCR$\gamma\delta^+$ and TCRβ^+ subsets from the skin tissue of SPF (■) and GF (□) mice Error bar is a mean of 4 mice ± SEM. Results are representative of 3 experiments. (**C-E**) Summarized proportions of eosinophils, mast cells, and various dendritic cell subsets from skin tissue of SPF (■) and GF (□) mice. Error bar is a mean of 4 mice ± SEM. Results are representative of 2-3 experiments.

Distinct commensal niches in the skin and gut locally regulate immunity.

The intestine is home to the body's most densely populated commensal niche, which is known to impact immunity both locally and systemically (Atarashi and Honda, 2011). For instance, Polysaccharide A (PSA) derived from gut-dwelling *Bacteroides fragilis*, is able to modulate the function of splenic $CD4^+$ T cells (Mazmanian et al., 2008). Additionally, signals emanating from intestinal microbiota have been shown to promote inflammation in mouse models of systemic autoimmunity. Alterations of the gut flora can impact immune pathology in the diabetic, arthritogenic, and encephalitogenic animals (Kriegel et al., 2011; Lee et al., 2011; Wu et al., 2010). The intestinal flora have also been shown to influence the development of immunity to pathogens in distal sites such as the lung (Ichinohe et al., 2011). Thus, it was likely that cutaneous T cells, which constantly recirculate throughout the body, may encounter and respond either directly or indirectly to signals derived from the intestinal microbiota.

Segmented filamentous bacteria (SFB) intimately associate with the intestinal epithelia and promote the maturation of effector T cells in the underlying lamina propria (Ivanov et al., 2009). Mice bred by Taconic farms contain high levels of intestinal SFB and consequently more IL-17A than mice bred by Jackson Laboratories (Ivanov et al., 2008). We assessed the skin of these animals to test whether intestinal SFB could impact cutaneous T cell function. In accord with previous reports, mice from the Jackson Laboratories harbored

significantly lower levels of IL-17A in their intestines than mice from Taconic Farms (**Figure 14A**). However, levels of IL-17A were comparable in the skin of these animals (**Figure 14A**). Consistent with these findings, association of germ-free mice with SFB was sufficient to raise intestinal IL-17A and IFN-γ production levels to those of SPF mice, but did not restore T cell cytokine production or impact T_{regs} in the skin (**Figure 14B though D**).

In addition to SFB, the intestine is colonized by a plethora of commensal species that could individually or cumulatively impact immunity in the skin. To test their role in modulating cutaneous T cell function, we treated mice with a commonly used cocktail of oral antibiotics (Ampicillin, Vancomycin, Metronidazole and Neomycin Sulfate) known to change the quantity and quality of the gut microbiota (Hill et al., 2010). To evaluate the impact of this regimen on the skin microbiota, we measured the density and characterized the composition of 16s rDNA genes isolated from the stool and skin after oral antibiotic treatment. While dramatic differences were observed in density (~ 7 fold decrease) and composition of the intestinal commensals, the cutaneous microbiota was not perturbed by this treatment (**Figure 15 A through C**). Thus, this intervention represents a valid approach for selectively manipulating gut flora while maintaining skin flora intact. Of note, residual 16S rDNA genes in the intestine of mice treated with an oral antibiotic have previously been shown to be derived from the food (Hill et al., 2010). Intestinal T cells have diminished potential to make inflammatory cytokines IL-17A and IFN-γ after oral antibiotic treatment (Hall

et al., 2008; Ivanov et al., 2008). IL-17A and IFN-γ production were reduced in the gut but not the skin after oral antibiotic treatment (**Figure 15D**). These findings demonstrate that the gut microbiota control T lymphocytes locally and that regulation of cutaneous T cell subsets is not dependent upon signals from the gut microbiota.

Diverse communities of commensal organisms are found in the peaks and valleys of the skin (Grice et al., 2009). We hypothesized that these resident organisms could directly control T cells in the underlying dermis. To test this, we made use of *Staphylococcus epidermidis* isolated from the normal human skin (Conlan et al., 2012). Of note, human commensal isolate *S. epidermidis* was used to address the contribution of cutaneous flora because of difficulties culturing commensals from our mouse colonies. We confirmed that this human commensal was able to stably colonize the skin of mice and could therefore serve as a model commensal organism (**data not shown**). This commensal was also used because it has no known function in processing of dietary factors in the intestine and therefore allowed us to dissociate the role of direct immune recognition from metabolic alterations in mono-colonized germ-free animals. Remarkably, associating germ-free mice with this single commensal organism topically was sufficient to restore IL-17A levels in the skin (**Figure 16A and B**). Accentuating the compartmentalized regulation of T cells in the skin and gut, *S. epidermidis* colonization did not alter intestinal IL-17A production in germ-free mice (**Figure 16A and B**). Germ-free mice mono-associated with *S. epidermidis*

were treated with oral antibiotics to confirm that *S. epidermidis* exerted its effects locally. Mono-associated mice treated with oral antibiotics had similar levels of IL-17A in their skin as animals treated with vehicle control (**Figure 16C**). These data reveal a non-redundant role for distinct commensal niches in the skin and gut in locally controlling T effector maturation.

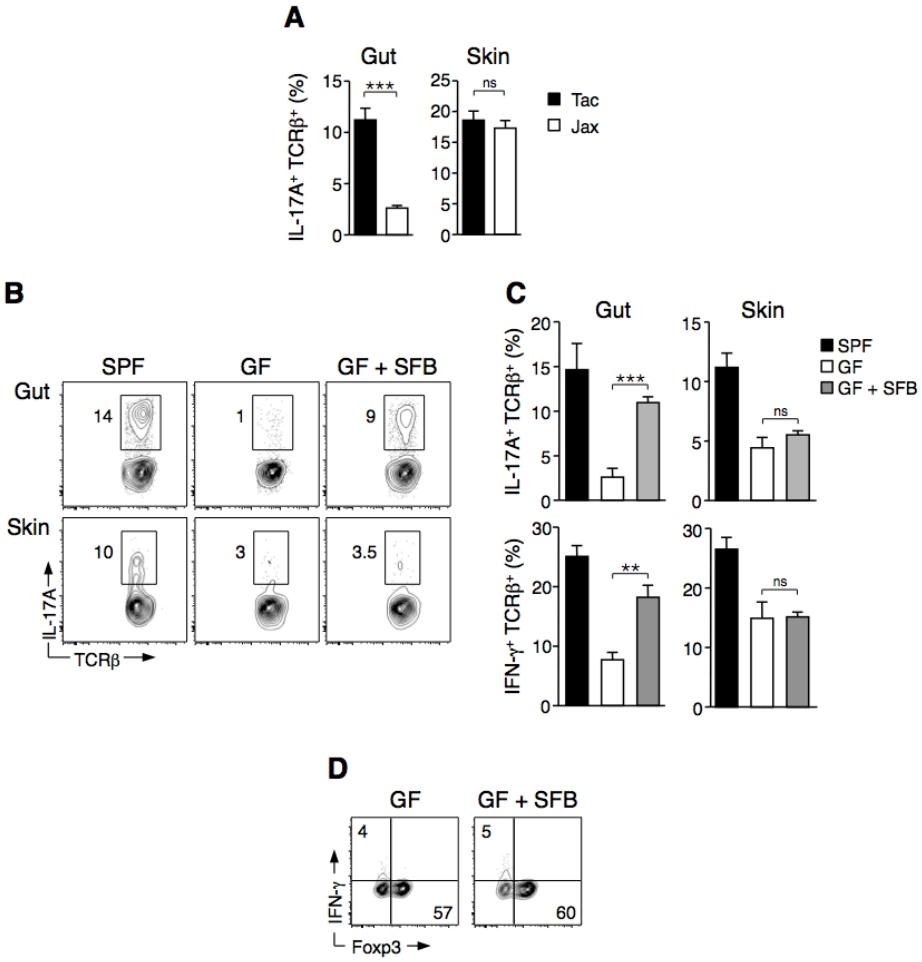

Figure 14: Gut resident *Segmented Filamentous Bacteria* (SFB) does not influence skin T lymphocyte subsets.

(**A**) Assessment of IL-17A expression by live CD45$^+$ TCRβ^+ cells from the intestine and skin of SPF mice from Taconic Farms and Jackson Laboratories (**B-C**) Comparative analysis of IL-17A and IFN-γ expression from the intestine and skin of SPF, GF, and GF mice mono-associated with Segmented Filamentous Bacteria (SFB). Flow cytometry plots are gated on live CD45$^+$ TCRβ^+ cells. Results are representative of 2 experiments. (**D**) Representative plots of IFN-γ and Foxp3 expression in the skin of SPF, and GF mice mono-associated with Segmented Filamentous Bacteria (SFB). Plots gated on live CD45$^+$ TCRβ^+ cells. Results are representative of 2 experiments. (***p<0.0005).

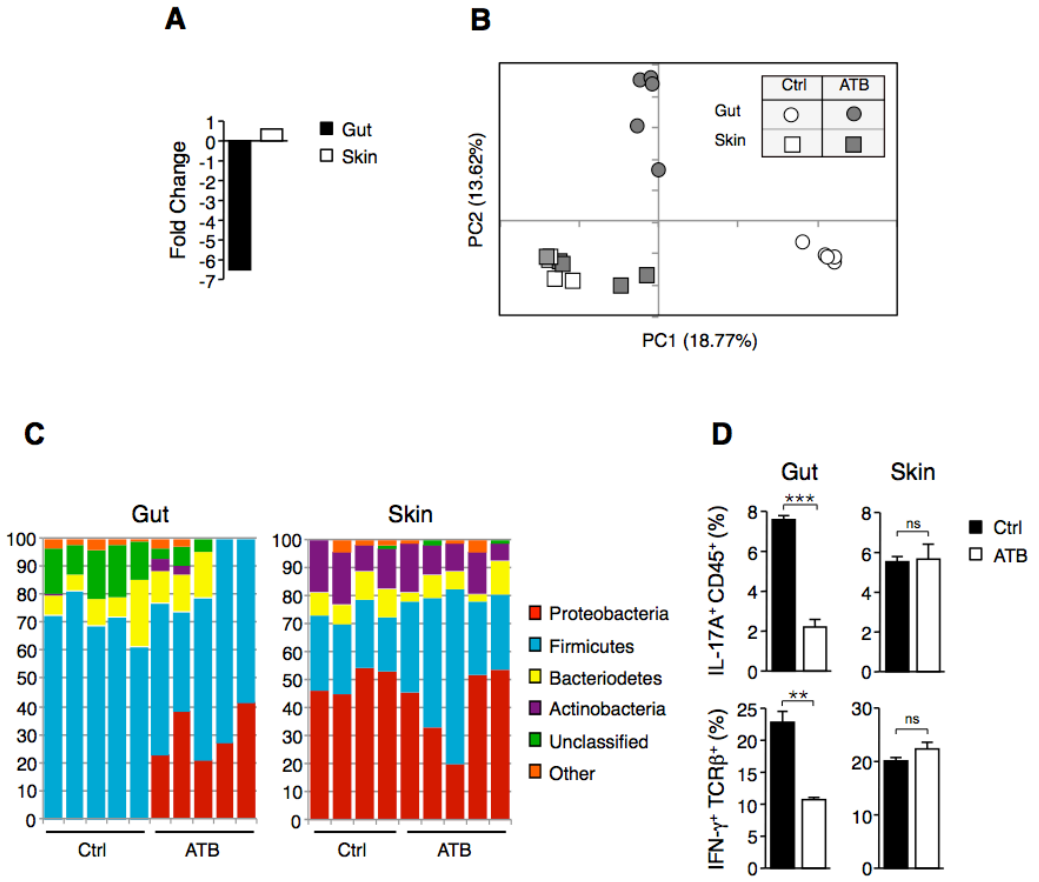

Figure 15: Inflammatory T cell cytokine production in the skin is not influenced by quality and quantity of intestinal commensals.

(**A**) Quantification of 16S rDNA copies in the skin tissue and fecal pellet of mice treated with oral antibiotic (ATB) or water control (Ctrl). Results are representative of 2 experiments. (**B**) Taxonomic affiliations at the phyla levels of 16s rDNA gene sequence data clustered at 97% identity from skin tissue and fecal pellet of control and antibiotic treated mice. Principal coordinate analysis (PCoA) of unweighted pairwise UniFrac distances from a phylogenetic tree of unique reads. (**C**) Taxonomic classifications at the phylum level for 16S rDNA gene sequence data clustered at 97% identity from skin tissue and fecal pellet of control and antibiotic-treated mice treated for 4 weeks. Each column represents an individual mouse. (**D**) Assessment of IFN-γ production in live $CD45^+$ $TCR\beta^+$ cells and IL-17A production in live $CD45^+$ cells from skin and intestine of mice treated with oral antibiotic cocktail (ATB ( )) or water (Ctrl (■)) for 4 weeks. Graphs represent a mean of four mice ± SEM. Results are representative of 2-3 experiments. (**p<0. 005, ***p<0. 0005).

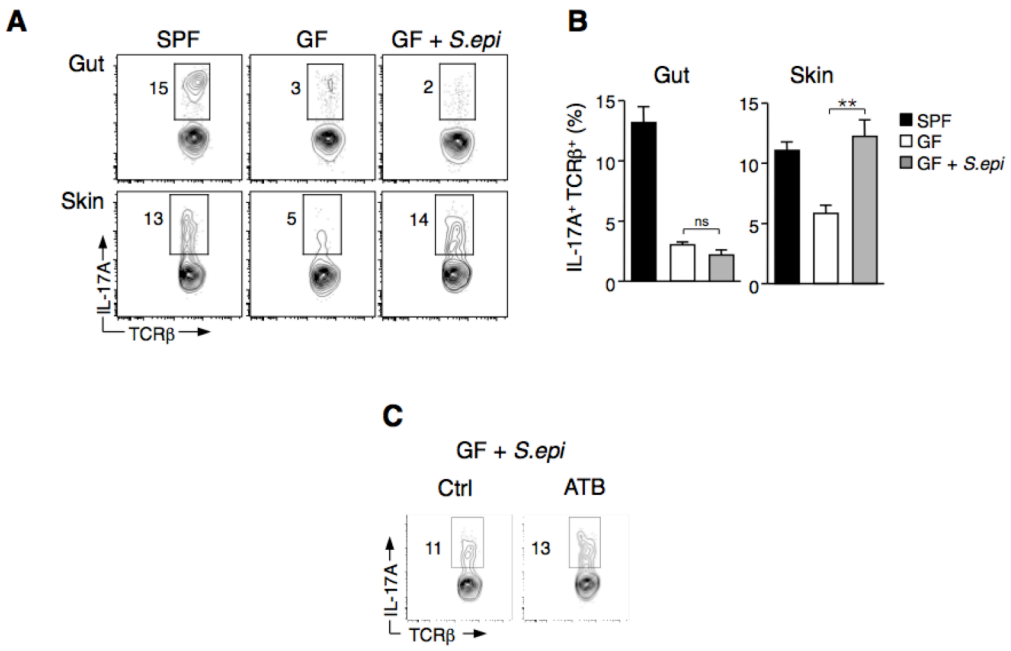

Figure 16: Resident commensal *Staphylococcus epidermidis* controls the levels IL-17A in skin tissue.

(**A-B**) Flow cytometric analysis of IL-17A production in live CD45$^+$ TCRβ^+ cells from the gut and skin of SPF, GF, and GF mice mono-associated with *S. epidermidis* (GF+*S.epi*) for 2-3 weeks. Graphs represent the mean of 3-5 mice ± SEM. Results are representative of 2 experiments. (**C**) Representative plots of IL-17A production in live CD45$^+$ TCRβ^+ cells from skin of GF mice associated with *S. epidermidis* and treated with oral vancomycin (ATB) or unsupplemented water (Ctrl). Results are representative of 1 experiment with 4 mice per group. (**p<0.005).

Cutaneous commensals control immunity to dermal pathogen Leishmania major.

The gut microbiota acts as a natural adjuvant and drives immunity and pathology during intestinal infections. Studies from our lab and others have shown that germ-free mice, mice treated with oral antibiotics, or mice deficient in commensal sensing toll-like receptors (TLRs) or nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are unable to control pathogen replication due to improper formation of protective immune responses (Ivanov and Honda, 2012). Thus the development of a proper immune response in the intestinal barrier relies heavily on the availability of stimulatory molecules from the resident microbiota.

The skin is routinely infected by a variety of bacterial, fungal, and protozoan pathogens. We therefore sought to evaluate if the resident microbiota were necessary for immunity to pathogens in the skin. To further probe the functional role of skin commensals in the control of protective and pathological responses to pathogens, we took advantage of a well-defined model of a non-disseminating dermal infection induced by the protozoan parasite *Leishmania major* (Belkaid et al., 2000). Immunity and pathology in this infectious model are critically dependent upon T-cell-derived IFN-γ. Intradermal inoculation of germ-free mice resulted in significantly smaller lesions with markedly reduced edema, necrosis, and immune infiltrate when compared to SPF mice (**Figure 17A and B**). We next evaluated the *Leishmania* specific inflammatory response in T cells

from the dermal lesions of SPF and GF animals. Germ-free mice had significantly fewer *Leishmania* specific IFN-γ^+ and TNFα^+ T cells in their skin when compared to commensally replete animals (**Figure 17C and D**). Consistent with an impaired immune response, germ-free mice harbored a greater number of parasites in their dermal lesions (**Figure 17E**). Diminished T cell immunity to *L. major* in germ-free mice could be a result of defective T cell polarization. However, T cells from GF and SPF mice expressed similar levels of canonical Th1 transcription factor T-bet and produced similar amounts of IFN-γ in response to non-specific phorbol myristate acetate and ionomycin stimulation (**data not shown**)**.** Although T cells from *L. major* lesions of germ-free mice did not exhibit defects in their potential to produce IFN-γ, they were unable to make IFN-γ in response to *Leishmania* antigen.

Persistent infection and impaired immune responses during *L. major* can be attributed to increased regulatory features, such as the production of anti-inflammatory cytokine IL-10 or the accumulation of regulatory T cells (Belkaid et al., 2002). However, we did not observe an increased in regulatory T cells frequencies or IL-10 production in the lesions of germ-free mice (**Figure 18A and B**). Often, strains of mice susceptible to *Leishmania* infection, such as BALB/c mice, fail to develop a proper Th1 response and instead harbor high levels Th2 cytokines in their skin (Jones et al., 1998). Per contra, susceptibility in the absence of commensals was not associated with increased Th2 polarization, as GF and SPF mice produced comparable amount of IL-4 (**data not shown**)**.**

These findings rule out excessive regulation or improper Th2 development as potential causes of increased susceptibility to *L. major* in germ-free mice.

We thus postulated that skin commensal signals promoted inflammatory responses locally in the dermis. Indeed, topical association of germ-free mice with *Staphylococcus epidermidis* at the time of infection was sufficient to restore both immunity and pathology to *L. major.* Monoassociated mice manifested larger and more necrotic lesions that resembled SPF controls (**Figure 19A and B**). Consistently, the addition of *S. epidermidis* to the skin of germ-free mice restored *Leishmania* specific INF-γ production and parasite control (**Figure 19B through D**). To test the contribution of the gut flora to the development of protective immunity, we infected orally antibiotic-treated mice with *L. major.* This treatment did not impact the development of dermal lesions or pathogen specific INF-γ responses (**data not shown**). We further confirmed a role for *S. epidermidis* in augmenting immunity locally by treating mono-associated animals with oral antibiotics during *L. major* infection (**Figure 19E and F**). This study underscored the role of distinct commensal niches in discretely controlling immune cells in adjacent tissue sites at steady state and during inflammation.

