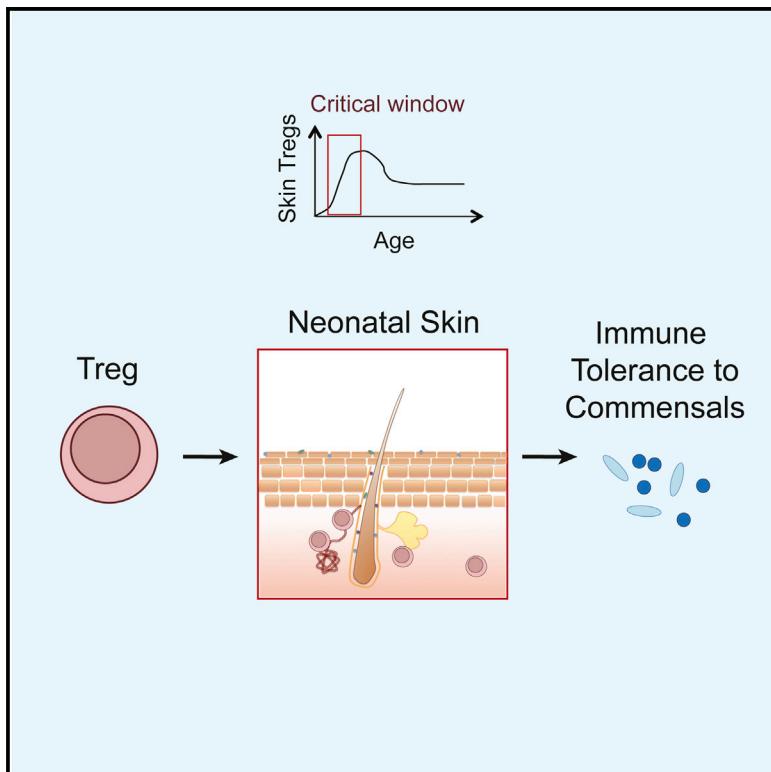


Immunity

A Wave of Regulatory T Cells into Neonatal Skin Mediates Tolerance to Commensal Microbes

Graphical Abstract



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In Brief

The mechanisms promoting immune tolerance to skin commensal bacteria are unknown. Using a model system to assay commensal-specific T cell responses, Rosenblum and colleagues demonstrate that an abrupt accumulation of regulatory T cells in neonatal skin mediates tolerance to skin commensal bacteria during this crucial developmental window.

Highlights

- Skin bacteria activate antigen-specific T cells across an intact skin barrier
- Tolerance to skin commensal bacteria is preferentially established in neonatal life
- A unique wave of activated regulatory T cells enters skin in this critical window
- Blocking entry of Treg cells into neonatal skin prevents tolerance to commensals

A Wave of Regulatory T Cells into Neonatal Skin Mediates Tolerance to Commensal Microbes

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SUMMARY

The skin is a site of constant dialog between the immune system and commensal bacteria. However, the molecular mechanisms that allow us to tolerate the presence of skin commensals without eliciting destructive inflammation are unknown. Using a model system to study the antigen-specific response to *S. epidermidis*, we demonstrated that skin colonization during a defined period of neonatal life was required for establishing immune tolerance to commensal microbes. This crucial window was characterized by an abrupt influx of highly activated regulatory T (Treg) cells into neonatal skin. Selective inhibition of this Treg cell wave completely abrogated tolerance. Thus, the host-commensal relationship in the skin relied on a unique Treg cell population that mediated tolerance to bacterial antigens during a defined developmental window. This suggests that the cutaneous microbiome composition in neonatal life is crucial in shaping adaptive immune responses to commensals, and disrupting these interactions might have enduring health implications.

INTRODUCTION

Our commensal microbiota reside primarily at barrier sites, such as the gastrointestinal tract, respiratory tract, urogenital tract, and skin, where they functionally tune our innate and adaptive immune systems (Belkaid and Hand, 2014; Belkaid and Segre, 2014; Hooper et al., 2012; Tremaroli and Bäckhed, 2012). Immune tolerance to these microbes must be established at each of these sites, but to date, the cellular and molecular mechanisms of how this occurs have almost exclusively been studied in the gastrointestinal tract. In this tissue, a simple columnar

epithelium is coated by a thick mucus layer that facilitates spatial segregation from luminal bacteria (Vaishnava et al., 2011) and also diminishes the immunogenicity of microbial antigens by delivering tolerogenic signals to resident dendritic cells (Shan et al., 2013). Innate lymphoid cells limit commensal-specific CD4⁺ T cell responses via a mechanism dependent on major histocompatibility complex (MHC) class II (Hepworth et al., 2013) and produce interleukin-22, which further promotes anatomical containment of microbes (Sonnenberg et al., 2012). Specialized gut-resident CD103⁺CD11b⁺ dendritic cells also play an important role in maintaining intestinal homeostasis by favoring induction of regulatory T (Treg) cells over pro-inflammatory CD4⁺ subsets (Coombes et al., 2007).

The cellular and molecular mechanisms that mediate tissue immune homeostasis differ between barrier sites (Maloy and Powrie, 2011; Pasparakis et al., 2014; Sather et al., 2007), and it follows that mechanisms to promote tolerance to commensals might also be tissue specific. The skin is a key barrier site and a rich immunologic organ, such that each square centimeter contains over a million lymphocytes and a million commensal bacteria (Clark et al., 2006; Grice et al., 2008). Unlike intestinal or lung mucosa, the skin is a stratified, cornified epithelium with a diverse topography studded by adenexal structures, including hair follicles, sweat ducts, and sebaceous glands. As our external body surface, the skin also sustains regular physical trauma that compromises its barrier integrity and facilitates interaction between immune cells and exogenous antigens. These unique properties pose discrete challenges for maintenance of a healthy immune dialog with commensal microbes. Currently, very little is known about how this process occurs in skin.

Treg cells play a major role in establishing and maintaining immune homeostasis in peripheral tissues, particularly at barrier sites (Powell et al., 1982; Russell et al., 1959), where they stably reside (Burzyn et al., 2013; Cipolletta et al., 2012; Sanchez Rodriguez et al., 2014). In the intestinal lamina propria, Treg cells not only maintain self-tolerance but also play a crucial role in mediating tolerance to commensal organisms. A large percentage of gut-resident Treg cells recognize commensal antigens (Lathrop et al., 2011b), and thymus-derived Treg (tTreg) cells support

tolerance to intestinal microbes (Cebula et al., 2013). In addition, certain bacterial species expand Treg cells in the lamina propria (Atarashi et al., 2011; Round and Mazmanian, 2010). Despite the clear role that Treg cells play in mediating tolerance to commensal microbes in the gut, it is currently unknown whether these cells play a role in establishing tolerance to commensal microbes at other barrier sites. Interestingly, commensal microbes do not appear to augment the numbers of Treg cells in the skin as they do in the gut or lungs (Naik et al., 2012).

In order to dissect the mechanisms by which adaptive immune tolerance to skin commensals is established, we engineered *Staphylococcus epidermidis* (*S. epidermidis*) to express a model T cell antigen, allowing us to comprehensively analyze commensal-specific CD4⁺ T cell responses in the context of both a polyclonal T cell repertoire and a complex microbiota. Using this system, we demonstrated that microbial antigens were continuously detected across an intact skin barrier. Skin colonization of adult animals did not establish tolerance. Instead, colonization during a defined period of neonatal life was required. Examination of neonatal skin revealed an abrupt wave of highly activated Treg cells accumulating in this tissue during the first weeks of life. Selective inhibition of Treg cell migration into skin during this period completely abrogated commensal-specific tolerance. Our results reveal that both a specific cell population and a specific window of time are required for establishing a healthy host-commensal relationship in skin.

RESULTS

Establishment of a Model System to Track the Antigen-Specific Immune Response to Skin Commensal Bacteria
 Tools for studying the antigen-specific response to commensal microbes residing in the gastrointestinal tract have led to significant advancements in our understanding of the host-commensal relationship at this barrier site (Hand et al., 2012; Yang et al., 2014). To date, similar tools have not been available for skin commensal bacteria, and therefore, work examining the immune response to skin microbes has primarily been limited to observations of bulk polyclonal immune cell populations. Immunologic tolerance is by definition an antigen-specific process. Thus, we developed a murine model of cutaneous commensalism in which T cells specific to a bacterial antigen could be assayed longitudinally in the context of both a complex microbiome and a polyclonal immune repertoire. *S. epidermidis* is a prevalent commensal bacterial species on human skin, and it can also stably colonize healthy mice (Garcia-Garcera et al., 2012; Grice et al., 2009; Naik et al., 2015). We engineered *S. epidermidis* (Augustin and Götz, 1990) to express the model peptide antigen 2W (Moon et al., 2007) linked to a fluorescent protein (Epi-2W) to allow for standardization of relative antigen expression (Figure S1). A single topical application of Epi-2W to the back skin of adult C57Bl/6 mice resulted in long-term persistence of the strain (Figure 1A). Flow-cytometric and histologic evaluation of Epi-2W-colonized skin failed to reveal evidence of tissue inflammation (Figures 1B and C), suggesting that a state of true commensalism was achieved.