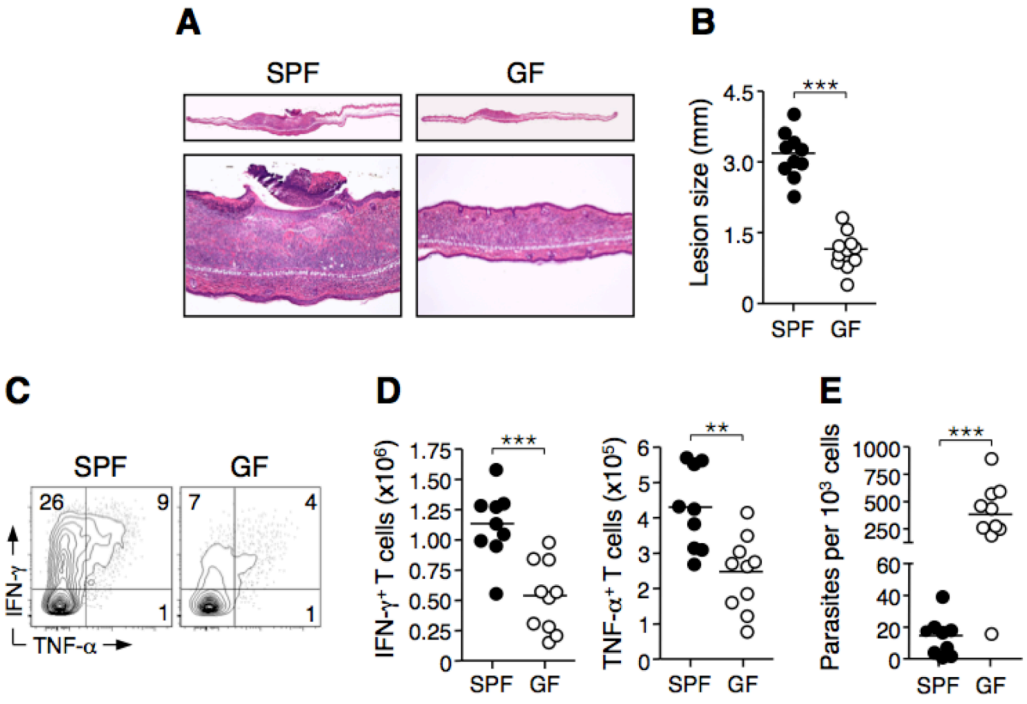

Figure 17: Immunity and pathology to *Leishmania major* is impaired in germ-free mice.

(**A-B**) Histopathological comparison of ear pinnae skin lesions from *L. major* infected SPF and GF mice. Assessment of lesion size in SPF (●) and GF (○) mice. Each data point represents an individual mouse. (**C-D**) Flow cytometric analysis of *Leishmania* antigen-specific IFN-γ and TNF-α production by TCRβ$^+$ CD4$^+$ dermal cells from *L. major* infected SPF (●) and GF (○) mice. Each data point represents an individual mouse. Results are representative of 3 experiments. (**E**) Number of *L. major* parasites per 1000 nucleated cells from dermal lesions of infected SPF (●) and GF (○) mice. Each data point represents an individual mouse. (**p< 0.005, ***p< 0.0005).

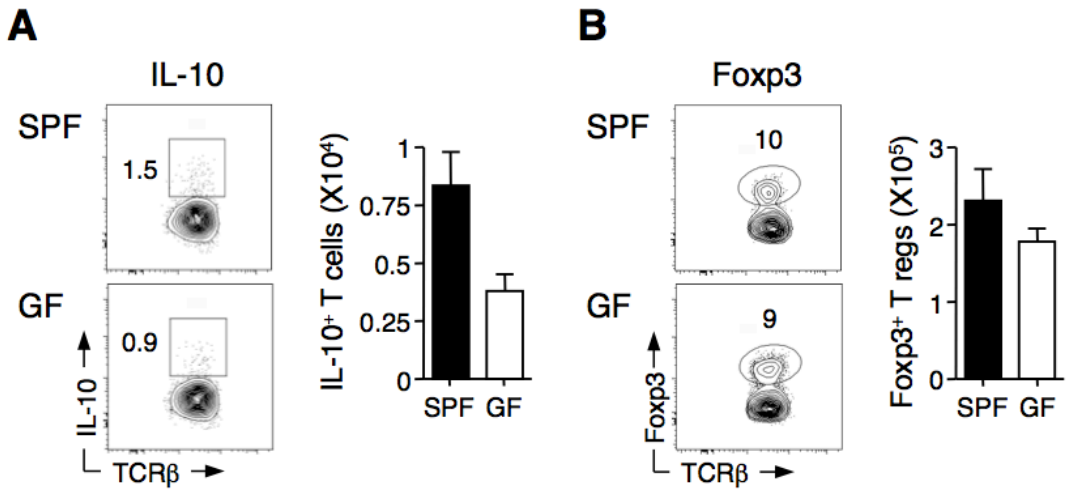

Figure 18: Regulatory signatures are not increased in the skin of germ-free mice during infectious challenge.

(**A**) Analysis of *Leishmania* specific IL-10 production by live $CD45^+$ $TCR\beta^+$ $CD4^+$ cells from SPF (●) and GF (○) mice. Each bar is a mean of 10 mice ± SEM. Results are representative of 3 experiments. (**B**) Frequency of regulatory T cells (live $CD45^+$ $TCR\beta^+$ $CD4^+$ $Foxp3^+$) in *L. major* infected skin lesions of SPF (●) and GF (○) mice. Each bar is a mean of 4 mice ± SEM. Results are representative of 3 experiments.

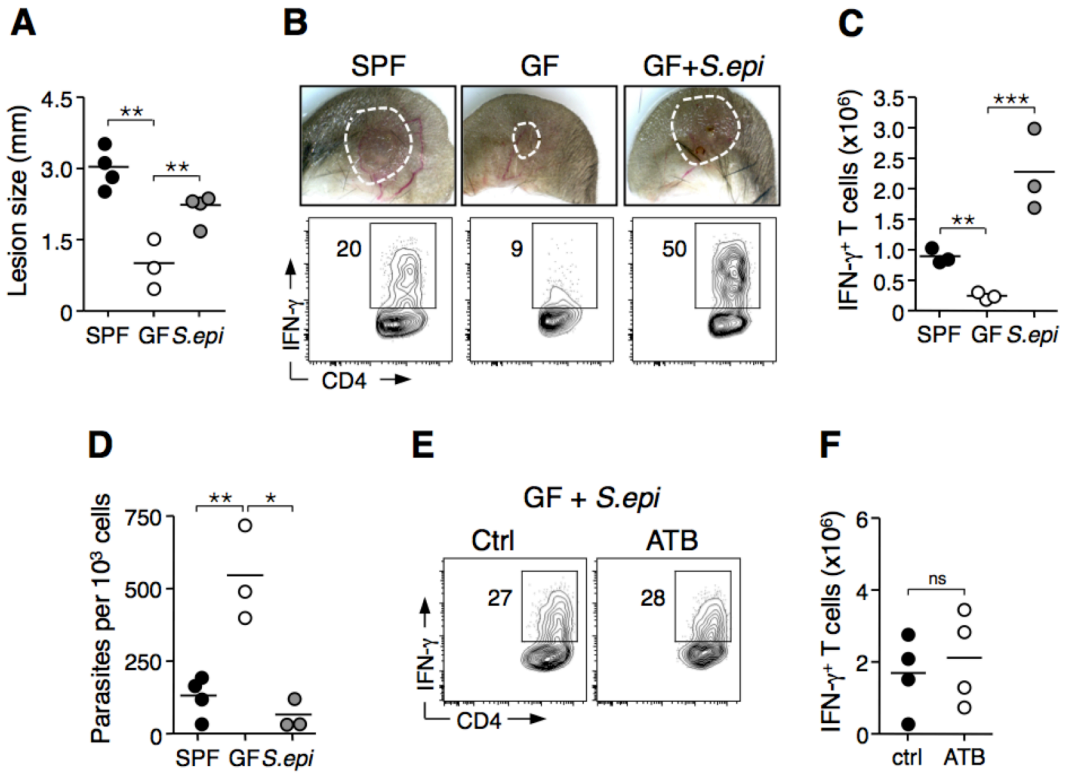

Figure 19: Cutaneous commensals drive immunity and promote pathology during *Leishmania major* infection.

(**A**) Assessment of lesion size in SPF (●), GF (○), and GF mice mono-associated with *Staphylococcus epidermidis* (GF +*S.epi* (●)). Each data point represents an individual mouse, (**p< 0.005). Results are representative of 2 experiments. (**B-C**) Representative image of *L. major* skin lesions and analysis of IFN-γ production by live $CD45^+$ $TCR\beta^+$ cells from SPF (●), GF (○) and GF + *S. epi* (●) mice. Each data point represents an individual mouse. Results are representative of 2 experiments (**D**) Number of *L. major* parasites per 1000 nucleated cells from dermal lesions of infected SPF (●), GF (○), and GF mice mono-associated with *Staphylococcus epidermidis* (GF +*S.epi* (●)). Results are representative of 2 experiments. Each data point represents an individual mouse (**E-F**) Assessment of *Leishmania* specific IFN-γ production by $TCR\beta^+$ $CD4^+$ in GF + *S. epi* mice treated with either oral vancomycin (ATB (○)) or water (Ctrl (●)). Each data point represents an individual mouse. Results are representative of 1 experiment. (*p< 0.05, **p< 0.005, ***p< 0.0005).

Chapter 4: Skin commensals control immune cells via a distinct IL-1/CD40-dependent mechanism

Abstract

The skin is densely populated with commensal organisms and the findings described in **Chapter 3** illustrate the importance of these resident organisms in the direct control of immunity. How cutaneous commensals dialog with the host immune system and the importance of these signals in ensuring the proper generation and regulation of immune responses is unclear. To date, the mechanisms underlying host commensal cross talk have only been examined in the intestinal mucosa. Furthermore, whether the modes of immune–commensal interactions along the body's epithelial barriers are ubiquitous or if there are unique mechanisms responsible for this dialogue in distinct tissue sites requires elucidation. This study aimed to identify the commensally driven factors that control T lymphocytes in skin tissue and revealed the following:

1. **T lymphocytes rely on local IL-1/CD40 signals within the skin for appropriate cytokine production.**
2. **Effector T cells in the skin and gut are controlled by distinct mechanisms.**
3. **Cutaneous commensals amplify the IL-1 pathway to tune the function of T cells locally.**
4. **Protective immunity to *Leishmania major* is critically dependent**

upon commensally driven IL-1.

Rationale

The host immune system senses pathogenic and commensal microbes through a serious of conserved receptors known as Pattern Recognition Receptors (PRR). In the intestine, several immune and metabolic receptors have been identified as essential mediators of the host commensal dialogue (Rakoff-Nahoum and Medzhitov, 2008). For instance, sensing of gut flora DNA via TLR9 is necessary for limiting T_{regs} and promoting effector T cell responses in the intestinal mucosa at steady state and during pathogen challenge (Hall et al., 2008).

Mechanisms underlying immune commensal cross talk outside the intestine, however, are largely unknown. Additionally, because of its primary role in nutrient absorption, the intestinal barrier is considerably more permissive than other epithelial sites (Chemical Society (Great Britain)). On the other hand, the skin has several layers of squamous epithelium that make up a more restrictive barrier. Moreover, several groups have uncovered the importance of intestinal IL-6 in controlling Th17 function in the gut (Hall et al., 2008; Ivanov et al., 2006). Yet the cytokine factors necessary for controlling T cell function in the skin have not been defined. Due to the restrictive nature of the keratinocyte barrier, it is likely that T cells in the skin may respond to secondary mediators, such as innate cytokines, rather than to pathways involving direct commensal sensing. Understanding how T lymphocytes in the skin are regulated and the means

by which cutaneous commensals modulate these pathways will provide novel insights into the tissue-specific regulation of immune cells. In **Chapter 3,** we described a distinct role for the cutaneous microbiota in locally promoting T lymphocyte function. Using a “bottom-up” approach, we first identified the innate mediators that direct T effector function in the skin and then assessed the role of commensals in amplifying these pathways. This study led to a better understanding of the tissue-specific pathways that control immune cells in distinct compartments and uncovered how unique commensal niches co-opt these pathways to locally promote immunity.

Results

IL-1 and CD40 signaling are necessary for IL-17A and IFN-γ production in the skin, but not the gut.

T effector lymphocytes in the skin are equipped with the potential to produce substantial amounts of inflammatory cytokines IL-17A and IFN-γ. However, the dominant factors that promote cytokine production in this tissue have not been defined. Studies using recombinant proteins and *in vitro* culture systems or experimental inflammatory models have identified several factors that can promote cytokine production from both γδ and αβ T cells (Zhu and Paul, 2010). Using a candidate approach, we examined cytokine production in the skin of mice that were deficient in each of these previously defined innate factors. Although optimal induction of Th17 may rely on synergistic signals, we found that IL-1 plays as dominant role in the skin tissue as IL-1R1, but not IL-6, or IL-23R deficient mice displayed a significant reduction in IL-17A production from both αβ and γδ T cells in skin tissue compared to control mice (**Figure 20A**). In addition, mice deficient in signaling molecules downstream of IL-1R1, MyD88, and MyD88/TRIF double-deficient mice (Muzio et al., 1997), phenocopied the defect in IL-17A production observed both in IL-1R1 deficient and germ-free mice (**Figure 20A**).

In addition to IL-1R1, MyD88 adaptor protein also promotes IL-18 and TLR

signaling. Because mice deficient in various TLRs and IL-18 did not have diminished IL-17A in the skin, we were able to exclude their role in controlling the production of this cytokine. (**Figure 20A and B**). Importantly, ligation of TLR2 with byproducts isolated from skin commensal *S. epidermidis* signaling results in the induction of anti-inflammatory responses in the context of tissue injury (Lai et al., 2009). However, this signaling pathway did not contribute to steady-state T cell regulation in the skin, and TLR2 knockout mice had levels of IL-17A similar to their WT counterparts (**Figure 20B**).

We next investigated the innate mediators that control IFN-γ production in the dermis. Strikingly, the capacity of $CD4^+$T cells to produce IFN-γ was lost in CD40-deficient animals, but not in MyD88, TRIF, TNFα, IL-6, IL-18, and IL-12p40-deficient animals. This revealed a non-redundant function for CD40 in the control of IFN-γ production by dermal $CD4^+$T cells (**Figure 21C**). Of note, mice deficient in TLR2, TLR3, TLR5, and TLR9 did not have impaired IFN-γ in the skin (**data not shown**). In contrast to *in vitro* reports (Perona-Wright et al., 2009), CD40 signaling was dispensable for IL-17A production by both $\alpha\beta$ and $\gamma\delta$T cells (**data not shown**).

In **Chapter 3,** we highlighted the role of flora in the local regulation of T cells in the skin and the intestine. Therefore, it was highly likely that innate signals that govern T cell function would also be segregated in distinct tissue sites. Indeed, IL-6, a critical regulator of IL-17A in the intestine, is nonessential for IL-17A production in the skin (**Figure 21A**). Conversely, levels of IL-17A in

MyD88/TRIF- or IL-1R1-deficient mice were unaffected in the intestinal lamina propria (**Figure 21A and B**). Additionally, CD40-deficient mice had no defect in IFN-γ production in the gut compartment (**Figure 21C**). These findings illustrated a compartmentalization of the signals that control T cells in distinct tissue sites and provided instructive clues to the possible mechanisms underlying the immune–commensal dialog in the skin.

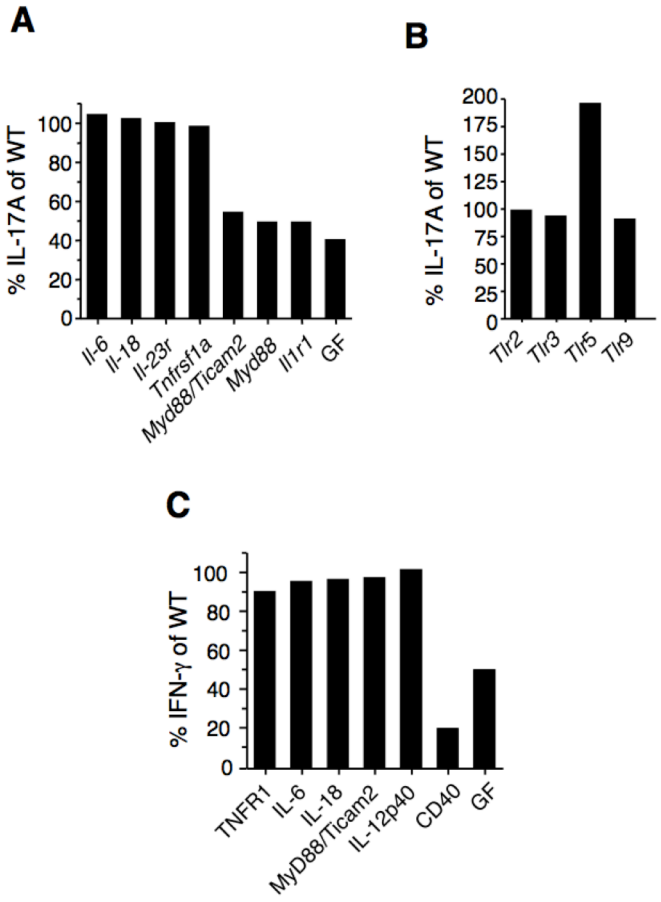

Figure 20: IL-R1 and CD40 signaling, respectively, are necessary for IL-17A and IFN-γ production in the skin.

(**A**) Expression of IL-17A by live CD45$^+$ skin cells from various knockout mice relative to co-housed wild-type control mice. Each bar is representative of 2-3 experiments with 2-4 mice per group. (**B**) Expression of IL-17A by live CD45$^+$ skin cells from *Tlr2*, *Tlr3*, *Tlr5,* and *Tlr9* $^{-/-}$ mice relative to co-housed wild-type control mice. Each bar is representative of 2-3 experiments with 2-4 mice per group. (**C**) Expression of IFN-γ by live CD45$^+$ TCRβ$^+$ skin cells from various knockout mice relative to co-housed wild-type controls. Each bar is representative of 2-5 experiments.

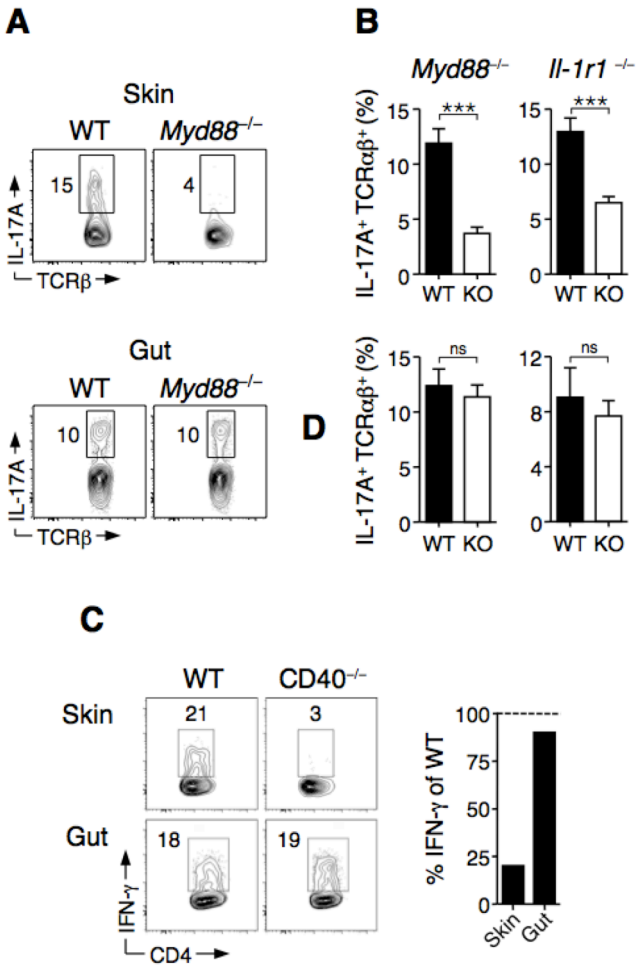

Figure 21: Under steady state conditions, IL-1R1 and CD40 control cutaneous, but not intestinal T cells

(**A**) Flow cytometric analysis of IL-17A production by live CD45$^+$ TCRβ$^+$ cells in skin and intestine of co-housed age matched *Myd88*$^{-/-}$ and *Il1r1*$^{-/-}$ mice. Graphs represent a mean of 3-4 mice ± SEM. Results are representative of 2-3 experiments. (***p< 0.0005). (**B**) Flow cytometric analysis of IFN-γ production by live CD45$^+$ TCRβ$^+$ cells in skin and intestine of co-housed age matched *CD40*$^{-/-}$ mice. Graphs represent a mean of 3-4 mice ± SEM. Results are representative of 2-3 experiments.

Commensally driven IL-1 signaling tunes the function of resident T cells in the skin.

A variety of cell types, critical for promoting skin immunity, are known to respond to microbial signals. These include radio-resistant epidermal keratinocytes and Langerhans cells as well as radio-sensitive hematopoietic cells such as T lymphocytes and DCs. To ascertain the importance of MyD88/TRIF signaling within these cellular compartments, we generated mixed bone marrow chimeras. Congenically marked 70% Myd88/TRIF-/-: 30% wild type chimeras were generated using Rag1-deficient animals as hosts (**Figure 22A**). Rag-deficient hosts were used in place of wild-types animals, as we have previously observed low chimerism in T cell compartment (<30%) of the skin (**data not shown**). Of note, this experimental setting leads to irradiation-induced inflammation and homeostatic proliferation of T cells. Under these conditions, MyD88 signaling was required for the production of both IFN-γ and IL-17A from $\alpha\beta$ T cells in the skin. (**Figure 22B and C**). Consistent with a partitioning of the dominant signals controlling the skin and gut environments, MyD88 deficiency in the radiosensitive compartment had no impact on T cell potential to produce IL-17A or IFN-γ in the intestine (**Figure 22B and C**). Thus, MyD88 signaling in the radiosensitive compartment is crucial for T cell activation in the skin.