To test whether commensal antigens were recognized across an intact skin barrier, we assayed the 2W-specific im-

mune response in mice colonized with Epi-2W. Adult animals were colonized with Epi-2W every 3 days for 1 week, and skin, skin-draining lymph nodes (SDLNs), and spleen were examined at day 10. In colonized animals, we observed a significant increase in the frequency and absolute number of activated (CD44⁺) antigen-specific (2W MHC class II tetramer⁺) CD4⁺ T cells in both SDLNs (Figures 1D and 1E) and spleen (Figures 1F and 1G). Taken together, these data suggest that we have established a model that closely recapitulates normal cutaneous bacterial commensalism, in that stable colonization occurred without resultant tissue inflammation. The results indicate that skin bacterial antigens were recognized by the adaptive immune system across an intact skin barrier. These findings are consistent with and build upon a recent report showing that skin commensal bacteria influence cutaneous immunity without causing tissue inflammation (Naik et al., 2015).

Bacterial Colonization during Adult Life Does Not Establish Tolerance

We hypothesized that the adaptive immune system plays a role in mediating tolerance to skin commensal bacteria. To test this, we colonized the skin of 6-week-old adult mice with Epi-2W. 3–4 weeks later, we challenged these mice alongside age-matched control animals (not colonized with Epi-2W) by applying Epi-2W in conjunction with lightly stripping the epidermis with tape to minimally abrade the skin surface (Figure S2A). We rationalized that this approach was the most physiologically appropriate method of elucidating anti-commensal immune responses because it recapitulated increased exposure to commensal antigens in the setting of frequent incidental skin trauma (e.g., abrasions and scratching), a mildly inflammatory context during which mechanisms of immune tolerance would need to be active.

Pre-colonization with Epi-2W did not attenuate skin inflammation upon challenge. 10 days after challenge was initiated, both pre-colonized and control groups had equivalently increased histologic evidence of skin inflammation (Figure 2A) and equivalent numbers and frequency of skin neutrophils (Figure 2B). Consistent with the above observations, pre-colonization did not alter the number of activated antigen-specific CD44⁺CD4⁺Foxp3⁻ effector T (Teff) cells in SDLNs (Figure 2C), nor did it preferentially enrich antigen-specific Treg cells in either the SDLNs or the skin (Figures 2D, 2E, and S2B). These results suggest that initial colonization by a skin commensal during adult life is not sufficient to establish tolerance to commensal antigens.

Neonatal Colonization Is Required for Establishing Tolerance to Skin Commensal Bacteria

Because the host-commensal relationship is formed immediately after birth (Dominguez-Bello et al., 2010; Rotimi and Duerden, 1981), we hypothesized that mechanisms required for establishing tolerance might be preferentially active during this period of time. To test this, we employed the previously outlined experimental approach, but instead we colonized mice early in the postnatal period. Neonatal mice were colonized with Epi-2W for 1 week beginning on postnatal day 7 and challenged (with the skin-abrasion technique previously described)

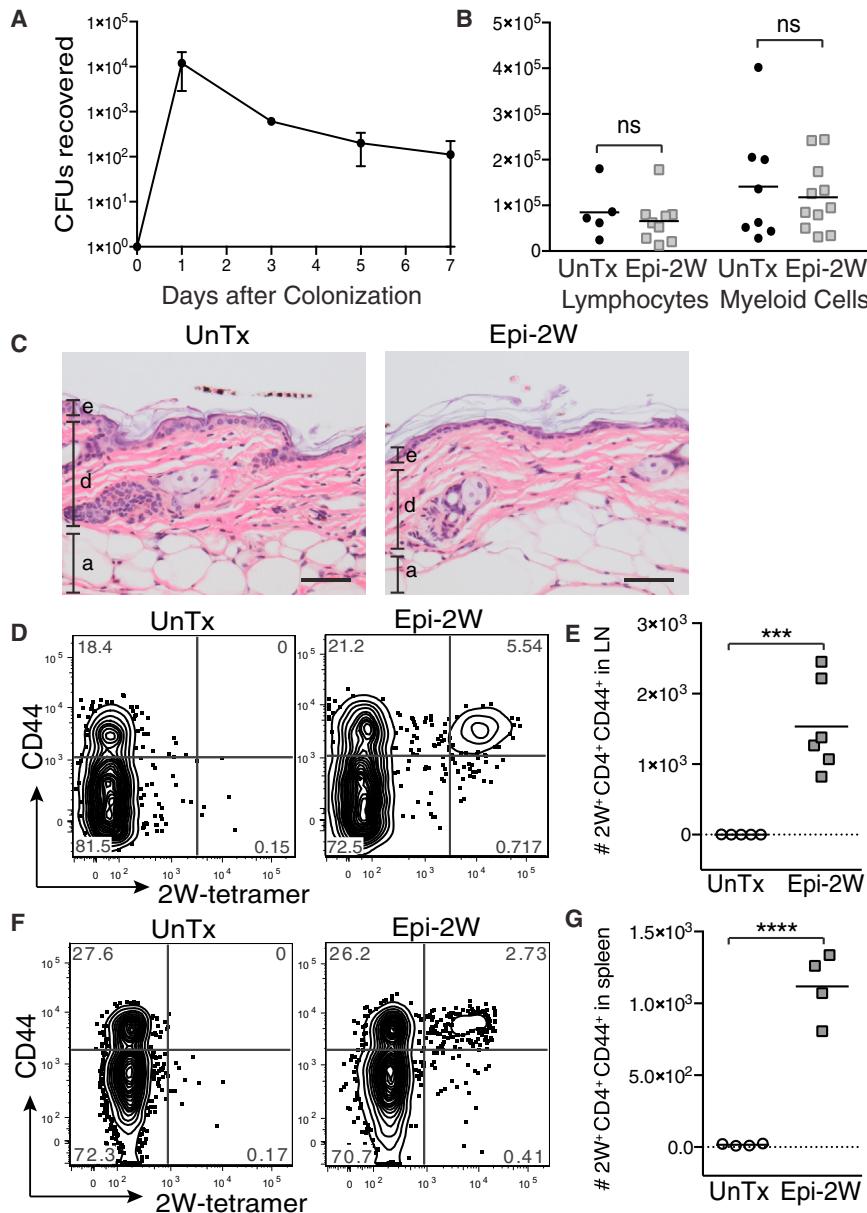


Figure 1. Antigen-Specific Recognition of Commensal Microbes across an Intact Skin Barrier

(A) Number of Epi-2W colony-forming units (CFUs) recovered via skin swab from mice colonized with Epi-2W once on day 0. Each data point represents an average of three mice. Adult mice were colonized with Epi-2W or left untreated (UnTx) every 3 days for three applications, and then skin, skin-draining lymph nodes (SDLNs), and spleen were harvested on day 10.

(B) Absolute number of lymphocytes (live CD45⁺CD3⁺) and myeloid cells (live CD45⁺CD3⁻) per gram of skin.

(C) Skin histology. Scale bars represent 50 μ m. Abbreviations are as follows: e, epidermis; d, dermis; a, adipose.

(D–G) Flow-cytometry plots of CD4⁺ T cells (gated on live CD45⁺CD3⁺CD4⁺ from a tetramer-enriched fraction) in SLDNs (D) and spleen (F) and absolute numbers of CD44⁺CD4⁺2W⁺ cells in SLDNs (E) and spleen (G) on day 10.

Data represent three (B–E) or two (A, F, and G) independent experiments. See also Figure S1.

these results suggest that skin colonization in the neonatal period uniquely promotes tolerance to commensal bacteria.

Neonatal Skin Is Characterized by Abrupt Accumulation of Activated Treg Cells

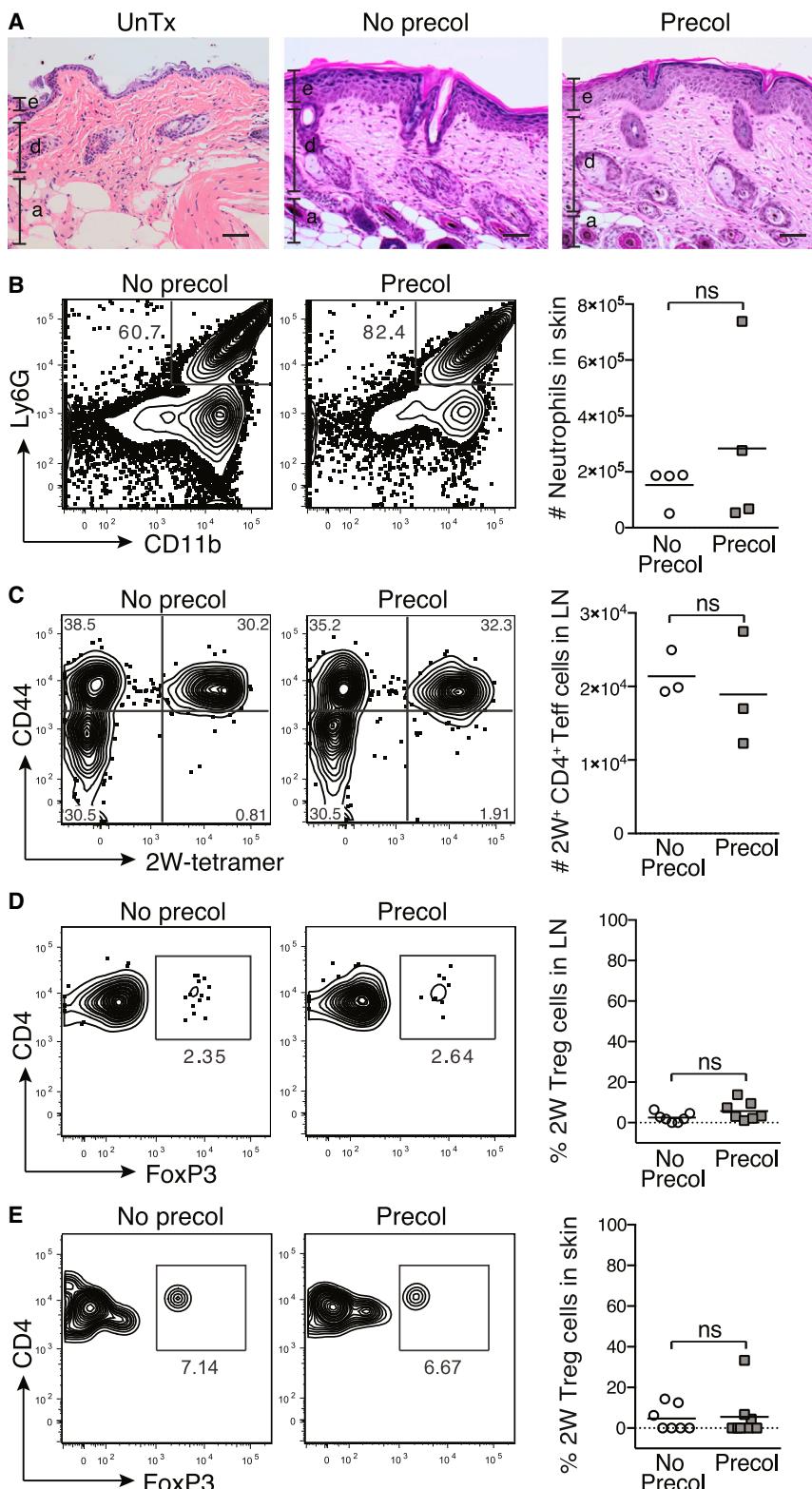
The observation that colonization of neonatal but not adult skin results in commensal-specific T cell tolerance prompted us to explore how neonatal and adult skin differ with respect to the major immune cell populations found in this tissue during these periods of life. Consistent with what is known about postnatal thymic development in mice (Adkins et al., 2004), we observed a marked accumulation of T cells express-