On the basis of the pleiotropic role of IL-1, this effect may result from direct IL-1 signaling in T cells and/or modulation of various innate inflammatory cells

such as neutrophils. Importantly, both αβ and γδ effector T cells are known to express IL-1R1, and produce IL-17A in response to IL-1 stimulation *in vitro (Gray et al., 2011; Guo et al., 2009; Shaw et al., 2012)*. We therefore sought to investigate the importance of IL-1 signaling directly on T cells isolated from the skin. To this end, purified dermal αβ and γδ lymphocytes were stimulated via their T cell receptors using anti-CD3 in the presence of recombinant IL-1α, IL-1β, or IL-6. Under these conditions, IL-1α, IL-1β, but not IL-6 potently increased the capacity of αβ and γδ T cells to release IL-17A (**Figure 23A and B**). Thus, T cells that reside at dermal sites can be functionally tuned by the local cytokine milieu and in particular by IL-1.

We next explored the possibility that IL-1 signaling may be diminished in the absence of commensals. We thus surveyed the spontaneous release of various innate cytokines from the skin of GF and SPF animals (**data not shown**). This screen revealed a specific impairment in cutaneous IL-1α production in the absence of commensals (**Figure 24A**). Additionally, mono-association of GF mice with *S. epidermidis* restored the production of this cytokine by cutaneous cells (**Figure 24A**). This screen also indicated that the skin of germ-free mice had greater levels of IL-1 receptor antagonist (IL-1ra), which were negatively regulated by the addition of *S. epidermidis* (**Figure 23B**). Although the dominant cellular source of IL-1 in the skin is not known, keratinocytes have been identified as the primary producers of IL-1ra under steady state conditions (Ishida et al.,

2006). Indeed, interfollicular keratinocytes isolated from the skin of GF mice displayed increased levels of the IL-1 receptor antagonist (IL-1ra) mRNA compared to SPF mice. These findings confirm that various aspects of IL-1 signaling are controlled by resident commensals and that optimal IL-1 signaling is essential for setting the T cell inflammatory set point in the skin.

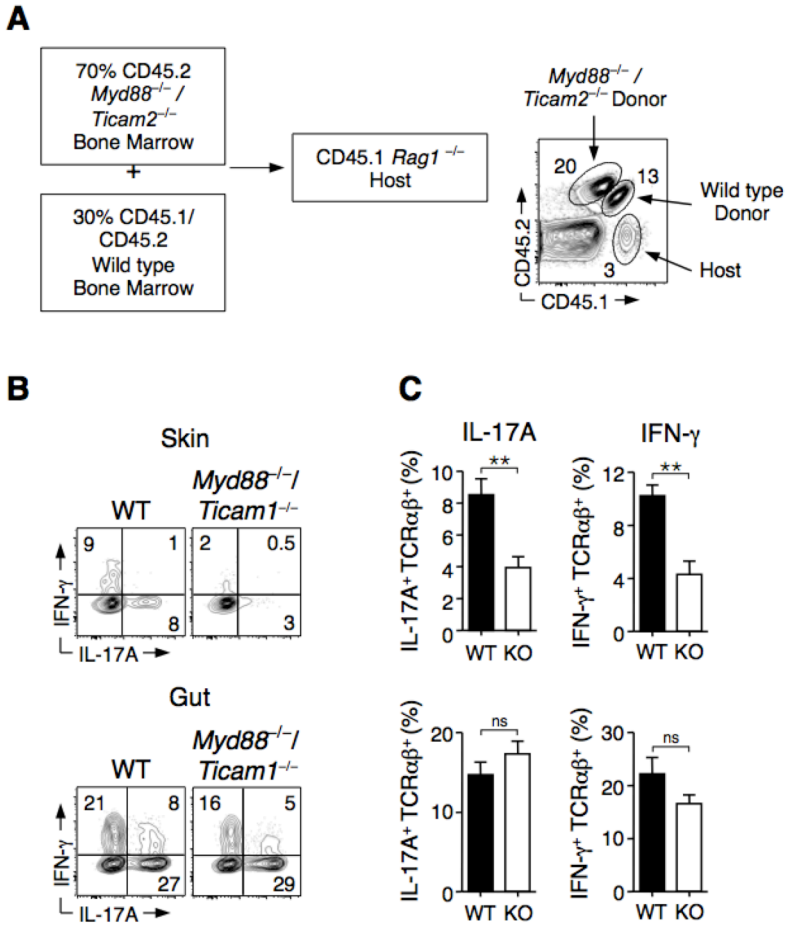

Figure 22: MyD88 signaling in radio-resistant cells is necessary for inflammatory cytokine production in the skin.

(**A**) Schematic for generation of bone marrow chimeras to evaluate immune cells in the skin. Gating strategy for identifying wild type and *Myd88*$^{-/-}$*/Ticam1*$^{-/-}$ (*Ticam1* encodes TRIF) cells in skin of bone marrow chimeric mice. (**B**) Comparative assessment of IFN-γ and IL-17A production from WT and *Myd88*$^{-/-}$*/Ticam1*$^{-/-}$ TCRβ^{+} cells from mixed bone marrow chimeric mice. Bar graphs show the frequency of cytokine production by knockout and wild type TCRβ^{+} cells ± SEM. Results are representative of 2 experiments in the skin and 1 experiment in the gut. (**p< 0.005).

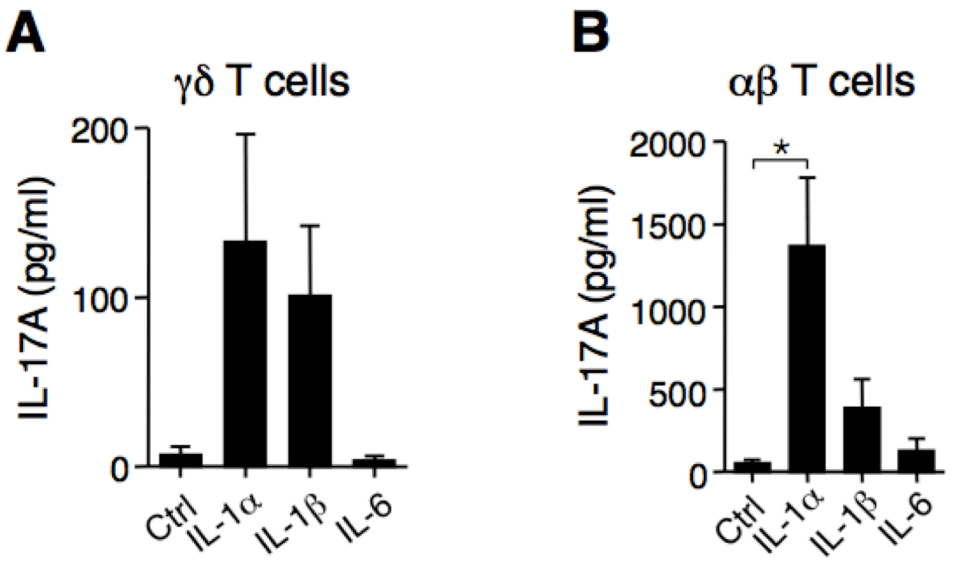

Figure 23: Direct IL-1 signaling in skin αβ and γδ T cells is sufficient to promote IL-17A production *in vitro*.

(**A**) Assessment of IL-17A production from purified dermal TCRgd^{low} cultured *in vitro* in the presence of anti-CD3 and either IL-1α, IL-1β or IL-6. Error bar is a mean of 3 experimental groups ± SEM. Results are representative of 3 experiments. (**B**) IL-17A production from purified skin CD45^{+} TCRβ^{+} cells cultured *in vitro* in the presence of anti-CD3 and either IL-1α, IL-1β or IL-6. Graphs represent the mean of 3 experimental groups ± SEM (*p< 0.05). Results are representative of 3 experiments.

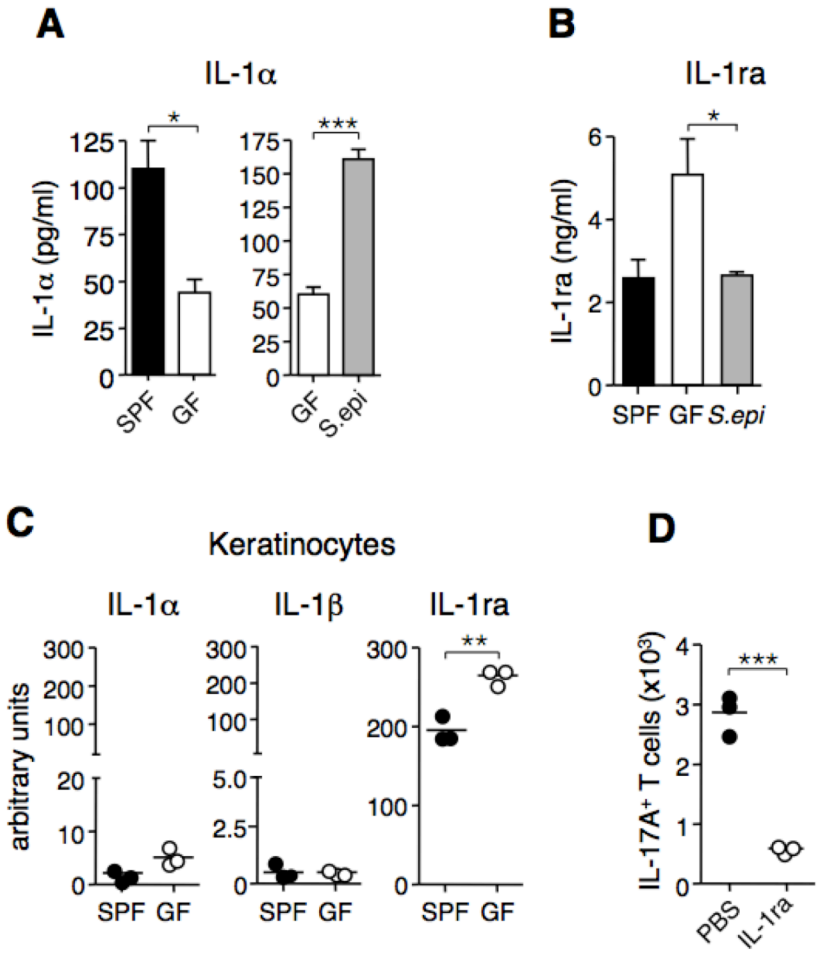

Figure 24: Skin commensals control steady state levels of IL-17A by locally promoting IL-1 signaling.

(**A**) Spontaneous release of IL-1α from skin-derived cells of SPF (■), GF (□), and GF + *S.epi* (■) ± SEM mice as measured by ELISA. (**B**) Spontaneous release of IL-1ra from skin-derived cells of naïve SPF (■), GF (□), and GF + *S.epi* (■) mice as measured by ELISA (**C**) Gene expression analysis of IL-1α, IL-1β and IL-1ra in interfollicular $a6^+$ keratinocytes from SPF (●) and GF (○) using NanoString nCounter analysis. (**D**) Number of $CD45^+$ $TCR\beta^+$ $IL\text{-}17A^+$ T cells from the skin of GF mice mono-associated with *S. epidermidis* treated with either IL-1ra (○) or PBS control (●) Results are representative of 1 experiment. Each data point represents an individual mouse. (*$p< 0.05$, **$p< 0.005$, ***$p< 0.0005$).

S. epidermidis modulates T cell function at steady state and during L. major infection in an IL-1 dependent manner.

The data presented above support a role for skin commensals in enhancing IL-1 signaling locally, which in turn augments inflammatory cytokine production from T cells. To functionally link commensally driven IL-1 signaling to T cell immunity in the skin, we blocked the downstream effects of *S. epidermidis* dependent IL-1 production by treating mono-associated animals with recombinant human IL-1ra (Anakinra). Of note, the neutralizing activity of IL-1ra administered intraperitoneally was confirmed in the skin by measuring the blockade of genes induced by intradermally injecting IL-1α (**data not shown**). Using this approach, we evaluated the importance of commensal-driven IL-1 in IL-17A production in the skin. Neutralizing IL-1 activity in mono-associated animals hindered this bacterium's ability to rescue IL-17A at steady state (**Figure 24D**).

Previous reports have attributed diminished immunity in MyD88-deficient animals to impaired sensing of *Leishmania* parasite (Muraille et al., 2003). However, to date, parasite-derived TLR ligands relevant for immunity *in vivo* have not been identified. To this end, we found that compromised immunity in MyD88-deficient animals was phenocopied by IL1R1-deficient animals, as these mice exhibited impaired ability to make *Leishmania* specific IFN-γ (**Figure 25A through C**). Complementing this, SPF mice treated with IL-1ra during the first

week of infection were unable to mount proper IFN-γ responses to *L. major* (**Figure 26A**). These data support a role for commensally driven IL-1 in promoting immunity to *Leishmania*. Indeed, treatment with IL-1ra impaired the ability of *S. epidermidis* to augment IFN-γ responses in germ-free mice infected with *L. major* (**Figure 26B**). Therefore, the adjuvant effect of skin commensals relies on their ability to locally boost IL-1 signaling.

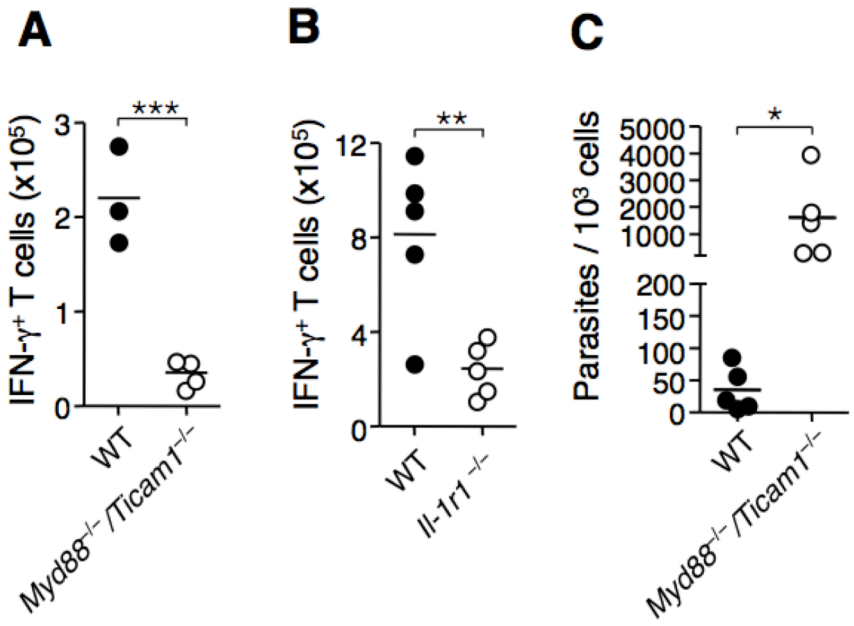

Figure 25: IL-1R1- and MyD88-deficient mice are highly susceptible to *Leishmania major* infection.

(**A-B**) Analysis of *L. major*-infected wild-type (●) and *Myd88*$^{-/-}$*/Ticam1*$^{-/-}$ or *Il1r1*$^{-/-}$ (○) mice. Results are representative of 2 experiments. (**C**) Number of *L. major* parasites per 1000 nucleated cells from dermal lesions of infected WT (●) and *Myd88*$^{-/-}$*/Ticam1*$^{-/-}$ (○) mice. Each data point represents an individual mouse (*p< 0.05).

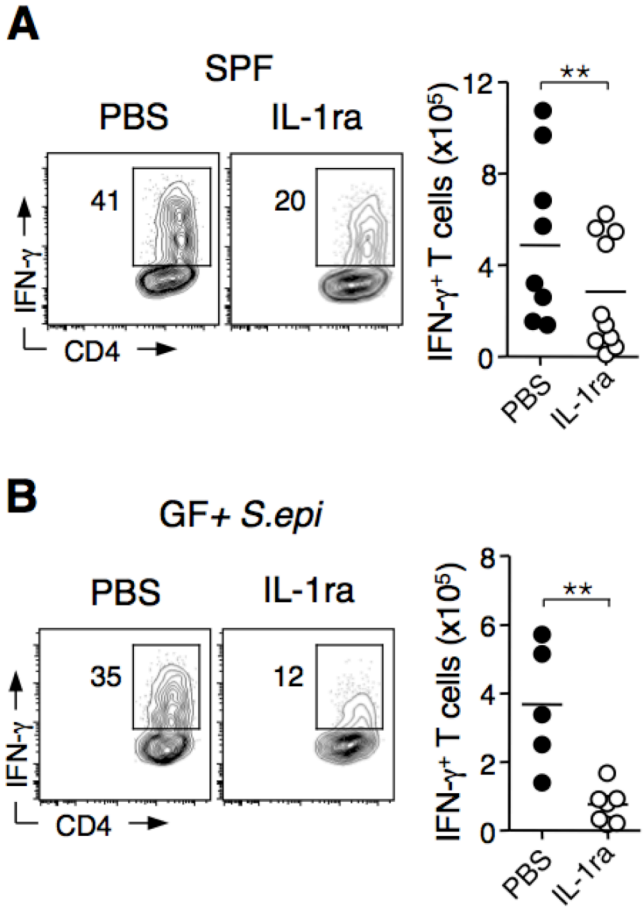

Figure 26: Commensally driven IL-1 augments immunity to *Leishmania major*.

(**A**) Flow cytometric assessment of *L. major*-specific IFN-γ production from TCRβ$^+$ CD4$^+$ T cells from the skin of SPF animals treated with either IL-1ra (○) or PBS (●) Results are a compilation of 2 experiments. (**B**) *L. major*-specific IFN-γ produced by TCRβ$^+$ CD4$^+$ T cells from the skin of GF mice mono-associated with *S. epidermidis* and treated with either IL-1ra (○) or PBS (●) Results are representative of 2 experiments. Each data point represents an individual mouse. (**p< 0.005)

Chapter 5: Discussion

Normal skin is immunologically active and heterogeneous.

The skin barrier forms a critical interface with the terrestrial environment and is therefore tasked with protecting our internal organs from harmful agents. To do so, layers of stratified squamous epithelia supported by a network of immune cells form a physical and chemically restricted barrier (Fuchs and Nowak, 2008; Kupper and Fuhlbrigge, 2004). Although it is widely accepted that skin is a primary immune effector site and has been extensively used as a model organ to study the induction of immune responses, little is known about the composition and regulation of immune cells in normal skin. A study by Clark and colleagues enumerated T cells from normal human skin biopsies and estimated that the total surface of the skin may have as many as 2×10^{10} resident T cells (Clark et al., 2006; Schaerli et al., 2006). However, the phenotype and functional capacity of T cells and their relative contribution to barrier protection in normal skin is largely unknown.

In **Chapter 2**, using novel cell extraction techniques, we find that the normal skin of mice and non-human primates (NHPs) contains a large number of T cells with the potential to produce the inflammatory cytokines IFNγ and IL-17A. The importance of these inflammatory T cell populations is evidenced by increased susceptibility to dermal pathogens in patients with deficiencies in IFNγ and IL-17A (Puel et al., 2011). In fact, previous studies have shown that resident T cells preferentially associate with appendages in skin tissue (Clark et al., 2006;

Schaerli et al., 2006). Consistent with this, we find that skin sites with a high numerical density of appendages also house significantly greater numbers of inflammatory T cells. Using an antibody that detects major bacterial and cell wall components, we confirmed that in line with previous reports, skin appendages are enriched in microbial byproducts. Thus, it is tempting to speculate that microbial-driven signals emanating from skin appendages drive inflammatory T cells specifically to these locations within the tissue. Indeed, Nagao and colleagues show that following inflammatory stress, hair follicle keratinocytes secrete chemokines to direct the migration of Langerhans cell progenitors to the epidermis (Nagao et al., 2012). How this process is influenced by commensals and if similar mechanisms direct T lymphocytes within the skin remains to be understood. Evaluating the composition and function of T cells in the skin of mice that lack hair follicles and other appendages is likely to shed light on the relative importance of these commensal niches in the maintenance of effector T cells in the skin.

T cells that localize to the skin appendages may also serve a vital role in regulating commensals within these specific anatomical sites. In support of this, 90% of patients with acquired immune deficiency syndrome (AIDS) that have specific defects in IL-17A production manifest inflammatory skin disorders that are associated with appendages, including seborrheic dermatitis. (Sampaio et al., 2011; Smith et al., 1994). Whether the depletion of IL-17A producing T cells in the skin of these patients results in the outgrowth of disease causing pathobionts

is unknown.

In addition to mapping the heterogeneous immune landscape of the skin, we were able to correlate the presence of effector T cells with certain microbial signatures. For instance, *Proteobacteria* are predominantly found on the back and belly skin of mice, and these sites also contain greater frequencies of IFN-γ– and IL-17A-producing TCRβ$^+$ cells. In contrast, ear skin dominantly housed *Firmicutes* and has fewer resident effector T lymphocytes with the capacity to produce inflammatory cytokines. It will be important to determine how the quality and quantity of these signatures impact the localization and function of immune cells in distinct niches within the skin. Additionally, it is unclear whether the specific microbes that reside in appendages are uniquely capable of modulating immunity locally, or if the increased concentration of microbial signals in these anatomical sites discretely enhances immune function. Moreover, further investigation is needed on the specific host receptors that facilitate commensal communication with the immune and stromal cells lining skin appendages and the downstream cellular responses to such interactions.

Previous studies have illustrated a site-specific difference in skin responsiveness to tissue stress. Mice infected with *Leishmania major* have altered immune and pathological responses based on the site of inoculation (Nabors and Farrell, 1994). Similarly, various immune-mediated dermal disorders, such as atopic dermatitis (AD) and psoriasis, have a characteristic pattern of expression (Guttman-Yassky et al., 2011a). For instance, AD often

manifests on the face and flexor skin, while psoriasis generally presents in the scalp and extensor skin. Based on the findings presented in **Chapter 2**, it is highly probable that heterogeneous immune responsiveness in this tissue may be due to steady-state variations in the seeding of the hematopoietic cells in distinct regions of the skin. Furthermore, this tissue serves as a reservoir for memory cells, which constantly traffic between the skin and draining lymph nodes (Egawa and Kabashima, 2011). Assessing if memory T cells preferentially migrate to certain skin sites, and if these sites are more prone to immune-mediated pathology in response to tissue injury or augmented protective responses to heterologous infections, will provide novel insights into immune function and regulation in the skin.

A comparative analysis of commensal communities from distinct murine skin sites revealed that similar to human skin microbiota, the specific ecology of a mouse skin site dictates its commensal colonization (Costello et al., 2009). Similarly, we found that murine skin and gut tissues also house unique communities of bacteria. Given that the skin and intestine of mice are ecologically distinct habitants, the former is exposed to light, maintains lower temperatures, moisture content, pH, and has limited nutrient availability compared to the latter, these findings accentuate the importance of environmental factors in controlling commensal colonization. Altogether, these analyses illustrate the site-specific differences in the composition of microbiota and uncover the immune heterogeneity in skin tissue. Although murine skin is structurally different from

human skin, the findings presented in **Chapter 2** validate the murine experimental model to study the complex relationship between the microbiota and immune cells in the skin. Because the skin is distant to the gut and has its own resident bacterial communities that heterogeneously colonize distinct physiological niches, it is highly likely that discrete interactions between indigenous flora and immune cells dictate the cutaneous immunological tone. Addressing how host commensal interactions in this heterogeneous tissue contribute to differential disease outcomes in distinct skin sites will be paramount to our understanding of the skin as an immunological barrier.

Compartmentalized control of skin immunity by resident commensals.

Numerous reports have stressed the importance of the gut flora in controlling immune cells locally in the intestinal lamina propria and at distal sites such as in the lung (Hooper et al., 2012). However, this gut-centric paradigm does not acknowledge the possible involvement of microbial communities residing in epithelial niches outside the intestine, such as those living in the skin (2012b). Grice and colleagues have shown that the skin houses its own unique flora and several studies have found that both immune and stromal cells from the skin are capable of sensing and responding to commensal signals (Gallo and Nakatsuji, 2011; Grice et al., 2009). The experiments described in **Chapter 3** show that the equilibrium between effector and regulatory T lymphocytes in the skin is critically dependent upon commensal signals. Further, our studies exclude a role for deficient priming or migratory capacity of T lymphocytes as comparable ratios of skin-homing effector and regulatory lymphocytes were isolated from draining lymph nodes as well as the circulation of germ-free mice and conventionally raised mice. While these experiments do not rule out the possibility that differential tissue signals may regulate the recruitment of T cells into the skin of conventionally raised and germ-free mice, our findings definitively show that circulating $CD44^{hi}$ skin-homing T cells are not influenced by the microbiota.

Germ-free mice are devoid of commensals on all their barrier surfaces.

Therefore, to delineate the relative contribution of the intestinal and cutaneous commensals to the regulation of T lymphocytes in the skin, we employed specific strategies to modulate either gut or skin flora. These studies allowed us to discern the local contribution of the skin microbiota in the discrete regulation of T cell cytokine production in the underlying tissue. Using gnotobiotic mice or oral antibiotic treatment strategies, several groups have implicated the intestinal microbiota in amplifying global inflammatory signatures in the context of systemic inflammation (Hooper et al., 2012). Specifically, the presence of SFB in the intestine results in more aggressive disease pathologies in mouse models of experimental autoimmune encephalitis, arthritis, and diabetes compared to mice devoid of this bacterium (Kriegel et al., 2011; Lee et al., 2011; Wu et al., 2010). While these findings draw associative models, they fail to define mechanistic pathways by which the commensals may modulate immune cells distally. Further, these studies involve exacerbated models of systemic inflammation, and do not examine barrier defects in the intestine or T cell trafficking patterns. Certain physiological conditions may make such interactions of the peripheral immune system with the intestinal microbiota more permissive, i.e. global inflammation. How the presence of SFB in the intestine may impact barrier function and systemic dissemination of microbial products remains an open question.

Under homeostatic conditions, the presence of SFB augmented T cell inflammatory potential in the gut, but not in the skin. Furthermore, we found that treating mice with a defined cocktail of oral antibiotics that alters the intestinal

microbiota while maintaining skin flora did not impact T lymphocyte function in the skin. The alterations in skin T lymphocytes of germ-free mice could be a result of impaired T cell development or global metabolic distress. To exclude these possibilities, we mono-associated germ-free animals with the ubiquitous skin commensal *Staphylococcus epidermidis* (Conlan et al., 2012). Importantly, *S. epidermidis* is a major constituent of normal human skin flora and has no known function in processing of dietary metabolites. Topical colonization with this skin tropic commensal was sufficient to restore T cell cytokine production in the skin, but not the gut, of germ-free mice. These experiments allowed us to conclude that distinct communities of organisms residing in the skin and gut locally tune T lymphocyte function. Whether skin commensals, like the intestinal microbiota, are able to influence immunity systemically in the context of inflammation is unclear. Challenging germ free mice that topically associated with skin commensals with diabetic, arthritogenic, and/or encephalitogenic autoimmune models of inflammation may reveal the contribution of this commensal niche in regulating immunity in distal sites.

While we utilized *S. epidermidis* as a model commensal to determine the role of cutaneous commensals in driving local immunity, it will be important to examine the contribution of other skin commensal organisms to tissue fitness. *S. epidermidis* is a facultative aerobic bacterium capable of colonizing various niches in the skin, including the outer cornified epithelium, and appendages (Conlan et al., 2012; Elsner, 2006). In contrast, certain bacterial species can only

survive in specific environments within the skin. For instance, *Propionibacterium* species are highly lipophilic bacteria that metabolize sebum and reside in sebaceous glands, and *Corynebacterium* species are particularly equipped to endure the harsh arid conditions of the stratum corneum (Elsner, 2006). It remains to be investigated whether *S. epidermidis* is unique in its ability to dialogue with the host immune system based on its capacity to colonize diverse environments within the skin, or if other bacterial species also have immune modulatory functions. Moreover, in the intestine, certain components of the microbiota can specifically program either inflammatory or regulatory immune pathways. For example, polysaccharide A produced by intestinal commensal *Bacteroides fragilis* directs mucosal IL-10 responses (Mazmanian et al., 2008). Specific cutaneous bacteria may have evolved similar instructive capacities to direct the maturation of defined immune pathways in the skin.

The resident microbiota shield the skin from colonization of potential pathogenic microbes or outgrowth of opportunistic pathogens already present. For instance, *S. epidermidis* provides the host with colonization resistance by limiting the growth of the pathobiont *S. aureus* (Iwase et al., 2010). The findings presented in **Chapter 3** support a previously unappreciated role for the skin microbiota in maintaining cutaneous immune homeostasis. In the absence of commensals, the balance of dermal effector and regulatory T cells is altered. While gut-tropic SFB did not impact cutaneous effecter and regulatory T cell populations, topical association with *S. epidermidis* was sufficient to reverse the

disequilibrium of cutaneous T lymphocytes in germ-free animals, indicating that localized interactions in the skin regulate immune function in this barrier. In the intestine, a reciprocal relationship between commensals and immune cells exists in which commensals stimulate immune function, which in turn controls commensal localization and composition (Round et al., 2011). Assessing the role of the skin immune surveillance system in regulating the localization, abundance, and composition of resident commensals is of critical importance. Such an understanding will help elucidate the host factors required for maintenance of a healthy microbiota.

A recent study has identified a central role for IL-17RA signaling in cutaneous wound healing (Lai et al., 2012). This effect of IL-17 signaling was attributed to production of the anti-microbial peptide regenerating islet-derived protein 3-alpha (REG3A) by keratinocytes (Lai et al., 2012). However, the contribution of this pathway in modulating skin commensals at steady state or during tissue stress is unknown. The IL-17 pathway is highly conserved across vertebrate evolution and has likely emerged at barrier sites as a specific mechanism to keep commensals at bay (Aggarwal and Gurney, 2002; Tsutsui et al., 2007). Indeed, a dysregulation of the IL-17 pathway is associated with a range of inflammatory dermal disorders and dysbiosis of the cutaneous microbiota. For instance, STAT3 deficient patients experience a loss of cutaneous IL-17A and develop severe forms of atopic dermatitis that is associated with an outgrowth of *Staphylococcal* species (Freeman and Holland,

2008). Similar alterations have been observed in the cutaneous flora of patients with psoriasis, a condition resulting from exuberant IL-17 responses. (Gao et al., 2008). Yet the exact mechanism by which host genetic factors, commensal microbiota, and environmental stress interact to trigger pathology in cutaneous disorders is not fully understood. Examining the function of commensally induced immune pathways in regulating resident bacteria will provide novel insights into the reciprocity of interactions between the host immune system and colonizing commensals along the skin barrier.

Elegant studies using bacterial 16s rDNA sequencing technology describe the bacterial garden that flourishes in and on our bodies (Costello et al., 2009). Importantly, these studies have laid the foundational groundwork for the understanding of immune-commensal interactions along our epithelial surfaces. While these barriers form a continuous tubular structure protecting our internal organs from the external environment, they are segregated into a range of habitats that house unique bacterial communities (Costello et al., 2009). The findings presented here support a non-redundant role for the indigenous microbes in each of these habitats in the local control of tissue immune function. Ascertaining the role of bacteria housed at other epithelial surfaces, such as the respiratory tract or oral mucosa, in driving immune and barrier function will be paramount for developing a global understanding of host-microbial mutualism.

Commensals control immunity and pathology during cutaneous infections.

Commensals are central orchestrators of immune responses in the intestine, where they stimulate immune cells and promote protection against invading pathogens. These natural adjuvants amplify immunity by interacting with various cellular components of the mucosal immune system to ensure a rapid response (Hall et al., 2008). Indeed, germ-free and orally-antibiotic-treated animals are more susceptible to infections from a variety of intestinal pathogens than commensally replete animals (Round and Mazmanian, 2009). Here, we find an analogous role for the skin microbiota in promoting immunity to the dermal pathogen *Leishmania major*. Dermal T cell production of IFN-γ in response to *Leishmania* antigen is greatly diminished in the absence of commensals. Consistent with this phenotype, lesional macrophages from germ-free mice are unable to control parasites residing in their cytoplasm. While fluctuations in gut flora do not impact immunity to *Leishmania*, association with the cutaneous commensal *S. epidermidis* is sufficient to reverse the defect in germ-free mice. Collectively, these results demonstrate an autonomous role for skin commensals in controlling protective immunity to *Leishmania major*.

The skin of germ-free mice contains greater frequencies and numbers of regulatory T cells than conventionally raised animals under homeostatic conditions. Although these differences in skin T_{reg} proportions are not maintained during the acute phase of the *L. major* infection, the imbalance of effector and regulatory T cells in the skin at steady state may impact the formation of

protective immunity in germ-free mice. Additionally, in our studies we mono-associate germ-free mice with commensals at the time of infection with *L. major*, as a result these mice have commensal signaling throughout the duration of the infection. To temporally dissect the role of skin commensals in the formation of an effector T cell response, it will be important to associate commensals at distinct time points during infectious challenge. Such studies will expand upon our findings to precisely dissect the role of commensals in priming and/or maintenance of effector T cells in skin inflammation.

The epidermal barrier is compromised when a pathogen invades the skin and establishes infection. Under such permissive conditions, commensals or their byproducts may penetrate into the underlying dermis and/or drain to the lymph node. It is not clear whether translocation of bacteria or their byproducts during infection is necessary for stimulating immunity to invading pathogens. Identifying the localization of commensals within the skin tissue and cutaneous lymph nodes during infection will provide additional clues to mechanisms by which commensals interact with specific cellular components of the skin immune system. After the resolution of skin infections, a population of effector memory T cells persists locally in the epidermis and provides protection upon pathogen reencounter (Gebhardt et al., 2011; Jiang et al., 2012). Assessing the role of skin commensals in the formation and persistence of such memory responses will be essential for the development of rational tissue-specific therapies and vaccine strategies.

A role for skin microbiota in the development of dermal pathologies has long been suspected. Indeed, we find that in addition to controlling protective responses, skin commensals also trigger pathology during *Leishmania major* infection. Commensally replete animals manifest necrotic dermal lesions with increased edema and immune infiltrate when compared to germ-free animals. These findings strongly implicate commensals in the etiology of pathological cutaneous inflammatory disorders such as atopic dermatitis and psoriasis (Gallo and Nakatsuji, 2011). Accordingly, shifts in the cutaneous microbiota are associated with both these disorders; yet it remains to be understood if there is a causal or associative relationship between such changes in the microflora and dermal disorders. Moreover, IL-17A-producing T cells are known to be major drivers of immune pathology in psoriasis. IL-17A and IL-17 receptor antibodies have been efficacious in clinical treatment of this disorder (Leonardi et al., 2012; Papp et al., 2012). The necessity for commensal signals in promoting these inflammatory pathways and the complex interactions between commensals and immune cells in human skin not yet been examined. Elucidation of such pathways could result in development of therapies to reinstate immune homeostasis by restoring a healthy skin flora.

While it is largely accepted that commensals modulate T cell function in the intestine by augmenting innate immune signals, recent studies have also demonstrated that cognate recognition of commensal antigens results in the formation of commensal-specific T cells. Such commensal-specific T cells adopt

either an effector or regulatory role in the context of the tissue microenvironment (Lathrop et al., 2011; Maynard et al., 2012). During acute gastrointestinal infection, commensal-specific effector T cells form in accord with the local cytokine milieu and persist long after resolution of inflammation (Hand et al., 2012). It is not known if effector T cells that recognize antigens derived from skin commensals form during cutaneous infection and whether these commensal-specific T cells are required for optimal immunity to invading pathogens. In further support of cognate recognition of skin commensal antigens by the host immune system, previous studies have found antibodies against cutaneous commensal antigens in the circulation of healthy humans (Haas et al., 2011). If commensal-specific cognate B and T cell responses form and persist in the skin, they may cause collateral tissue damage during subsequent infections or in response to tissue trauma as has been suggested in the gut (Hand et al., 2012). Furthermore, understanding their role in the etiology and pathology of uncontrolled inflammatory skin conditions such as psoriasis or rosacea will provide novel insights into these complex disorders. Mice chronically infected with *Leishmania major* exhibit psoriatic pathologies in areas of the skin distal to the initial site of inoculation (Y. Belkaid, unpublished observation), supporting the notion that commensal-specific T cell responses may form during cutaneous infection and reactivate to cause disease in response to tissue stress. Additionally, it has been proposed that infections in other tissues that result in the priming of bacterial-specific T cells may also promote dermal pathologies. Indeed, CLA^+ T cells that

recognize streptococcal M protein and keratin are primed in the tonsils during streptococcal infection. (Valdimarsson et al., 2009). These cells can migrate into the skin and have been linked to psoriatic skin flares (Valdimarsson et al., 2009). Moreover, inflammatory T cells responding to bacterial and host antigens released by the tissue trauma may be the underlying cause of the Koebner response. Genome-wide association studies have determined that in addition to an inflammatory microenvironment, alterations of the epidermal barrier and increased permeability are closely linked to psoriasis. Barrier dysfunction and the resultant translocation of commensal bacteria or bacterial products may lead to dysregulated commensal-specific immune responses and aberrant pathologies in the skin.

Resident commensals co-opt tissue specific pathways to modulate T lymphocytes in the skin and gut tissue.

The cytokine signals required for the generation and maintenance of specific T helper subsets have been clearly defined using both *in vitro* systems and in the context of inflammation *in vivo (Zhu and Paul, 2010).* However, the factors controlling T cell effector capacity in extralymphoid tissues under steady state conditions have been limited to the intestine. Gut dwelling Th17 cells are regulated locally by lamina propria DCs via the production of IL-6 (Hall et al., 2008; Ivanov et al., 2006). To date, no such analysis of the cytokine factors controlling resident T cells in the skin had been conducted. Using a candidate approach, we uncovered a bifurcation in the signals that control inflammatory cytokine production in the skin. IL-17A production from both $\alpha\beta$ and $\gamma\delta$ T cells was critically dependent upon IL-1 signaling, while CD40 signaling in the skin promoted IFN-γ production from $\alpha\beta$ T cells under steady state conditions. Importantly, these signals were dispensable for T cell function in the intestine, indicating that T cells in these two tissue compartments are modulated by unique tissue specific cues (**Figure 27**). These findings support the premise that T cell function in a particular tissue site is tailored to the unique cytokine signatures of that microenvironment.

It is well appreciated that T cells primed in distinct tissue draining lymph nodes are differentially programed with expression of homing markers that prompt their migration into the associated tissue site (Kunkel and

Butcher, 2002). For instance, the priming of T cells in skin draining lymph nodes results in the induction of skin homing receptor CCR10, while cells primed in the mesenteric lymph nodes upregulate gut homing receptors CCR9 and $\alpha 4\beta 7$ (Sigmundsdottir and Butcher, 2008; Sigmundsdottir et al., 2007). It remains to be investigated whether effector T cells are similarly imprinted with the ability to respond to the cytokine cues of the corresponding tissue environment during priming, or if a particular tissue site selects for cells with the capacity to respond to local factors.

Isolating antigen-specific effector T cells that are primed in a given tissue microenvironment, and assessing their maintenance/functional capacity when introduced to a different tissue environment in the presence of cognate antigen may reveal the responsiveness of effector T cells to distinct microenvironmental cues. For example, examining the persistence and function of effector T cells that are isolated from the intestine and then reintroduced into the skin and vice versa will provide insight into the instructive role of these two disparate microenvironments and the ability of an effector cell to function in distinct environments. The data presented here support a role for IL-1/MyD88 signaling in T cells for inflammatory cytokine production in the skin, but not the gut. Although we find that IL-1R1 signaling is dispensable for optimal homeostasis of intestinal effector T cells, a recent report demonstrated that intestinal Th17 cells are capable of producing IL-17A in response to IL-1 *in vitro* (Shaw et al., 2012). Of note, following resolution of cutaneous viral infection a subset of CD4 effector

memory cells was shown to re-circulates in and out of the skin tissue (Gebhardt et al., 2011). Long-term effector memory cells may recirculate though various tissue microenvironments and the ability of these effector T cells to provide optimal protection during pathogen re-encounter at distinct barriers may be critically dependent upon its responsiveness to local cues. Thus, it is likely that effector T cells generated in any site are capable of responding to an array of cytokine cues, some of which may be more functionally relevant in certain tissue sites.