alongside Epi-2W-naive age-matched animals 3–4 weeks later in adult life. In contrast to our observations with adult animals, mice colonized with Epi-2W in the neonatal period demonstrated markedly diminished histologic skin inflammation and substantially reduced neutrophilic infiltration upon challenge with Epi-2W (Figures 3A and 3B). This was associated with significantly fewer activated 2W-specific CD44⁺CD4⁺ Teff cells in the SLDNs (Figure 3C) and a marked enrichment of 2W-specific Foxp3⁺ Treg cells within the antigen-specific CD4⁺ population in the SLDNs (Figures 3D and S2C). Enrichment of antigen-specific Treg cells was also observed in skin (Figure 3E). Notably, bacterial burden of Epi-2W after initial colonization was similar between neonatal and adult mice, suggesting that the distinct effects of Epi-2W exposure in these two windows was not due to differential antigen load or preferential colonization in these different periods of life (Figure S2D). Collectively,

ing the $\alpha\beta$ T cell receptor in skin between postnatal days 6 and 13 (Figures 4A and 4B).

CD4⁺ Treg cells constituted the vast majority of $\alpha\beta$ T cells accumulating in skin during this period (Figures 4C and S3A). By day 13, Treg cells accounted for >80% of CD4⁺ T cells in skin (whereas Treg cells constituted only ~50% in adult skin; Figure 4D), and their density was more than double that observed in adults (Figure S3A). In contrast, other $\alpha\beta$ and $\gamma\delta$ T cell subsets did not significantly accumulate in skin during this window (Figures S3B–S3D). Treg cells infiltrating neonatal skin were highly activated, as evidenced by higher expression of CTLA-4 and ICOS in these cells than in Treg cells in adult skin (Figures 4E and 4F).

To determine whether this wave of activated Treg cells was unique to the skin, we examined Treg cells in the intestinal lamina propria and SLDNs of 13-day-old neonates. In both these sites,



Treg cells constituted fewer than 20% of CD4⁺ T cells at day 13 and were not more abundant in neonatal tissues than in adult tissues (Figures 4G and S3E). Moreover, neonatal Treg cells isolated from the lamina propria and SDLNs expressed significantly

Figure 2. Colonization with Commensal Bacteria in Adult Mice Does Not Establish Immune Tolerance

Adult mice were not colonized (No precol) or colonized with Epi-2W (Precol) every 3 days for 1 week and then challenged 3–4 weeks later with Epi-2W and superficial skin abrasion.

(A) Representative histology of skin 10 days after challenge and healthy age-matched skin. Scale bars represent 50 μ m. Abbreviations are as follows: e, epidermis; d, dermis; a, adipose.