The downstream effects of IL-1-dependent MyD88 signaling in skin T cells that result in augmented effector function are unclear. The IL-1/MyD88 pathway is known to play a role in the priming of T lymphocytes both by maturing antigen-presenting cells and by directly signaling into T cells (Hu et al., 2011). However, we find that alterations in T cells of both germ-free and IL-1R1 deficient animals are restricted to the skin tissue and are not found in the draining lymph node. Additionally, circulating T cells from germ-free and conventionally raised animals express equivalent levels of skin homing markers. Collectively, these data exclude the possibility that alterations in skin T cells are a result of defective priming or improper induction of skin homing makers in the cutaneous lymph nodes in the absence of commensal driven IL-1. The differences in the proportions of cutaneous effector and regulatory T cells in germ-free mice are likely due to alterations that occur locally in the skin tissue. To this end, IL-1 in the skin may control cutaneous T cells by the following non-mutually exclusive

mechanisms: 1) controlling the entry, retention, and/or exit of lymphocytes, 2) differentially regulating the survival, and/or proliferation of effector and regulatory lymphocytes (**Figure 28**)

The composition of T cells in an extralymphoid compartment is in part dependent upon their entry, retention, and egress from that tissue. Using bone marrow chimeric mice we have demonstrated that MyD88 signaling in T cells is necessary for optimal IL-17A production in the skin. Therefore, it is possible that upon entry into the dermis, an effector T cell receives IL-1 signals that positively regulate the expression of integrins and promotes their retention in the tissue. Alternatively, IL-1 acting on T regulatory cells may promote their egress from the skin to the draining lymph node. Indeed, several groups have observed that both effector and regulatory T cells constitutively exit skin tissue via the afferent lymph using defined chemokine receptors such as CCR7 (Debes et al., 2005; Tomura et al., 2010). Assessing the differential expression of retention receptors such as CD103 or egress receptors such as CCR7 on purified skin tissue T cells after IL-1 stimulation and in T cells isolated from germ-free animals may reveal a role for commensally driven IL-1 in modulating the flux of lymphocytes in skin tissue. Furthermore, measuring egress of effector and regulatory T cells from skin using mice genetically engineered to express photo-activator reporter proteins after topical association with skin commensals may provide mechanistic insights into the local modulation of T cells by IL-1 and skin commensals (Tomura et al., 2010).

The survival and/or local proliferation of effector T cells in the skin tissue may also be augmented by IL-1 signals. Indeed, IL-1 signaling has been shown to promote the phosphorylation of mTOR and JNK in effector T cells, thus stimulating their antigen driven expansion (Ben-Sasson et al., 2009; Gulen et al., 2010). Ligation of the TCR and IL-1R1 on effector T cells in the skin may induce their local proliferation. In further support of this possibility, a recent report demonstrated that effector and regulatory T cells isolated from the normal human skin expand based on context depend cues, whereby effector T cells multiply in response to inflammatory stimuli and regulatory T cells expand in the absence of inflammatory signals (Seneschal et al., 2012). Commensally driven IL-1 may similarly modulate the proportions of effector and regulatory cells in the skin. On the other hand, IL-1 may negatively regulate the survival of regulatory T cells by limiting the production of T_{reg} maintenance factors such as IL-10/TGFβ in the cutaneous microenvironment (Murai et al., 2009). Effector T cells may also be better equipped in the presence of IL-1, either on a per cell basis or on the population level, to compete for common survival factors such as IL-2, IL-7, and IL-15, without which the pool of regulatory T cells in the skin may contract (Rochman et al., 2009). Thus, the dynamic equilibrium of effector of regulatory T cells in the skin is likely to be controlled by several non-mutually exclusive pathways downstream of commensal driven IL-1. Future studies will need to address the mechanism by which IL-1 controls T cells in the skin and whether analogous functions are served by IL-6 in the intestine.

Importantly, the T cell intrinsic role of MyD88 signaling at steady state and during inflammation appears to be distinct. At steady state, MyD88 signaling in T cells is necessary for optimal IL-17A production, but does not regulate IFN-γ production in the skin. In contrast, the inflammatory milieu of mixed bone marrow chimeric animals revealed a role for MyD88 signaling in T cells for IFN-γ production. Consistent with this, mice deficient in IL-1R1 and MyD88 as well as mice treated with IL-1 receptor antagonist are unable to mount an appropriate *Leishmania* specific IFN-γ response (Muraille et al., 2003). Although the T cell intrinsic role for IL-1 signaling was not examined during *L. major* infection, based on the bone marrow chimeric experiments it is highly probable that absence of MyD88 signaling within the T cell compartment contributes to the defect observed in germ-free, and MyD88/IL-1R1 deficient animals.

The mechanistic basis for cell intrinsic IL-1 control of effector T cell function during inflammation is likely to be similar to that outlined above for steady state conditions (**Figure 28**). In addition to T cell intrinsic sensing of MyD88, this pathway may also control the flux of lymphocytes by acting on vascular and lymphatic endothelial cells. Zhu and colleagues recently demonstrated that vascular permeability is increased by IL-1/MyD88 signaling in endothelial cells (Zhu et al., 2012). Hence, IL-1 downstream of commensal signaling acting on T cells as well as accessory cells may manipulate the composition of T cells in skin tissue. A comparative analysis of T cell potential to produce IFN-γ versus *Leishmania* specific IFN-γ production revealed while T cells

from the skin of germ-free mice maintain the capacity to produce IFN-γ, they are unable to make it in response to *Leishmania* antigen stimulation *ex vivo* (S. Naik unpublished observation). This defect in *ex vivo* cytokine production may be due to impaired IL-1 signaling in T cells or improper activation of accessory cells that are necessary for optimally stimulating T cell function within the dermal lesion. To this end, the IL-1 pathway is also known to promote the activation of both innate immune cells as well as skin stromal cells such as keratinocytes and fibroblasts (Miller and Cho, 2011; Sims and Smith, 2010). For example, IL-1 promotes the maturation of dendritic cells, enhancing their antigen presentation capacity, co-stimulatory molecule expression, and cytokine production (Eriksson et al., 2003). Thus, in addition to a lack of T cell intrinsic MyD88 signaling, defects in activation of innate immune cells such as dendritic cells as well as stromal cells such as keratinocytes in the absence of commensal driven IL-1 is likely to contribute to the susceptibility phenotype in germ-free animals infected with *Leishmania major*.

A defect in IL-1 production was observed in the skin of germ-free mice and was reversed by the addition of *S. epidermidis*. Treatment of *S. epidermidis* mono-associated mice with an IL-1 receptor antagonist was able prevent this commensal from restoring skin immunity in germ-free mice, demonstrating that commensally driven IL-1 signaling directs T cell function in the skin. Identifying the specific commensal signals that drive IL-1 in the skin and the dominant cellular source of IL-1 both at steady state and during infection will be the aim of future studies. By integrating these data into a working model, we posit that

resident skin commensals drive IL-1 signaling in the skin, which in turn tunes the function of effector T cells in the skin (**Figure 29**). The IL-1 pathway is an evolutionarily conserved arm of the innate immune system that may have arisen as an early mediator of host-commensal cross talk. This pathway is also linked to a multitude of chronic inflammatory disorders such as arthritis and asthma (Sims and Smith, 2010). Of relevance to our present study, IL-1 is implicated in the etiology and pathology of psoriasis and other cutaneous disorders and is essential for providing protection against dermal pathogens (Miller and Cho, 2011; Murphy et al., 2000; Pazyar et al., 2012). Thus, via their capacity to promote IL-1 signaling and consequently effector T cell function, skin commensals are likely important drivers and amplifiers of skin immunity.

How interactions between commensals and their hosts at distinct anatomical locations create unique cytokine milieus and set up site-specific thresholds for T cell activation is unclear. Importantly, the availability of commensal signals within a given tissue environment and differences in the expression of innate factors in that response to these signals require elucidation. For example, lamina propria dendritic cells produce substantial amounts of IL-6 and other inflammatory cytokines in response to commensal ligands, while dermal dendritic cells stimulated with the same ligands do not (S. Naik, unpublished observation) Given the extreme heterogeneity of skin commensals, the mechanisms by which commensals in distinct topographical locations control immunity will need to be explored. Understanding the role of the skin

microbiota in maintaining tissue function is of primary importance for human health. Furthermore, altered diets, widespread antibiotic use, and other lifestyle choices may result in unnatural shifts in the cutaneous flora. The assessment of how such host factors impact the cutaneous microbiota and potentially lead to disease states will enable the development of therapies that promote the maintenance of a healthy microbiota.

Concluding Remarks

The results presented in this thesis provide novel insights into the localized regulation of T cells in the skin and gut by resident commensals. Our findings show that in addition to having a heterogeneous commensal landscape, physiologically distinct skin sites also have unique immune signatures. These data support the premise that discrete interactions between resident commensal and host cells in unique cutaneous microenvironments dictate the local inflammatory tone. Indeed, the experiments presented in **Chapters 3 and 4** allow for the emergence of such a paradigm by underscoring the role of indigenous commensals and tissue-specific cytokine milieus in setting the immunological tone of a given tissue microenvironment. More specifically, our data reveal an essential role for IL-1 signaling, driven by resident commensal organisms, in maintaining skin immune fitness. Furthermore, our findings demonstrate that the formation of protective immune responses and pathology during infectious challenge is critically dependent upon resident microbiota. Collectively, these studies unveil a non-redundant role for the cutaneous microbiota in shaping

skin immunity in health and disease.

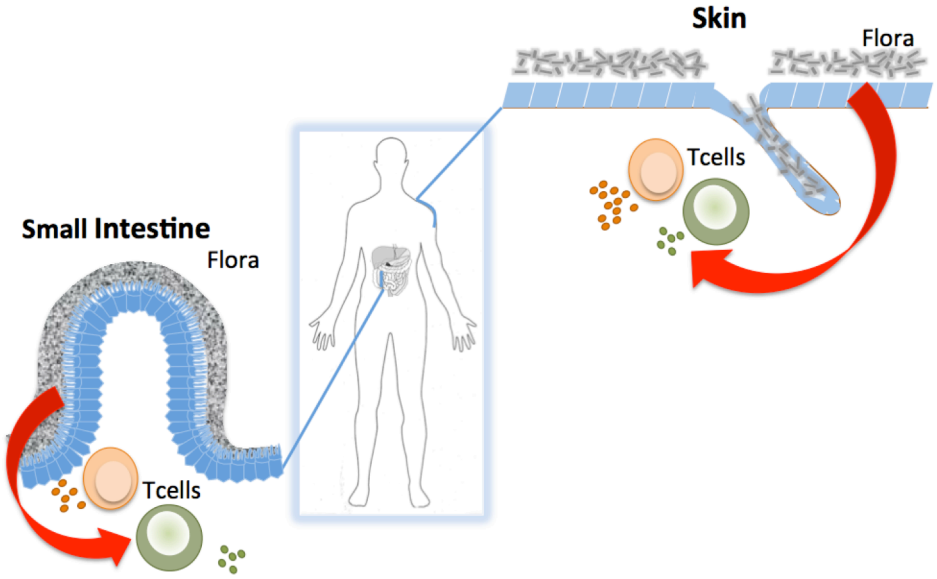

Figure 27: Model 1- Distinct commensal communities residing in the skin and gut locally control T cell function in the underlying tissue.

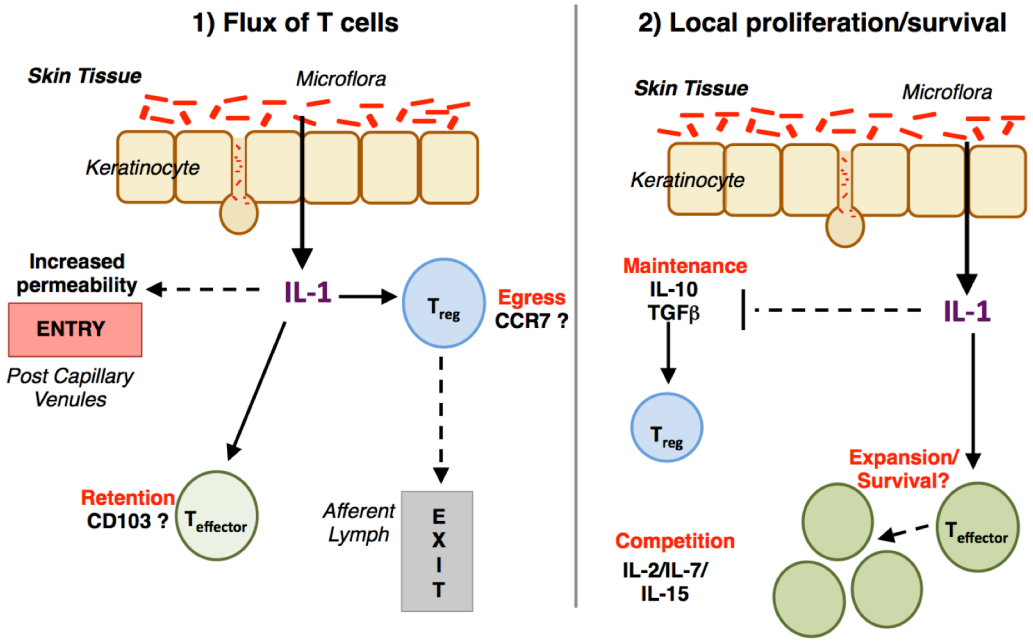

Figure 28: IL-1 dependent control of cutaneous T cells: Possible Mechanisms of Action

1) Flux of cells: IL-1 may act on effector T cells by promoting the expression of retention molecules such as CD103 and/or augmenting egress of regulatory T cells by enhancing the expression of CCR7. During inflammation, IL-1 may also act on the vascular endothelium to increase permeability of endothelial cells, thus facilitating the entry of effector T cells into the skin. **2) Local proliferation/survival:** IL-1 signaling may negatively regulate T_{reg} maintenance factors IL-10 and TGFβ in the skin. IL-1 may also direct the local expansion of cutaneous effector T cells. The local proliferation of effector T cells may lead to increased competition for local survival factors such as IL-2/IL-7/IL-15 and may have indirect effects on the pool of regulatory T cells in the skin

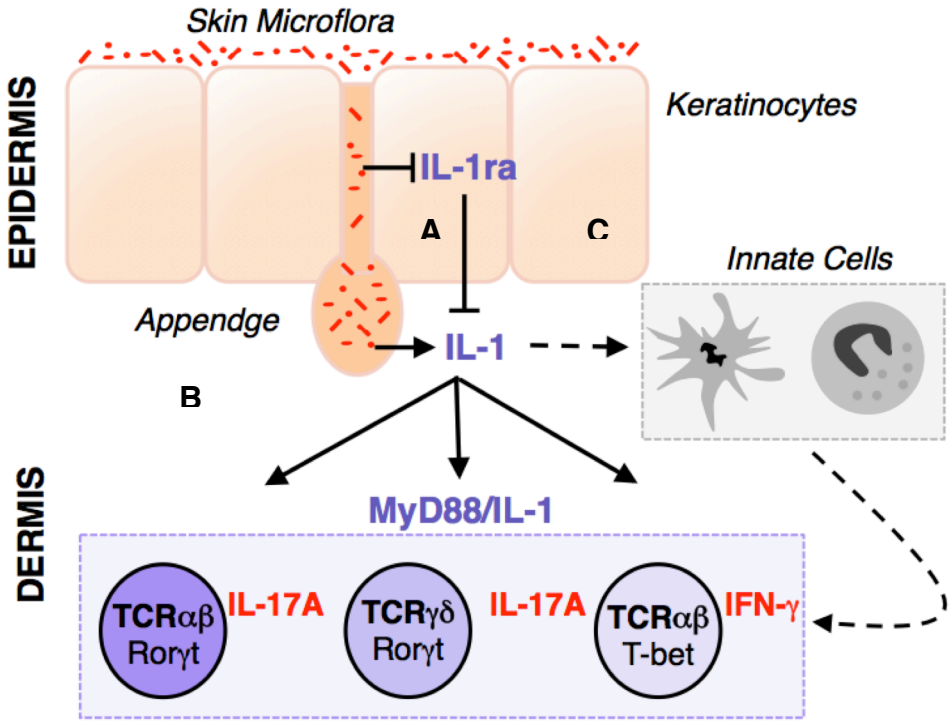

Figure 29: Model 2 - Commensals tune the function of skin T cells in an IL-1 dependent manner.

(**A**) Skin commensals promote the production of IL-1a and limit IL-1 receptor antagonist production by interfollicular keratinocytes. (**B**) Cutaneous T cells produce IL-17A and IFN-γ in response to commensally drive IL-1. (**C**) The role of innate cells in the IL-1 dependent control of T cells is not known.

Chapter 6 Materials and Methods

Mouse Strains:

C57BL/6 specific pathogen-free mice were purchased from Taconic Farms and The Jackson Laboratory. Germ-free C57BL/6 mice were bred at Taconic Farms and maintained in the NIAID gnotobiotic facility. B6.129S1-*Tlr3*^{tm1Flv}/J (*Tlr3*$^{-/-}$), *Il-1r1*$^{-/-}$, and B6.129S7-*Rag1*^{tm1Mom} (*Rag1*$^{-/-}$) were obtained through the NIAID Taconic exchange program from Taconic Farms. B6.129S2-*Il6*^{tm1Kopf}/J (*Il-6*$^{-/-}$) mice were obtained from The Jackson Laboratory. *Il-23r*$^{-/-}$ animals were a kind gift from Dr. M. Oukka (Seattle Children's Research Institute). B6.129-*Tlr2*^{tm1Kir}/J (*Tlr2*$^{-/-}$), B6.129P2-*Tlr5*^{tm1Aki} (*Tlr5*$^{-/-}$), B6.129P2-*Tlr9*^{tmAki} (*Tlr9*$^{-/-}$), B6.129P2-*Myd88*^{tmAki} (*Myd88*$^{-/-}$) and *Myd88*$^{-/-}$*/Ticam2*$^{-/-}$ mice were generous gifts from Dr. A. Sher (NIAID/NIH). B6.129P2-*Il18*^{tmAki}/J (*Il-18*$^{-/-}$) and C57BL/9-*Tnfrsf1a*$^{tm2.1Rsie}$ (*Tnfr1*$^{-/-}$) mice were obtained from Dr. G. Trinchieri (NCI/NIH) and Dr. R. Siegel (NIAMS/NIH), respectively. All mice were maintained at and all experiments were performed in an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at the National Institute for Allergy and Infectious Diseases (NIAID) and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the NIAID Animal Care and Use Committee. Gender- and age-matched mice between 8-12 weeks of age were used.

Non-human primate Species:

Skin tissue blood and lymph node samples from normal uninfected rhesus macaques, pig-tailed macaques, and cynomolgus monkeys, were obtained in collaboration with Dr. Jason Brenchley. Animals were housed and cared in accordance with the American Association for Accreditation of Laboratory Animal Care standards in AAALAC-accredited facilities, and all animal procedures were performed according to the protocols approved by the Institutional Animal Care and Use Committees of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Murine Tissue preparation:

In order to attain cells from various skin sites the following techniques were employed. Ears were excised and separated into the ventral and dorsal sheets. Flank (dorsal) skin was shaved with chrom mini (Wahl), adipose tissue was removed with a number 10 scalpel, and skin was cut in 1cm by 1cm pieces. Tissue samples were digested in RPMI containing 100 U/ml penicillin, 100 µg/ml streptomycin, 55 mM b-mercaptoethanol, 20 mM HEPES (HyClone), and 0.25 mg/ml Liberase purified enzyme blend (Roche Diagnostic Corp.), and incubated for 2 hours at 37°C and 5% CO_2. Digested skin sheets were homogenized using the Medicon/Medimachine tissue homogenizer system (Becton Dickinson). Digested tissue was then immediately diluted in RPMI-1640 medium containing: DNase I (500 **µ**g/ml, Sigma-Aldrich), 10% FCS, 100**µ**g/ml Penn/Strep, 25 mM HEPES and 50 **µ**M **β**-mercaptoethanol, and filtered through 70-**µ**m cell strainers (BD Biosciences). Cell suspensions were spun down and re-suspended in

complete RPMI-1640 medium (Complete) containing: 10% FBS, 100**μ**g/ml Penn/Strep, 25mM HEPES, 2mM L-glutamine, 1 mM Na Pyruvate (cellgro), 1X MEM nonessential amino acids (Roozendaal et al.) (cellgro) and 50 **μ**M of **β**-ME prior to use.

For isolation of keratinocytes, flank (dorsal) skin was shaved, adipose tissue was removed and skin was placed on 0.25% Trysin EDTA (Invitrogen) for 35-60 min at 37°C and 5% CO_2. Epidermal cells were manually scraped using a number 10 scalpel.

To prepare small intestinal lamina propria (Lp) tissue cell suspensions, adventitial fat was carefully stripped away from the whole small intestine. Peyer's patches (Pp) were then carefully excised, and the remaining tissue was cut longitudinally, extensively washed in cold HBSS w/o $Ca2^+$ and $Mg2^+$ (cellgro), then cut into ~ 1cm segments. Segments were treated with RPMI-1640 medium (Hyclone) containing: 3% FCS, 100**μ**g/ml Penn/Strep, 25 mM HEPES (Hyclone), 50 **μ**M **β**-mercaptoethanol (**β**-ME)146 (Gibco 1000X), 5mM EDTA, and 145 **μ**g/ml of Dithiothreitol (DTT) for 20 min in an incubator at 37ºC/5% CO2 with constant magnetic stirring. The IEL fraction was removed by filtration through a hand-held, fine mesh, sieve placed atop a collection beaker on ice. The pieces were then collected and placed into a 50ml conical containing 10-15ml of serum free media. Tissue was then further digested in serum free media containing: 25 mM HEPES

and 50 **μ**M **β**-ME with liberase CI (200 **μ**g/ml, Roche) in conjunction with DNase I (500 **μ**g/ml, Sigma-Aldrich) with continuous magnetic stirring in an incubator at 37ºC/5% CO2 for 26-30 min. Digested tissue was then immediately diluted in RPMI-1640 medium containing: 3% FCS, 100**μ**g/ml Penn/Strep, 25 mM HEPES and 50 **μ**M **β**-mercaptoethanol, and serially mashed through 70- and 40-**μ**m cell strainers (BD Biosciences). Cell suspensions were spun down and resuspended in complete RPMI-1640 medium (Complete) containing: 10% FBS, 100**μ**g/ml Penn/Strep, 25mM HEPES, 2mM L-glutamine, 1 mM Na Pyruvate (cellgro), 1X MEM nonessential amino acids (cellgro) and 50 **μ**M of **β**-ME prior to use.

Cells from Spleen (Sp) and cutaneous lymph nodes (cln), were prepared by teasing and gently smashing the tissue through a 70-**μ**m cell strainer with the rubber end of a plunger from a 3ml syringe.

Non-human primate Tissue preparation:

In order to attain cells from various NHP skin sample first adipose tissue was removed with a number 10 scalpel and tissue was weighted prior to processing. Using a 31 gauge insulin syringe samples were evenly injected with 100μl of RPMI containing 100 U/ml penicillin, 100 μg/ml streptomycin, 55 mM b-mercaptoethanol, 20 mM HEPES (HyClone), and 0.25 mg/ml Liberase purified enzyme blend (Roche Diagnostic Corp.) in 10μl doses. Injected tissues were incubated at 4°C for an hour in 5ml of the above mentioned digest media. Tissue

samples were minced into 0.5-1 am pieces and digested in RPMI containing 100 U/ml penicillin, 100 µg/ml streptomycin, 55 mM b-mercaptoethanol, 20 mM HEPES (HyClone), and 0.25 mg/ml Liberase purified enzyme blend (Roche Diagnostic Corp.), and incubated for 2 hours at 37°C and 5% CO_2. Digested tissue was then immediately diluted in RPMI-1640 medium containing: DNase I (500 **µ**g/ml, Sigma-Aldrich), 10% FCS, 100**µ**g/ml Penn/Strep, 25 mM HEPES and 50 **µ**M **β**-mercaptoethanol, and mashed through 70-**µ**m cell strainers (BD Biosciences). Cell suspensions were spun down and re-suspended in complete RPMI-1640 medium (Complete) containing: 10% FBS, 100**µ**g/ml Penn/Strep, 25mM HEPES, 2mM L-glutamine, 1 mM Na Pyruvate (cellgro), 1X MEM nonessential amino acids (cellgro) and 50 **µ**M of **β**-ME prior to use

Cellular Phenotypic Analysis:

Single cell suspensions were stained with either LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen) or 4', 6-diamidino-2-phenylindol (DAPI, Sigma) in HBSS to exclude dead cells. For detection of transcription factors, cells were stained using the Foxp3 staining set (eBioscience) according to the manufacturer's protocol. For detection of intracellular cytokines or langerin expression, cells were fixed and permeabilized with BD Cytofix/Cytoperm and stained in BD Perm Wash buffer (BD Biosciences). Cells were stained with the following antibodies purchased from either eBioscience, BD Biosciences, or Dendritics corp: CD45.1 (A20), CD45.2 (104), gdTCR (GL3), TCRb (H57-57), CD4 (RM4-5), IL-10

(JES5-16E3), IL-17A (ebio17B7), IFN-g (XMG1.2), TNF-a (MP6-XT22), Foxp3 (FJK-16a), Siglec-F (E50-2440), MHCII (M5/114.15.2) CD11b (M1/70), CD11c (N418), FceRI (Mar-1), C-kit (2B8), Langerin (929F3.01), CD103 (2E7), α6 (eBioGoH3), CD34 (RAM34), CD44 (IM7) and/or CD25 (PC61.5). Staining was performed in the presence of FcBlock (eBioscience), 0.2 mg/ml purified rat IgG and 1 mg/ml of normal mouse serum (Jackson Immunoresearch). Stain for skin homing markers was performed as previously described (Dudda et al., 2008).

Flow cytometry:

All cell acquisition was performed using an LSRII machine with FACSDiVa software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar). To calculate absolute numbers, the fraction of a particular subset to singlet gated, total living cells was multiplied by the total cellularity of the tissue based on trypan blue exclusion.

Immunofluorescense microscopy:

Mouse skin samples were fixed in 10% formalin and paraffin embedded. Paraffin sections were dewaxed and washed with 95% ethanol followed by methanol hydrogen peroxide. The sections were then treated with a heat induced epitope retrieval procedure using rodent Decloaker solution (Biocare Medical, RD913) and the Biocare decloaking chamber. After being washed in Tris pH 7.4, sections were incubated in the presence of rat serum and FcBlock (24G2) followed by

rabbit anti-Escherichia coli B (DAKO, B0357) diluted in the blocking solution. Samples were washed in Tris and then incubated with goat anti-rabbit IgG-Texas Red antibody (Invitrogen, T2767). The tissue was then counterstained with HOECSHT, and imaged using a Leica DM IRBE fluorescent microscope.

Lymph nodes were harvested and fixed in 0.05 M PBS containing 0.1 M L-lysine (pH 7.4), 2 mg/ml NaIO4, and 10mg/ml paraformaldehyde for 12 hrs. Samples were washed in phosphate buffer and dehydrated in 30% sucrose phosphate buffer. Spleens were snap frozen in Tissue-Tek (Sakura Finetek). Sections were cut on cryostat. Lymphoid tissue samples were stained with LYVE-1 (Novus Biologicals), B220, CD4 (BD Biosciences), CD8 (Caltag), Collagen IV (Abcam), FDC-M2 (Immunokontact) followed by secondary staining with goat anti-rabbit or goat anti-rat antibodies (Invitrogen).

Antibiotic treatment:

Female 3-week-old C57BL/6 mice were provided ampicillin (1 g/l), vancomycin (500 mg/l), neomycin trisulfate (1 g/l), and metronidazole (1 g/l) in drinking water for 4 to 8 weeks as previously described (Hall et al., 2008). Mice were infected with *L. major* 4 weeks post-antibiotic treatment and maintained on antibiotics for the duration of the infection. All antibiotics were purchased from Sigma-Aldrich. Germ free animals were treated with vancomycin (500 mg/l) in drinking

water starting one week prior to topical association with *S. epidermidis* maintained on antibiotic water for the duration of the experiment.

DNA extraction and Bacterial 454 analysis:

Ear and flank skin samples were sterilely obtained and processed using a protocol adapted from Grice et al (Grice et al., 2009). DNA was extracted from fecal pellets using QIAamp DNA stool mini kit (Qiagen). For quantitative analysis of 16S rDNA, real time PCR was performed using primers BacF (5'-CGGCAACGAGCGCAACCC-3') and BacR (5'-CCATTGTAGCACGTGTGTAGCC-3') (Pei et al., 2010). For sequencing of 16S rDNA amplicon libraries were prepared from sample DNA using Accuprime High Fidelity Taq polymerase (Invitrogen) and universal primers flanking variable regions V1 (primer 27F; 5'-AGAGTTTGATCCTGGCTCAG-3') and V3 (primer 534R; 5'-ATTACCGCGGCTGCTGG-3'). For each sample, the universal primers were tagged with unique sequences ("barcodes") to allow for multiplexing/demultiplexing (Lennon et al., 2010) PCR products were then purified using the Agencourt Ampure XP Kit (Beckman Counter Genomics) and quantitated using the QuantIT dsDNA High-Sensitivity Assay Kit (Invitrogen). Approximately equivalent amounts of each PCR product were then pooled and purified with a Qiagen minElute column (Qiagen) into 30 ml TE buffer prior to sequencing at the NIH Intramural Sequencing Center. Amplicon libraries were sequenced on a 454 FLX instrument using Titanium chemistry. Flowgrams

were processed using the 454 Basecalling pipeline (v2.5.3). *Sequence pre-processing, alignment and chimera removal*: mothur (version 1.21.0)(Schloss et al., 2009) was used for all 16S rRNA gene sequence analysis steps. Prior to analysis, sequences were trimmed of low quality ends and filtered to retain sequences with a minimum length of 200 bp. After alignment to a bacterial reference alignment (Sampaio et al.), chimeras were removed using the chimera slayer implementation in the mothur package. *Biodiversity and phylogenetic analyses:* From the alignment, a distance matrix was calculated and sequences were clustered into OTUs using the average neighbor algorithm at a cutoff of 0.03. Taxonomic classification of reads clustered in OTUs was done using the RDP Classifier included in mothur. A phylogenetic tree was generated from a relaxed neighbor-joining algorithm using the Clearcut program available via mothur. Unweighted and weighted UniFrac analyses (mothur & FastUnifrac http://bmf.colorado.edu/fastunifrac/) were subsequently done from the phylogenetic data to evaluate community differences. Principal coordinate analysis (PCoA) was used to visualize distribution patters from UniFrac distances.

Mouse cohousing and microbial reconstitution:

Wild-type age matched females were tail tattooed and housed in the same cage with equal number of respective knockout mice for at least two weeks. For association of germ-free mice with SFB (a kind gift from Dr. Yoshinori

Umesaki) (Umesaki et al., 1995)), fecal pellets isolated from SFB mono-associated mice were reconstituted in sterile PBS and 200 µl of this suspension was administered to each germ-free mouse by gavage in sterile isolator. SFB reconstitution was confirmed by qPCR of fecal 16S rDNA relative to negative GF controls as previously described (Salzman et al., 2010). Mono-associated mice were maintained for 1-2 weeks prior to analysis. For mono-association of germ-free mice with *Staphylococcus epidermidis*, clinical isolate NIHLM087 (Grice et al., 2009) was cultured for 18 hrs in tryptic soy broth at 37°C. Germ free mice were associated by placing100 – 500 ml of overnight *S. epidermidis* bacterial suspension on ear and flank skin using a sterile cotton swab every 3 days for one to two weeks. For infectious studies, mice were topically associated either a week prior or at time of *L. major* inoculation. *S. epidermidis* reconstitution was confirmed by sterilely homogenizing ear skin and plating on tryptic soy agar for 18 hrs.

ELISA:

Naïve or infected skin tissue homogenates were cultured for 18 hrs in RPMI containing 100 U/ml penicillin, 100 µg/ml streptomycin, 55 mM b-mercaptoethanol, 20 mM HEPES (HyClone) and 10% FBS at 37°C. Supernatants were collected and levels of inflammatory mediators were measured either ELISA (R&D Systems) or multiplex (Millipore).

NanoString nCounter analysis:

nCounter Gene Expression Assay was performed using two specific probes (capture and reporter) for each gene of interest. In brief, cell lysates from 10,000 cells per sample were hybridized with customized Reporter CodeSet and Capture ProbeSet according to manufacturer's instructions (NanoString Technologies, Seattle, USA), for direct labeling of mRNAs of interest with molecular barcodes without the use of reverse transcription or amplification. Then, the hybridized samples were recovered with the NanoString Prep Station and the mRNA molecules counted with the NanoString nCounter. The resulting counts were corrected by subtracting the average value of the negative control (alien probes from the CodeSet, lacking spiked transcript) from the raw counts obtained for each RNA. Values less than zero were considered equal to 1. The corrected raw data were finally normalized using *Gapdh* as housekeeping gene.

***Leishmania major* infection and parasite enumeration:**

Mice were infected in the ear dermis with 10^4-10^5 *L. major* metacyclic promastigotes clone V1 (MHOM/IL/80/Friedlin) in a volume of 5 µl, using a 27 1/2 G needle as previously described (Belkaid et al., 2000). Immune responses were assessed between 4 and 5 weeks post infection. Lesion sizes were measured using an engineer caliper (Mitutoyo). To enumerate parasite, cytospin slides prepared from single cell suspensions of dermal lesions were stained with diff-quick (Fisher Scientific). Parasites and total number of nucleated on each

slide were counted using a light microscope.

Generation of Bone Marrow Chimeras:

Bone marrow was extracted from hind legs of knockout and wild type animals and T cell depleted using CD90.2 microbeads (Miltenyi). 5 week old Rag1 deficient/ CD45.1 animals were lethally irradiated and reconstituted with either 10 million mixed WT: KO bone marrow cells in a 30: 70 ratio or individually into separate hosts. Animals were maintained on antibiotics for up to one week after reconstitution. Cutaneous and intestinal lymphocytes were assessed 12-16 weeks after reconstitution.

***In vitro* T cell re-stimulation:**

For detection of basal cytokine potential, single cell suspensions from various tissues were stimulated directly *ex vivo* with 50 ng/ml phorbol myristate acetate (PMA)/ (Sigma) and 5 µg/ml ionomycin (Sigma) in the presence of brefeldin A (GolgiPlug, BD Biosciences) in RPMI 1640 supplemented with 10% FBS, penicillin, streptomycin, HEPES, glutamine, nonessential amino acids, and 50 mM of β-mercaptoethanol for 4 hours at 37°C and 5% CO2. For *Leishmania* specific stimulations, single cells suspensions were stimulated in the presence of soluble *Leishmania* antigen for 18 hrs. Brefeldin A (GolgiPlug, BD Biosciences) was added to cultures for the last 8 hrs. For *ex vivo* cytokine production analysis

post *L. major* infection, skin tissue was digested in the presence of Brefeldin A and isolated cells were then incubated in the presence of Brefeldin A and RPMI 1640 supplemented with 10% FBS, penicillin, streptomycin, HEPES, glutamine, nonessential amino acids, and 50 mM of β-mercaptoethanol for 4 hrs at 37°C and 5% CO2. $CD4^+$ T cells, based on the expression of TCRβ and CD4 and dermal gd T cells, based on intermediate expression levels of the pan γδ TCR, were sorted from the skin of SPF mice. Purified cells T cells were cultured in αCD3 (1mg/ml) coated plates in the presence or absence of IL-1α, IL-1β (10ng/ml) or IL-6 (15 ng/ml) and supplemented with 10% FBS, penicillin, streptomycin, HEPES, glutamine, nonessential amino acids, and 50 mM of β-mercaptoethanol for 48hrs at 37°C and 5% CO2. Cytokine expression in the cell culture supernatants was assayed using FlowCytomix Multiplex Technology (ebioscience) and was adjusted to the plated density of $5x10^3$ cells in 50μl total culture volume.

***In vivo* cytokine administration:**

Naïve or *L. major* infected animals were treated intraperitoneally with either 250 mg/kg of recombinant human IL-1ra / Kineret (Biovitrum) or PBS control daily for 7 days starting at the time of infection and/or bacterial mono-association.

Statistics:

Paired or Unpaired Student *t* test was used to compare the corresponding populations. Error bars represent standard error of the mean.

Bibliography

(2012a). A framework for human microbiome research. Nature *486*, 215-221.

(2012b). Structure, function and diversity of the healthy human microbiome. Nature *486*, 207-214.

Abrams, G.D., Bauer, H., and Sprinz, H. (1963). Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germ-free and conventional mice. Lab Invest *12*, 355-364.

Abt, M.C., Osborne, L.C., Monticelli, L.A., Doering, T.A., Alenghat, T., Sonnenberg, G.F., Paley, M.A., Antenus, M., Williams, K.L., Erikson, J.*, et al.* (2012). Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity *37*, 158-170.

Aggarwal, S., and Gurney, A.L. (2002). IL-17: prototype member of an emerging cytokine family. J Leukoc Biol *71*, 1-8.

Aksentijevich, I., Masters, S.L., Ferguson, P.J., Dancey, P., Frenkel, J., van Royen-Kerkhoff, A., Laxer, R., Tedgard, U., Cowen, E.W., Pham, T.H.*, et al.* (2009). An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. N Engl J Med *360*, 2426-2437.

Allenspach, E.J., Lemos, M.P., Porrett, P.M., Turka, L.A., and Laufer, T.M. (2008). Migratory and lymphoid-resident dendritic cells cooperate to efficiently prime naive CD4 T cells. Immunity *29*, 795-806.

Atarashi, K., and Honda, K. (2011). Microbiota in autoimmunity and tolerance. Curr Opin Immunol *23*, 761-768.

Barbee, S.D., Woodward, M.J., Turchinovich, G., Mention, J.J., Lewis, J.M., Boyden, L.M., Lifton, R.P., Tigelaar, R., and Hayday, A.C. (2011). Skint-1 is a highly specific, unique selecting component for epidermal T cells. Proc Natl Acad Sci U S A *108*, 3330-3335.

Bedoui, S., Whitney, P.G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R.S., Wojtasiak, M., Shortman, K., Carbone, F.R.*, et al.* (2009). Cross-

presentation of viral and self antigens by skin-derived CD103+ dendritic cells. Nat Immunol *10*, 488-495.

Belkaid, Y., Kamhawi, S., Modi, G., Valenzuela, J., Noben-Trauth, N., Rowton, E., Ribeiro, J., and Sacks, D.L. (1998). Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of Leishmania major infection in the mouse ear dermis. J Exp Med *188*, 1941-1953.

Belkaid, Y., Mendez, S., Lira, R., Kadambi, N., Milon, G., and Sacks, D. (2000). A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. J Immunol *165*, 969-977.

Belkaid, Y., Piccirillo, C.A., Mendez, S., Shevach, E.M., and Sacks, D.L. (2002). CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature *420*, 502-507.

Ben-Sasson, S.Z., Hu-Li, J., Quiel, J., Cauchetaux, S., Ratner, M., Shapira, I., Dinarello, C.A., and Paul, W.E. (2009). IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. Proc Natl Acad Sci U S A *106*, 7119-7124.

Bergboer, J.G., Oostveen, A.M., de Jager, M.E., Zeeuwen, P.L., Joosten, I., Seyger, M.M., and Schalkwijk, J. (2012). Koebner phenomenon in psoriasis is not associated with deletion of late cornified envelope genes LCE3B and LCE3C. J Invest Dermatol *132*, 475-476.

Berin, M.C., Zheng, Y., Domaradzki, M., Li, X.M., and Sampson, H.A. (2006). Role of TLR4 in allergic sensitization to food proteins in mice. Allergy *61*, 64-71.

Bisgaard, H., Li, N., Bonnelykke, K., Chawes, B.L., Skov, T., Paludan-Muller, G., Stokholm, J., Smith, B., and Krogfelt, K.A. (2011). Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. J Allergy Clin Immunol *128*, 646-652 e641-645.

Blanpain, C., and Fuchs, E. (2009). Epidermal homeostasis: a balancing act of

stem cells in the skin. Nat Rev Mol Cell Biol *10*, 207-217.

Blaser, M.J., Dominguez-Bello, M.G., Contreras, M., Magris, M., Hidalgo, G., Estrada, I., Gao, Z., Clemente, J.C., Costello, E.K., and Knight, R. (2012). Distinct cutaneous bacterial assemblages in a sampling of South American Amerindians and US residents. Isme J.

Brandl, K., Plitas, G., Mihu, C.N., Ubeda, C., Jia, T., Fleisher, M., Schnabl, B., DeMatteo, R.P., and Pamer, E.G. (2008). Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature *455*, 804-807.

Braverman, I.M. (1997). The cutaneous microcirculation: ultrastructure and microanatomical organization. Microcirculation *4*, 329-340.

Butcher, E.C., and Picker, L.J. (1996). Lymphocyte homing and homeostasis. Science *272*, 60-66.

Campbell, J.J., Haraldsen, G., Pan, J., Rottman, J., Qin, S., Ponath, P., Andrew, D.P., Warnke, R., Ruffing, N., Kassam, N.*, et al.* (1999). The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. Nature *400*, 776-780.

Caricilli, A.M., Picardi, P.K., de Abreu, L.L., Ueno, M., Prada, P.O., Ropelle, E.R., Hirabara, S.M., Castoldi, A., Vieira, P., Camara, N.O.*, et al.* (2011). Gut microbiota is a key modulator of insulin resistance in TLR 2 knockout mice. PLoS Biol *9*, e1001212.

Cataisson, C., Salcedo, R., Hakim, S., Moffitt, B.A., Wright, L., Yi, M., Stephens, R., Dai, R.M., Lyakh, L., Schenten, D.*, et al.* (2012). IL-1R-MyD88 signaling in keratinocyte transformation and carcinogenesis. J Exp Med *209*, 1689-1702.

Cebra, J.J. (1999). Influences of microbiota on intestinal immune system development. Am J Clin Nutr *69*, 1046S-1051S.

Charkoudian, N. (2003). Skin blood flow in adult human thermoregulation: how it works, when it does not, and why. Mayo Clin Proc *78*, 603-612.

Chemical Society (Great Britain) Foreign compound metabolism in

mammals. In Specialist periodical report (London,, Chemical Society.), p. v.

Cho, I., Yamanishi, S., Cox, L., Methe, B.A., Zavadil, J., Li, K., Gao, Z., Mahana, D., Raju, K., Teitler, I.*, et al.* (2012). Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature *488*, 621-626.