(B) Flow cytometry and absolute numbers of skin neutrophils. Plots are gated on a live CD45⁺CD3⁻ population.

(C) Flow cytometry and absolute numbers of CD44⁺CD4⁺2W⁺FoxP3⁻ cells in SDLN. Plots are gated on a live D⁻CD45⁺CD3⁺CD4⁺FoxP3⁻ population in a tetramer-enriched fraction.

(D and E) Flow cytometry and percentage of 2W-specific Treg cells in SDLN (D) and skin (E). Plots are gated on a live D⁻CD45⁺CD3⁺CD4⁺CD44⁺2W⁺ population in a tetramer-enriched fraction for SDLN and a total unenriched fraction for skin. Each point represents pooled data from two mice.

Data represent three independent experiments with at least six mice per group. See also Figure S2.

lower levels of CTLA-4 and ICOS than did Treg cells infiltrating the skin (Figures 4H and 4I), indicating that they were less activated than skin Treg cells. Thus, the influx of highly activated Treg cells seems to be unique to the skin during this postnatal time period.

Neonatal Treg Cells Are Required for Establishing Tolerance to Skin Commensal Microbes

The abrupt accumulation of Treg cells in neonatal skin suggested seeding of the tissue by a migratory wave from developing lymphoid organs. To test this, we treated neonatal mice with the sphingosine-1-phosphate receptor antagonist FTY720 between postnatal days 5 and 11 to block the egress of lymphocytes from the thymus and SDLN (Matloubian et al., 2004). On postnatal day 13, FTY720-treated neonates had substantially reduced percentages of Treg cells in skin; these percentages were similar to those observed in untreated mice at postnatal day 6 (Figures 5A and 5B). Absolute numbers of skin Treg cells were

also markedly reduced in FTY720-treated mice at day 13, whereas CD4⁺FoxP3⁻ Teff cells were not significantly changed, consistent with our observation that Treg cells make up >80% of the CD4⁺ T cells accumulating in skin in this window (Figure 5C).

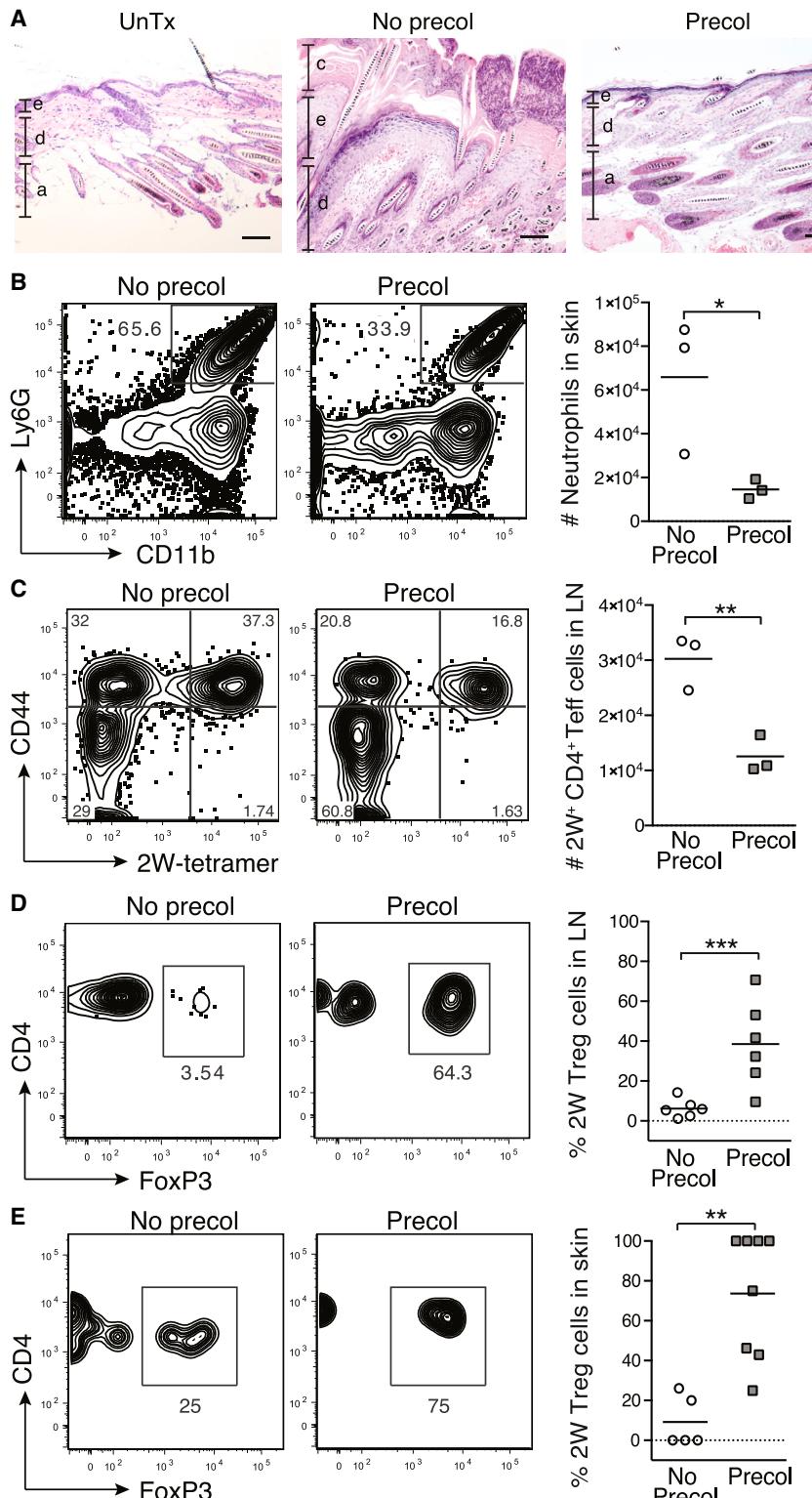


Figure 3. Colonization of Neonatal Mice with Commensal Bacteria Results in Antigen-Specific Immune Tolerance

Neonatal mice were not colonized (No Precol) or colonized with Epi-2W (Precol) on postnatal day 7 and then challenged 3–4 weeks later with Epi-2W and superficial skin abrasion.

(A) Representative histology of skin 10 days after challenge and untreated age-matched skin. Scale bars represent 50 μ m. Abbreviations are as follows: e, epidermis; d, dermis; a, adipose; c, crust.

(B) Flow cytometry and numbers of skin neutrophils. Plots are gated on a live CD45⁺CD3⁻ population.

(C) Flow cytometry and absolute numbers of CD44⁺CD4⁺2W⁺Foxp3⁻ cells in SDLNs. Plots are gated on a live D⁻CD45⁺CD3⁺CD4⁺Foxp3⁻ population in a tetramer-enriched fraction.

(D) Flow cytometry and percentage of 2W-specific Treg cells in SDLNs (D) and skin (E). Plots are gated on a live D⁻CD45⁺CD3⁺CD4⁺CD44⁺2W⁺ population in a tetramer-enriched fraction for SDLNs and a total unenriched fraction for skin. Each point represents pooled data from two mice.

Data represent three independent experiments with at least six mice per group. See also Figure S2.

populations in the skin at postnatal day 13 (Figures 5E–H). These data are consistent with our prior observation that these populations did not significantly accumulate in skin between postnatal days 6 and 13 and demonstrate that FTY720 treatment in this window preferentially blocked migration of Treg cells into skin while leaving other T cell populations relatively unchanged.

The abrupt accumulation of activated Treg cells in neonatal skin in conjunction with the preferential ability to establish tolerance to Epi-2W in this time period suggested that this population might play a major role in mediating tolerance to skin commensal microbes. To test this, we transiently blocked migration of Treg cells into skin by treating mice with FTY720 on postnatal days 5 and 7, immediately before colonization with Epi-2W (Figure S4A). These mice were compared to age-matched controls colonized with Epi-2W but not treated with FTY720. Both groups

FTY720 treatment produced preferential accumulation of Treg cells in the thymus rather than in SDLNs, suggesting that these cells migrate to the tissue directly from the thymus (Figure 5D). Notably, FTY720 treatment in this window did not significantly alter absolute numbers of other T cell or myeloid

were then challenged 3–4 weeks later with Epi-2W plus skin abrasion. Blocking migration of skin Treg cells into neonatal skin resulted in increased histologic evidence of skin inflammation upon challenge with Epi-2W in adult life (Figure 6A). Concomitantly, there were increased numbers of