Cho, J.S., Pietras, E.M., Garcia, N.C., Ramos, R.I., Farzam, D.M., Monroe, H.R., Magorien, J.E., Blauvelt, A., Kolls, J.K., Cheung, A.L.*, et al.* (2010). IL-17 is essential for host defense against cutaneous Staphylococcus aureus infection in mice. J Clin Invest *120*, 1762-1773.

Chow, J., Tang, H., and Mazmanian, S.K. (2011). Pathobionts of the gastrointestinal microbiota and inflammatory disease. Curr Opin Immunol *23*, 473-480.

Clark, R., and Kupper, T. (2005). Old meets new: the interaction between innate and adaptive immunity. J Invest Dermatol *125*, 629-637.

Clark, R.A., Chong, B., Mirchandani, N., Brinster, N.K., Yamanaka, K., Dowgiert, R.K., and Kupper, T.S. (2006). The vast majority of CLA+ T cells are resident in normal skin. J Immunol *176*, 4431-4439.

Clark, R.A., and Kupper, T.S. (2007). IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin. Blood *109*, 194-202.

Conlan, S., Mijares, L.A., Comp Seq Program, N., Becker, J., Blakesley, R.R., Bouffard, G.G., Brooks, S., Coleman, H.L., Gupta, J., Gurson, N.*, et al.* (2012). Staphylococcus epidermidis pan-genome sequence analysis reveals diversity of skin commensal and hospital infection-associated isolates. Genome Biol *13*, R64.

Costello, E.K., Lauber, C.L., Hamady, M., Fierer, N., Gordon, J.I., and Knight, R. (2009). Bacterial community variation in human body habitats across space and time. Science *326*, 1694-1697.

Debes, G.F., Arnold, C.N., Young, A.J., Krautwald, S., Lipp, M., Hay, J.B., and Butcher, E.C. (2005). Chemokine receptor CCR7 required for T lymphocyte exit

from peripheral tissues. Nat Immunol *6*, 889-894.

Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., and Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A *107*, 11971-11975.

Donahue, B.A., McArthur, J.G., Spratt, S.K., Bohl, D., Lagarde, C., Sanchez, L., Kaspar, B.A., Sloan, B.A., Lee, Y.L., Danos, O., and Snyder, R.O. (1999). Selective uptake and sustained expression of AAV vectors following subcutaneous delivery. J Gene Med *1*, 31-42.

Dowlen, H., and Owers, K. (2011). Koebner phenomenon following steroid injection for trigger finger. J Hand Surg Eur Vol *36*, 517.

Dudda, J.C., Perdue, N., Bachtanian, E., and Campbell, D.J. (2008). Foxp3+ regulatory T cells maintain immune homeostasis in the skin. J Exp Med *205*, 1559-1565.

Duhen, T., Geiger, R., Jarrossay, D., Lanzavecchia, A., and Sallusto, F. (2009). Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. Nat Immunol *10*, 857-863.

Egawa, G., and Kabashima, K. (2011). Skin as a peripheral lymphoid organ: revisiting the concept of skin-associated lymphoid tissues. J Invest Dermatol *131*, 2178-2185.

Egles, C., Garlick, J.A., and Shamis, Y. (2010). Three-dimensional human tissue models of wounded skin. Methods Mol Biol *585*, 345-359.

Elinav, E., Strowig, T., Kau, A.L., Henao-Mejia, J., Thaiss, C.A., Booth, C.J., Peaper, D.R., Bertin, J., Eisenbarth, S.C., Gordon, J.I., and Flavell, R.A. (2011). NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell *145*, 745-757.

Elsner, P. (2006). Antimicrobials and the skin physiological and pathological flora. Curr Probl Dermatol *33*, 35-41.

Eriksson, U., Kurrer, M.O., Sonderegger, I., Iezzi, G., Tafuri, A., Hunziker, L., Suzuki, S., Bachmaier, K., Bingisser, R.M., Penninger, J.M., and Kopf, M. (2003). Activation of dendritic cells through the interleukin 1 receptor 1 is critical for the induction of autoimmune myocarditis. J Exp Med *197*, 323-331.

Fagarasan, S., Muramatsu, M., Suzuki, K., Nagaoka, H., Hiai, H., and Honjo, T. (2002). Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. Science *298*, 1424-1427.

Foulongne, V., Sauvage, V., Hebert, C., Dereure, O., Cheval, J., Gouilh, M.A., Pariente, K., Segondy, M., Burguiere, A., Manuguerra, J.C*., et al.* (2012). Human skin microbiota: high diversity of DNA viruses identified on the human skin by high throughput sequencing. PLoS One *7*, e38499.

Franke, A., McGovern, D.P., Barrett, J.C., Wang, K., Radford-Smith, G.L., Ahmad, T., Lees, C.W., Balschun, T., Lee, J., Roberts, R.*, et al.* (2010). Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet *42*, 1118-1125.

Freeman, A.F., and Holland, S.M. (2008). The hyper-IgE syndromes. Immunol Allergy Clin North Am *28*, 277-291, viii.

Fuchs, E., and Nowak, J.A. (2008). Building epithelial tissues from skin stem cells. Cold Spring Harb Symp Quant Biol *73*, 333-350.

Gallo, R.L., and Hooper, L.V. (2012). Epithelial antimicrobial defence of the skin and intestine. Nat Rev Immunol *12*, 503-516.

Gallo, R.L., and Nakatsuji, T. (2011). Microbial Symbiosis with the Innate Immune Defense System of the Skin. J Invest Dermatol.

Gao, Z., Tseng, C.H., Strober, B.E., Pei, Z., and Blaser, M.J. (2008). Substantial alterations of the cutaneous bacterial biota in psoriatic lesions. PLoS One *3*, e2719.

Garrett, W.S., Lord, G.M., Punit, S., Lugo-Villarino, G., Mazmanian, S.K., Ito, S., Glickman, J.N., and Glimcher, L.H. (2007). Communicable ulcerative colitis

induced by T-bet deficiency in the innate immune system. Cell *131*, 33-45.

Gebhardt, T., Whitney, P.G., Zaid, A., Mackay, L.K., Brooks, A.G., Heath, W.R., Carbone, F.R., and Mueller, S.N. (2011). Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. Nature *477*, 216-219.

Gevers, D., Knight, R., Petrosino, J.F., Huang, K., McGuire, A.L., Birren, B.W., Nelson, K.E., White, O., Methe, B.A., and Huttenhower, C. (2012). The human microbiome project: a community resource for the healthy human microbiome. PLoS Biol *10*, e1001377.

Godin, B., and Touitou, E. (2007). Transdermal skin delivery: predictions for humans from in vivo, ex vivo and animal models. Adv Drug Deliv Rev *59*, 1152-1161.

Gray, E.E., Friend, S., Suzuki, K., Phan, T.G., and Cyster, J.G. (2012). Subcapsular sinus macrophage fragmentation and CD169+ bleb acquisition by closely associated IL-17-committed innate-like lymphocytes. PLoS One *7*, e38258.

Gray, E.E., Suzuki, K., and Cyster, J.G. (2011). Cutting edge: Identification of a motile IL-17-producing gammadelta T cell population in the dermis. J Immunol *186*, 6091-6095.

Gregorio, J., Meller, S., Conrad, C., Di Nardo, A., Homey, B., Lauerma, A., Arai, N., Gallo, R.L., Digiovanni, J., and Gilliet, M. (2010). Plasmacytoid dendritic cells sense skin injury and promote wound healing through type I interferons. J Exp Med *207*, 2921-2930.

Grice, E.A., Kong, H.H., Conlan, S., Deming, C.B., Davis, J., Young, A.C., Bouffard, G.G., Blakesley, R.W., Murray, P.R., Green, E.D*., et al.* (2009). Topographical and temporal diversity of the human skin microbiome. Science *324*, 1190-1192.

Grice, E.A., and Segre, J.A. (2011). The skin microbiome. Nat Rev Microbiol *9*, 244-253.

Grice, E.A., Snitkin, E.S., Yockey, L.J., Bermudez, D.M., Liechty, K.W., and

Segre, J.A. (2010). Longitudinal shift in diabetic wound microbiota correlates with prolonged skin defense response. Proc Natl Acad Sci U S A *107*, 14799-14804.

Gros, E., Bussmann, C., Bieber, T., Forster, I., and Novak, N. (2009). Expression of chemokines and chemokine receptors in lesional and nonlesional upper skin of patients with atopic dermatitis. J Allergy Clin Immunol *124*, 753-760 e751.

Groscurth, P. (2002). Anatomy of sweat glands. Curr Probl Dermatol *30*, 1-9.

Guilliams, M., Crozat, K., Henri, S., Tamoutounour, S., Grenot, P., Devilard, E., de Bovis, B., Alexopoulou, L., Dalod, M., and Malissen, B. (2010). Skin-draining lymph nodes contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. Blood *115*, 1958-1968.

Gulen, M.F., Kang, Z., Bulek, K., Youzhong, W., Kim, T.W., Chen, Y., Altuntas, C.Z., Sass Bak-Jensen, K., McGeachy, M.J., Do, J.S.*, et al.* (2010). The receptor SIGIRR suppresses Th17 cell proliferation via inhibition of the interleukin-1 receptor pathway and mTOR kinase activation. Immunity *32*, 54-66.

Guo, L., Wei, G., Zhu, J., Liao, W., Leonard, W.J., Zhao, K., and Paul, W. (2009). IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. Proc Natl Acad Sci U S A *106*, 13463-13468.

Gustafsson, B.E. (1948). Germ-free rearing of rats: general technique (Lund,: Berlingska boktryckeriet).

Guttman-Yassky, E., Nograles, K.E., and Krueger, J.G. (2011a). Contrasting pathogenesis of atopic dermatitis and psoriasis--part I: clinical and pathologic concepts. J Allergy Clin Immunol *127*, 1110-1118.

Guttman-Yassky, E., Nograles, K.E., and Krueger, J.G. (2011b). Contrasting pathogenesis of atopic dermatitis and psoriasis--part II: immune cell subsets and therapeutic concepts. J Allergy Clin Immunol *127*, 1420-1432.

Haas, A., Zimmermann, K., Graw, F., Slack, E., Rusert, P., Ledergerber, B., Bossart, W., Weber, R., Thurnheer, M.C., Battegay, M.*, et al.* (2011). Systemic antibody responses to gut commensal bacteria during chronic HIV-1 infection.

Gut *60*, 1506-1519.

Hall, J.A., Bouladoux, N., Sun, C.M., Wohlfert, E.A., Blank, R.B., Zhu, Q., Grigg, M.E., Berzofsky, J.A., and Belkaid, Y. (2008). Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. Immunity *29*, 637-649.

Han, T., Abdel-Motal, U.M., Chang, D.K., Sui, J., Muvaffak, A., Campbell, J., Zhu, Q., Kupper, T.S., and Marasco, W.A. (2012). Human Anti-CCR4 Minibody Gene Transfer for the Treatment of Cutaneous T-Cell Lymphoma. PLoS One *7*, e44455.

Hand, T.W., Dos Santos, L.M., Bouladoux, N., Molloy, M.J., Pagan, A.J., Pepper, M., Maynard, C.L., Elson, C.O., 3rd, and Belkaid, Y. (2012). Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses. Science *337*, 1553-1556.

Hapfelmeier, S., Lawson, M.A., Slack, E., Kirundi, J.K., Stoel, M., Heikenwalder, M., Cahenzli, J., Velykoredko, Y., Balmer, M.L., Endt, K.*, et al.* (2010). Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science *328*, 1705-1709.

Havran, W.L., and Jameson, J.M. (2010). Epidermal T cells and wound healing. J Immunol *184*, 5423-5428.

He, B., Xu, W., Santini, P.A., Polydorides, A.D., Chiu, A., Estrella, J., Shan, M., Chadburn, A., Villanacci, V., Plebani, A.*, et al.* (2007). Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. Immunity *26*, 812-826.

Heinecke, H. (1990). [The origins of the gnotobiotic technic--Kuster/Nuttall/Schottelius/ Thierfelder]. Z Versuchstierkd *33*, 19-22.

Helft, J., and Merad, M. (2010). Isolation of cutaneous dendritic cells. Methods Mol Biol *595*, 231-233.

Hill, D.A., Hoffmann, C., Abt, M.C., Du, Y., Kobuley, D., Kirn, T.J., Bushman, F.D., and Artis, D. (2010). Metagenomic analyses reveal antibiotic-induced

temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. Mucosal Immunol *3*, 148-158.

Hill, D.A., Siracusa, M.C., Abt, M.C., Kim, B.S., Kobuley, D., Kubo, M., Kambayashi, T., Larosa, D.F., Renner, E.D., Orange, J.S.*, et al.* (2012). Commensal bacteria-derived signals regulate basophil hematopoiesis and allergic inflammation. Nat Med *18*, 538-546.

Hofer, D. (2006). Antimicrobial textiles, skin-borne flora and odour. Curr Probl Dermatol *33*, 67-77.

Hooper, L.V., Littman, D.R., and Macpherson, A.J. (2012). Interactions between the microbiota and the immune system. Science *336*, 1268-1273.

Hooper, L.V., Midtvedt, T., and Gordon, J.I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr *22*, 283-307.

Hu, W., Troutman, T.D., Edukulla, R., and Pasare, C. (2011). Priming microenvironments dictate cytokine requirements for T helper 17 cell lineage commitment. Immunity *35*, 1010-1022.

Huzaira, M., Rius, F., Rajadhyaksha, M., Anderson, R.R., and Gonzalez, S. (2001). Topographic variations in normal skin, as viewed by in vivo reflectance confocal microscopy. J Invest Dermatol *116*, 846-852.

Ichinohe, T., Pang, I.K., Kumamoto, Y., Peaper, D.R., Ho, J.H., Murray, T.S., and Iwasaki, A. (2011). Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A *108*, 5354-5359.

Igyarto, B.Z., Haley, K., Ortner, D., Bobr, A., Gerami-Nejad, M., Edelson, B.T., Zurawski, S.M., Malissen, B., Zurawski, G., Berman, J., and Kaplan, D.H. (2011). Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses. Immunity *35*, 260-272.

Imaoka, A., Matsumoto, S., Setoyama, H., Okada, Y., and Umesaki, Y. (1996). Proliferative recruitment of intestinal intraepithelial lymphocytes after microbial

colonization of germ-free mice. Eur J Immunol *26*, 945-948.

Ishida, Y., Kondo, T., Kimura, A., Matsushima, K., and Mukaida, N. (2006). Absence of IL-1 receptor antagonist impaired wound healing along with aberrant NF-kappaB activation and a reciprocal suppression of TGF-beta signal pathway. J Immunol *176*, 5598-5606.

Ivanov, II, Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V.*, et al.* (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell *139*, 485-498.

Ivanov, II, Frutos Rde, L., Manel, N., Yoshinaga, K., Rifkin, D.B., Sartor, R.B., Finlay, B.B., and Littman, D.R. (2008). Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe *4*, 337-349.

Ivanov, II, and Honda, K. (2012). Intestinal commensal microbes as immune modulators. Cell Host Microbe *12*, 496-508.

Ivanov, II, McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell *126*, 1121-1133.

Iwase, T., Uehara, Y., Shinji, H., Tajima, A., Seo, H., Takada, K., Agata, T., and Mizunoe, Y. (2010). Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. Nature *465*, 346-349.

Jameson, J., and Havran, W.L. (2007). Skin gammadelta T-cell functions in homeostasis and wound healing. Immunol Rev *215*, 114-122.

Jameson, J., Ugarte, K., Chen, N., Yachi, P., Fuchs, E., Boismenu, R., and Havran, W.L. (2002). A role for skin gammadelta T cells in wound repair. Science *296*, 747-749.

Jarmuda, S., O'Reilly, N., Zaba, R., Jakubowicz, O., Szkaradkiewicz, A., and Kavanagh, K. (2012). Potential role of Demodex mites and bacteria in the

induction of rosacea. J Med Microbiol *61*, 1504-1510.

Jiang, X., Clark, R.A., Liu, L., Wagers, A.J., Fuhlbrigge, R.C., and Kupper, T.S. (2012). Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. Nature *483*, 227-231.

Jones, D.E., Elloso, M.M., and Scott, P. (1998). Host susceptibility factors to cutaneous leishmaniasis. Front Biosci *3*, D1171-1180.

Kadow, S., Jux, B., Zahner, S.P., Wingerath, B., Chmill, S., Clausen, B.E., Hengstler, J., and Esser, C. (2011). Aryl hydrocarbon receptor is critical for homeostasis of invariant gammadelta T cells in the murine epidermis. J Immunol *187*, 3104-3110.

Kalayciyan, A., Aydemir, E.H., and Kotogyan, A. (2007). Experimental Koebner phenomenon in patients with psoriasis. Dermatology *215*, 114-117.

Kambayashi, T., Assarsson, E., Lukacher, A.E., Ljunggren, H.G., and Jensen, P.E. (2003). Memory CD8+ T cells provide an early source of IFN-gamma. J Immunol *170*, 2399-2408.

Kastenmuller, W., Torabi-Parizi, P., Subramanian, N., Lammermann, T., and Germain, R.N. (2012). A spatially-organized multicellular innate immune response in lymph nodes limits systemic pathogen spread. Cell *150*, 1235-1248.

Kaye, P., and Scott, P. (2011). Leishmaniasis: complexity at the host-pathogen interface. Nat Rev Microbiol *9*, 604-615.

Kennedy-Crispin, M., Billick, E., Mitsui, H., Gulati, N., Fujita, H., Gilleaudeau, P., Sullivan-Whalen, M., Johnson-Huang, L.M., Suarez-Farinas, M., and Krueger, J.G. (2012). Human keratinocytes' response to injury upregulates CCL20 and other genes linking innate and adaptive immunity. J Invest Dermatol *132*, 105-113.

Khavari, P.A. (2006). Modelling cancer in human skin tissue. Nat Rev Cancer *6*, 270-280.

Kong, H.H., Oh, J., Deming, C., Conlan, S., Grice, E.A., Beatson, M.A.,

Nomicos, E., Polley, E.C., Komarow, H.D., Murray, P.R.*, et al.* (2012). Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. Genome Res *22*, 850-859.

Kriegel, M.A., Sefik, E., Hill, J.A., Wu, H.J., Benoist, C., and Mathis, D. (2011). Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. Proc Natl Acad Sci U S A *108*, 11548-11553.

Kubo, A., Nagao, K., Yokouchi, M., Sasaki, H., and Amagai, M. (2009). External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. J Exp Med *206*, 2937-2946.

Kullberg, M.C., Ward, J.M., Gorelick, P.L., Caspar, P., Hieny, S., Cheever, A., Jankovic, D., and Sher, A. (1998). Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism. Infect Immun *66*, 5157-5166.

Kunkel, E.J., and Butcher, E.C. (2002). Chemokines and the tissue-specific migration of lymphocytes. Immunity *16*, 1-4.

Kupper, T.S., and Fuhlbrigge, R.C. (2004). Immune surveillance in the skin: mechanisms and clinical consequences. Nat Rev Immunol *4*, 211-222.

Lai, Y., Di Nardo, A., Nakatsuji, T., Leichtle, A., Yang, Y., Cogen, A.L., Wu, Z.R., Hooper, L.V., Schmidt, R.R., von Aulock, S.*, et al.* (2009). Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. Nat Med *15*, 1377-1382.

Lai, Y., Li, D., Li, C., Muehleisen, B., Radek, K.A., Park, H.J., Jiang, Z., Li, Z., Lei, H., Quan, Y.*, et al.* (2012). The antimicrobial protein REG3A regulates keratinocyte proliferation and differentiation after skin injury. Immunity *37*, 74-84.

Lathrop, S.K., Bloom, S.M., Rao, S.M., Nutsch, K., Lio, C.W., Santacruz, N., Peterson, D.A., Stappenbeck, T.S., and Hsieh, C.S. (2011). Peripheral education of the immune system by colonic commensal microbiota. Nature *478*, 250-254.

Lee, Y.K., Menezes, J.S., Umesaki, Y., and Mazmanian, S.K. (2011).

Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A *108 Suppl 1*, 4615-4622.

Leeming, J.P., Holland, K.T., and Cunliffe, W.J. (1984). The microbial ecology of pilosebaceous units isolated from human skin. J Gen Microbiol *130*, 803-807.

Lennon, N.J., Lintner, R.E., Anderson, S., Alvarez, P., Barry, A., Brockman, W., Daza, R., Erlich, R.L., Giannoukos, G., Green, L.*, et al.* (2010). A scalable, fully automated process for construction of sequence-ready barcoded libraries for 454. Genome Biol *11*, R15.

Leonardi, C., Matheson, R., Zachariae, C., Cameron, G., Li, L., Edson-Heredia, E., Braun, D., and Banerjee, S. (2012). Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. N Engl J Med *366*, 1190-1199.

Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R., and Gordon, J.I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol *6*, 776-788.

Mabuchi, T., Chang, T.W., Quinter, S., and Hwang, S.T. (2012). Chemokine receptors in the pathogenesis and therapy of psoriasis. J Dermatol Sci *65*, 4-11.

Macfarlane, A.J., Kon, O.M., Smith, S.J., Zeibecoglou, K., Khan, L.N., Barata, L.T., McEuen, A.R., Buckley, M.G., Walls, A.F., Meng, Q.*, et al.* (2000). Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. J Allergy Clin Immunol *105*, 99-107.

Mackay, C.R. (1993). Homing of naive, memory and effector lymphocytes. Curr Opin Immunol *5*, 423-427.

Mackay, C.R., Marston, W.L., and Dudler, L. (1990). Naive and memory T cells show distinct pathways of lymphocyte recirculation. J Exp Med *171*, 801-817.

Macpherson, A.J., and Uhr, T. (2004a). Compartmentalization of the mucosal immune responses to commensal intestinal bacteria. Ann N Y Acad Sci *1029*, 36-

43.

Macpherson, A.J., and Uhr, T. (2004b). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science *303*, 1662-1665.

Manolova, V., Flace, A., Bauer, M., Schwarz, K., Saudan, P., and Bachmann, M.F. (2008). Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol *38*, 1404-1413.

Mauel, J., Ransijn, A., and Buchmuller-Rouiller, Y. (1991). Killing of Leishmania parasites in activated murine macrophages is based on an L-arginine-dependent process that produces nitrogen derivatives. J Leukoc Biol *49*, 73-82.

Maynard, C.L., Elson, C.O., Hatton, R.D., and Weaver, C.T. (2012). Reciprocal interactions of the intestinal microbiota and immune system. Nature *489*, 231-241.

Mazmanian, S.K., and Kasper, D.L. (2006). The love-hate relationship between bacterial polysaccharides and the host immune system. Nat Rev Immunol *6*, 849-858.

Mazmanian, S.K., Round, J.L., and Kasper, D.L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. Nature *453*, 620-625.

Menon, G.K. (2002). New insights into skin structure: scratching the surface. Adv Drug Deliv Rev *54 Suppl 1*, S3-17.

Merad, M., Ginhoux, F., and Collin, M. (2008). Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. Nat Rev Immunol *8*, 935-947.

Merad, M., Manz, M.G., Karsunky, H., Wagers, A., Peters, W., Charo, I., Weissman, I.L., Cyster, J.G., and Engleman, E.G. (2002). Langerhans cells renew in the skin throughout life under steady-state conditions. Nat Immunol *3*, 1135-1141.

Michel, M.L., Pang, D.J., Haque, S.F., Potocnik, A.J., Pennington, D.J., and Hayday, A.C. (2012). Interleukin 7 (IL-7) selectively promotes mouse and

human IL-17-producing gammadelta cells. Proc Natl Acad Sci U S A.

Miller, L.S., and Cho, J.S. (2011). Immunity against Staphylococcus aureus cutaneous infections. Nat Rev Immunol *11*, 505-518.

Miller, L.S., and Modlin, R.L. (2007). Human keratinocyte Toll-like receptors promote distinct immune responses. J Invest Dermatol *127*, 262-263.

Moreau, M.C., Ducluzeau, R., Guy-Grand, D., and Muller, M.C. (1978). Increase in the population of duodenal immunoglobulin A plasmocytes in axenic mice associated with different living or dead bacterial strains of intestinal origin. Infect Immun *21*, 532-539.

Murai, M., Turovskaya, O., Kim, G., Madan, R., Karp, C.L., Cheroutre, H., and Kronenberg, M. (2009). Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol *10*, 1178-1184.

Muraille, E., De Trez, C., Brait, M., De Baetselier, P., Leo, O., and Carlier, Y. (2003). Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to Leishmania major infection associated with a polarized Th2 response. J Immunol *170*, 4237-4241.

Murphy, J.E., Robert, C., and Kupper, T.S. (2000). Interleukin-1 and cutaneous inflammation: a crucial link between innate and acquired immunity. J Invest Dermatol *114*, 602-608.

Muzio, M., Ni, J., Feng, P., and Dixit, V.M. (1997). IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science *278*, 1612-1615.

Nabors, G.S., and Farrell, J.P. (1994). Site-specific immunity to Leishmania major in SWR mice: the site of infection influences susceptibility and expression of the antileishmanial immune response. Infect Immun *62*, 3655-3662.

Nabors, G.S., Nolan, T., Croop, W., Li, J., and Farrell, J.P. (1995). The influence of the site of parasite inoculation on the development of Th1 and Th2 type immune responses in (BALB/c x C57BL/6) F1 mice infected with Leishmania

major. Parasite Immunol *17*, 569-579.

Naderer, T., and McConville, M.J. (2011). Intracellular growth and pathogenesis of Leishmania parasites. Essays Biochem *51*, 81-95.

Nagao, K., Kobayashi, T., Moro, K., Ohyama, M., Adachi, T., Kitashima, D.Y., Ueha, S., Horiuchi, K., Tanizaki, H., Kabashima, K.*, et al.* (2012). Stress-induced production of chemokines by hair follicles regulates the trafficking of dendritic cells in skin. Nat Immunol *13*, 744-752.

Nakajima, K. (2012). Critical role of the interleukin-23/T-helper 17 cell axis in the pathogenesis of psoriasis. J Dermatol *39*, 219-224.

Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., and Pettersson, S. (2012). Host-gut microbiota metabolic interactions. Science *336*, 1262-1267.

Niess, J.H., and Adler, G. (2010). Enteric flora expands gut lamina propria CX3CR1+ dendritic cells supporting inflammatory immune responses under normal and inflammatory conditions. J Immunol *184*, 2026-2037.

Olszak, T., An, D., Zeissig, S., Vera, M.P., Richter, J., Franke, A., Glickman, J.N., Siebert, R., Baron, R.M., Kasper, D.L., and Blumberg, R.S. (2012). Microbial exposure during early life has persistent effects on natural killer T cell function. Science *336*, 489-493.

Ortner, U., Inaba, K., Koch, F., Heine, M., Miwa, M., Schuler, G., and Romani, N. (1996). An improved isolation method for murine migratory cutaneous dendritic cells. J Immunol Methods *193*, 71-79.

Palmer, C.N., Irvine, A.D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S.P., Goudie, D.R., Sandilands, A., Campbell, L.E., Smith, F.J.*, et al.* (2006). Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet *38*, 441-446.

Pantelyushin, S., Haak, S., Ingold, B., Kulig, P., Heppner, F.L., Navarini, A.A., and Becher, B. (2012). Rorgammat+ innate lymphocytes and gammadelta T cells

initiate psoriasiform plaque formation in mice. J Clin Invest *122*, 2252-2256.

Papp, K.A., Leonardi, C., Menter, A., Ortonne, J.P., Krueger, J.G., Kricorian, G., Aras, G., Li, J., Russell, C.B., Thompson, E.H., and Baumgartner, S. (2012). Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis. N Engl J Med *366*, 1181-1189.

Parfrey, L.W., and Knight, R. (2012). Spatial and temporal variability of the human microbiota. Clin Microbiol Infect *18 Suppl 4*, 8-11.

Pasteur, L. (1885). Observations relatives a la note de M. Duclaux. Comptes Rendus De L'academie des Sciences
100, 68.

Pazyar, N., Feily, A., and Yaghoobi, R. (2012). An overview of interleukin-1 receptor antagonist, anakinra, in the treatment of cutaneous diseases. Curr Clin Pharmacol *7*, 271-275.

Pei, C.X., Liu, Q., Dong, C.S., Li, H., Jiang, J.B., and Gao, W.J. (2010). Diversity and abundance of the bacterial 16S rRNA gene sequences in forestomach of alpacas (Lama pacos) and sheep (Ovis aries). Anaerobe *16*, 426-432.

Perona-Wright, G., Jenkins, S.J., O'Connor, R.A., Zienkiewicz, D., McSorley, H.J., Maizels, R.M., Anderton, S.M., and MacDonald, A.S. (2009). A pivotal role for CD40-mediated IL-6 production by dendritic cells during IL-17 induction in vivo. J Immunol *182*, 2808-2815.

Puel, A., Cypowyj, S., Bustamante, J., Wright, J.F., Liu, L., Lim, H.K., Migaud, M., Israel, L., Chrabieh, M., Audry, M.*, et al.* (2011). Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. Science *332*, 65-68.

Rahat, M., and Dimentman, C. (1982). Cultivation of bacteria-free Hydra viridis: missing budding factor in nonsymbiotic hydra. Science *216*, 67-68.

Rakoff-Nahoum, S., and Medzhitov, R. (2008). Innate immune recognition of the indigenous microbial flora. Mucosal Immunol *1 Suppl 1*, S10-14.

Reinhardt, C., Bergentall, M., Greiner, T.U., Schaffner, F., Ostergren-Lunden, G., Petersen, L.C., Ruf, W., and Backhed, F. (2012). Tissue factor and PAR1 promote microbiota-induced intestinal vascular remodelling. Nature *483*, 627-631.

Rennert, P.D., Hochman, P.S., Flavell, R.A., Chaplin, D.D., Jayaraman, S., Browning, J.L., and Fu, Y.X. (2001). Essential role of lymph nodes in contact hypersensitivity revealed in lymphotoxin-alpha-deficient mice. J Exp Med *193*, 1227-1238.

Renz, H., Brandtzaeg, P., and Hornef, M. (2012). The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. Nat Rev Immunol *12*, 9-23.

Reyniers, J.A., Trexler, P.C., and Ervin, R.F. (1946). Rearing germ-free albino rats. Lobund reports, 1-84.

Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E., and Chang, H.Y. (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell *129*, 1311-1323.

Rochman, Y., Spolski, R., and Leonard, W.J. (2009). New insights into the regulation of T cells by gamma(c) family cytokines. Nat Rev Immunol *9*, 480-490.

Roozendaal, R., Mempel, T.R., Pitcher, L.A., Gonzalez, S.F., Verschoor, A., Mebius, R.E., von Andrian, U.H., and Carroll, M.C. (2009). Conduits mediate transport of low-molecular-weight antigen to lymph node follicles. Immunity *30*, 264-276.

Round, J.L., Lee, S.M., Li, J., Tran, G., Jabri, B., Chatila, T.A., and Mazmanian, S.K. (2011). The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science *332*, 974-977.

Round, J.L., and Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol *9*, 313-323.

Sagi, L., and Trau, H. (2011). The Koebner phenomenon. Clin Dermatol *29*, 231-

236.

Salzman, N.H., Hung, K., Haribhai, D., Chu, H., Karlsson-Sjoberg, J., Amir, E., Teggatz, P., Barman, M., Hayward, M., Eastwood, D.*, et al.* (2010). Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol *11*, 76-83.

Sampaio, A.L., Mameri, A.C., Vargas, T.J., Ramos-e-Silva, M., Nunes, A.P., and Carneiro, S.C. (2011). Seborrheic dermatitis. An Bras Dermatol *86*, 1061-1071; quiz 1072-1064.

Sather, B.D., Treuting, P., Perdue, N., Miazgowicz, M., Fontenot, J.D., Rudensky, A.Y., and Campbell, D.J. (2007). Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. J Exp Med *204*, 1335-1347.

Savage, D.C. (1977). Microbial ecology of the gastrointestinal tract. Annu Rev Microbiol *31*, 107-133.

Schaerli, P., Ebert, L.M., and Moser, B. (2006). Comment on "The vast majority of CLA+ T cells are resident in normal skin". J Immunol *177*, 1375-1376; author reply 1376-1377.

Scharschmidt, T.C., List, K., Grice, E.A., Szabo, R., Renaud, G., Lee, C.C., Wolfsberg, T.G., Bugge, T.H., and Segre, J.A. (2009). Matriptase-deficient mice exhibit ichthyotic skin with a selective shift in skin microbiota. J Invest Dermatol *129*, 2435-2442.

Scharton-Kersten, T., and Scott, P. (1995). The role of the innate immune response in Th1 cell development following Leishmania major infection. J Leukoc Biol *57*, 515-522.

Schlake, T. (2007). Determination of hair structure and shape. Semin Cell Dev Biol *18*, 267-273.

Schlickum, S., Sennefelder, H., Friedrich, M., Harms, G., Lohse, M.J., Kilshaw, P., and Schon, M.P. (2008). Integrin alpha E(CD103)beta 7 influences cellular

shape and motility in a ligand-dependent fashion. Blood *112*, 619-625.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J*., et al.* (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol *75*, 7537-7541.

Seneschal, J., Clark, R.A., Gehad, A., Baecher-Allan, C.M., and Kupper, T.S. (2012). Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells. Immunity *36*, 873-884.

Shaw, M.H., Kamada, N., Kim, Y.G., and Nunez, G. (2012). Microbiota-induced IL-1beta, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. J Exp Med *209*, 251-258.

Sigmundsdottir, H., and Butcher, E.C. (2008). Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking. Nat Immunol *9*, 981-987.

Sigmundsdottir, H., Pan, J., Debes, G.F., Alt, C., Habtezion, A., Soler, D., and Butcher, E.C. (2007). DCs metabolize sunlight-induced vitamin D3 to 'program' T cell attraction to the epidermal chemokine CCL27. Nat Immunol *8*, 285-293.

Silvers, W.K., and Mintz, B. (1998). Differences in latency and inducibility of mouse skin melanomas depending on the age and anatomic site of the skin. Cancer Res *58*, 630-632.

Sims, J.E., and Smith, D.E. (2010). The IL-1 family: regulators of immunity. Nat Rev Immunol *10*, 89-102.

Singer, S.M., and Nash, T.E. (2000). The role of normal flora in Giardia lamblia infections in mice. J Infect Dis *181*, 1510-1512.

Smith, K.J., Skelton, H.G., Yeager, J., Ledsky, R., McCarthy, W., Baxter, D., Turiansky, G.W., Wagner, K.F., and Turianski, G. (1994). Cutaneous findings in HIV-1-positive patients: a 42-month prospective study. Military Medical Consortium for the Advancement of Retroviral Research (MMCARR). J Am Acad

Dermatol *31*, 746-754.

Springer, T.A. (1995). Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. Annu Rev Physiol *57*, 827-872.

Steeber, D.A., and Tedder, T.F. (2000). Adhesion molecule cascades direct lymphocyte recirculation and leukocyte migration during inflammation. Immunol Res *22*, 299-317.

Strauss, M.B. (2012). The effect of hyperbaric oxygen in crush injuries and skeletal muscle-compartment syndromes. Undersea Hyperb Med *39*, 847-855.

Streilein, J.W. (1989). Skin-associated lymphoid tissue. Immunol Ser *46*, 73-96.

Suffia, I., Reckling, S.K., Salay, G., and Belkaid, Y. (2005). A role for CD103 in the retention of CD4+CD25+ Treg and control of Leishmania major infection. J Immunol *174*, 5444-5455.

Sumaria, N., Roediger, B., Ng, L.G., Qin, J., Pinto, R., Cavanagh, L.L., Shklovskaya, E., Fazekas de St Groth, B., Triccas, J.A., and Weninger, W. (2011). Cutaneous immunosurveillance by self-renewing dermal gammadelta T cells. J Exp Med *208*, 505-518.

Swanson, P.A., 2nd, Kumar, A., Samarin, S., Vijay-Kumar, M., Kundu, K., Murthy, N., Hansen, J., Nusrat, A., and Neish, A.S. (2011). Enteric commensal bacteria potentiate epithelial restitution via reactive oxygen species-mediated inactivation of focal adhesion kinase phosphatases. Proc Natl Acad Sci U S A *108*, 8803-8808.

Thiboutot, D. (2004). Regulation of human sebaceous glands. J Invest Dermatol *123*, 1-12.

Tian, T., Liu, L., Freyschmidt, E.J., Murphy, G.F., Kupper, T.S., and Fuhlbrigge, R.C. (2009). Overexpression of IL-1alpha in skin differentially modulates the immune response to scarification with vaccinia virus. J Invest Dermatol *129*, 70-78.

Tilg, H., and Kaser, A. (2011). Gut microbiome, obesity, and metabolic

dysfunction. J Clin Invest *121*, 2126-2132.

Tomura, M., Honda, T., Tanizaki, H., Otsuka, A., Egawa, G., Tokura, Y., Waldmann, H., Hori, S., Cyster, J.G., Watanabe, T.*, et al.* (2010). Activated regulatory T cells are the major T cell type emigrating from the skin during a cutaneous immune response in mice. J Clin Invest *120*, 883-893.

Tsutsui, S., Nakamura, O., and Watanabe, T. (2007). Lamprey (Lethenteron japonicum) IL-17 upregulated by LPS-stimulation in the skin cells. Immunogenetics *59*, 873-882.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P.*, et al.* (2009). A core gut microbiome in obese and lean twins. Nature *457*, 480-484.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., and Gordon, J.I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. Nature *444*, 1027-1031.

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., and Roy, N.C. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. J Nutr *141*, 769-776.

Umesaki, Y., Okada, Y., Matsumoto, S., Imaoka, A., and Setoyama, H. (1995). Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. Microbiol Immunol *39*, 555-562.

Vaishnava, S., Yamamoto, M., Severson, K.M., Ruhn, K.A., Yu, X., Koren, O., Ley, R., Wakeland, E.K., and Hooper, L.V. (2011). The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science *334*, 255-258.

Valdimarsson, H., Thorleifsdottir, R.H., Sigurdardottir, S.L., Gudjonsson, J.E., and Johnston, A. (2009). Psoriasis--as an autoimmune disease caused by molecular mimicry. Trends Immunol *30*, 494-501.

Vijay-Kumar, M., Aitken, J.D., Carvalho, F.A., Cullender, T.C., Mwangi, S., Srinivasan, S., Sitaraman, S.V., Knight, R., Ley, R.E., and Gewirtz, A.T. (2010). Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science *328*, 228-231.

Villadangos, J.A., and Heath, W.R. (2005). Life cycle, migration and antigen presenting functions of spleen and lymph node dendritic cells: limitations of the Langerhans cells paradigm. Semin Immunol *17*, 262-272.

Wanke, I., Steffen, H., Christ, C., Krismer, B., Gotz, F., Peschel, A., Schaller, M., and Schittek, B. (2011). Skin commensals amplify the innate immune response to pathogens by activation of distinct signaling pathways. J Invest Dermatol *131*, 382-390.

Weinstock, G.M. (2012). Genomic approaches to studying the human microbiota. Nature *489*, 250-256.

Wen, L., Ley, R.E., Volchkov, P.Y., Stranges, P.B., Avanesyan, L., Stonebraker, A.C., Hu, C., Wong, F.S., Szot, G.L., Bluestone, J.A.*, et al.* (2008). Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature *455*, 1109-1113.

Wildin, R.S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J.L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L.*, et al.* (2001). X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet *27*, 18-20.

Wilson, N.S., Young, L.J., Kupresanin, F., Naik, S.H., Vremec, D., Heath, W.R., Akira, S., Shortman, K., Boyle, J., Maraskovsky, E.*, et al.* (2008). Normal proportion and expression of maturation markers in migratory dendritic cells in the absence of germs or Toll-like receptor signaling. Immunol Cell Biol *86*, 200-205.

Wostmann, B.S. (1996). Germfree and gnotobiotic animal models : background and applications (Boca Raton: CRC Press).

Wostmann, B.S., Larkin, C., Moriarty, A., and Bruckner-Kardoss, E. (1983). Dietary intake, energy metabolism, and excretory losses of adult male germfree

Wistar rats. Lab Anim Sci *33*, 46-50.

Wu, H.J., Ivanov, II, Darce, J., Hattori, K., Shima, T., Umesaki, Y., Littman, D.R., Benoist, C., and Mathis, D. (2010). Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity *32*, 815-827.

Wysocki, A.B. (1999). Skin anatomy, physiology, and pathophysiology. Nurs Clin North Am *34*, 777-797, v.

Zheng, Y., Danilenko, D.M., Valdez, P., Kasman, I., Eastham-Anderson, J., Wu, J., and Ouyang, W. (2007). Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature *445*, 648-651.

Zhu, J., and Paul, W.E. (2010). Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. Immunol Rev *238*, 247-262.

Zhu, W., London, N.R., Gibson, C.C., Davis, C.T., Tong, Z., Sorensen, L.K., Shi, D.S., Guo, J., Smith, M.C., Grossmann, A.H.*, et al.* (2012). Interleukin receptor activates a MYD88-ARNO-ARF6 cascade to disrupt vascular stability. Nature.

Zielinski, C.E., Mele, F., Aschenbrenner, D., Jarrossay, D., Ronchi, F., Gattorno, M., Monticelli, S., Lanzavecchia, A., and Sallusto, F. (2012). Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. Nature *484*, 514-518.