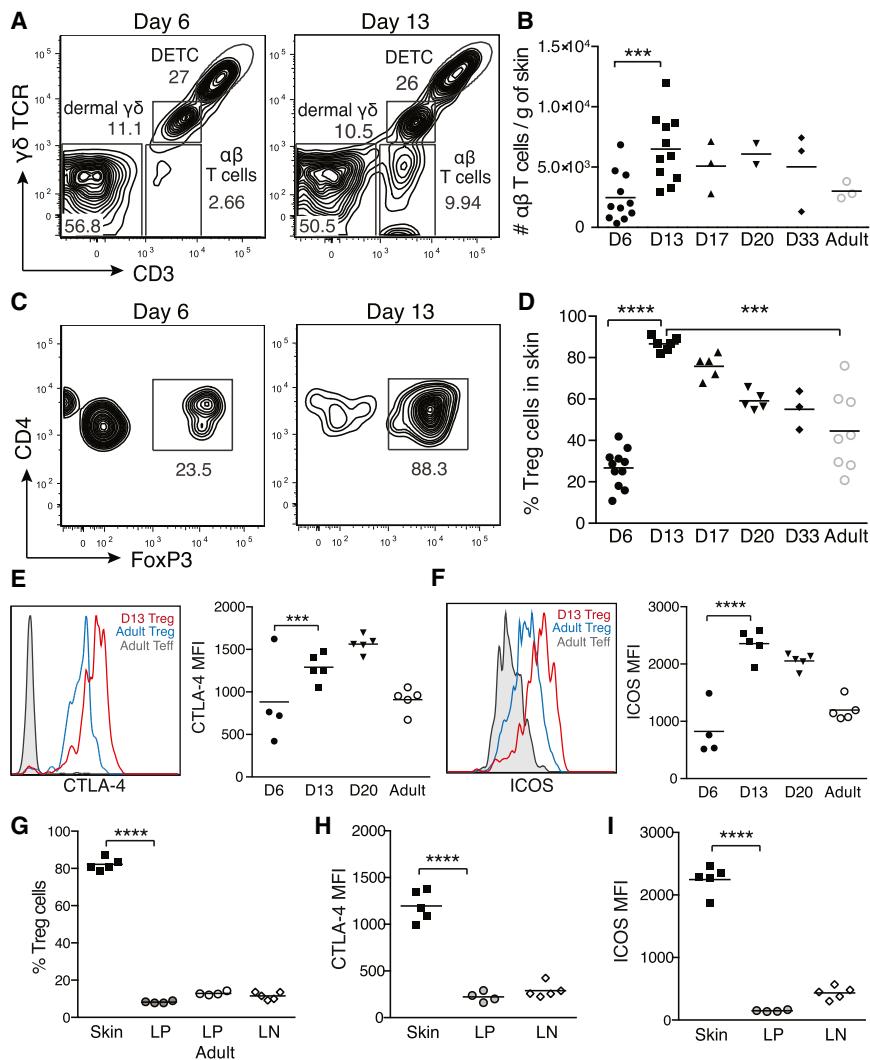


Figure 4. Activated Treg Cells Abruptly Accumulate in Neonatal Skin

(A) Representative flow-cytometry plots outline T cell subsets in murine skin at postnatal days 6 and 13. Plots are gated on live CD45⁺ cells.

(B) Absolute numbers of skin $\alpha\beta$ T cells by age.

(C) Flow cytometry of skin CD4⁺ cells at days 6 and 13.

(D) Percentage of Treg cells in skin by age.

(E and F) Expression of total CTLA-4 (E) and ICOS (F) by flow cytometry and mean fluorescent intensity (MFI) on skin Treg cells by age. Teff cells are Foxp3⁻ CD4⁺ T cells.

(G–I) Percentage of Treg cells (G), MFI of CTLA-4 (H), and MFI of ICOS (I) in day 13 Treg cells from skin, lamina propria (LP), and SDLNs. Each point represents data from an individual mouse.

Data represent three (A–D) or two (E–I) independent experiments with at least three mice per group. See also Figure S3.

DISCUSSION

We generated a system to study how adaptive immune tolerance to commensal bacteria is established in skin. Using this system, we found that even across an intact skin barrier, commensal antigens were recognized both locally and systemically, as evidenced by the expansion of commensal-specific CD4⁺ T cells in both the SDLNs and spleen. Although colonization of adult animals did not induce immune tolerance, neonatal colonization led to antigen-specific tolerance characterized by enrichment of commensal-specific Treg cells in skin

and SDLNs, reduced commensal-specific CD4⁺ Teff cells, and diminished tissue inflammation. Examination of neonatal skin revealed an abrupt accumulation of activated Treg cells, which might preferentially migrate directly to skin from the thymus. Attenuating Treg cell accumulation in neonatal skin prevented the development of commensal-specific tolerance. Utilizing this model system to quantitatively assay commensal-specific T cell responses, we have elucidated a cellular mechanism by which the skin establishes tolerance to commensal microbes. In doing so, we have found that the timing of colonization is critical for promoting a healthy host-commensal relationship in this tissue.

Our findings illustrate important differences between the mechanisms that support tolerance to commensals in the skin and those that support tolerance in the intestine. Although Treg cells are a critical population at both barrier sites, the relative contributions of tTreg cells and peripherally derived Treg cells might be distinct. Both subsets play a role in promoting tolerance to gut commensals (Cebula et al., 2013; Lathrop et al., 2011a). Our ability to prevent commensal-specific tolerance by blocking Treg cell migration to skin (with a concomitant accumulation of

antigen-specific CD4⁺ Teff cells in the SDLNs (Figure 6B) and a relative reduction of antigen-specific Treg cells in both the SDLNs (Figure 6C) and skin (Figure 6D). Importantly, Treg cells not specific to the 2W antigen (i.e., polyclonal Treg cells) were able to migrate into the skin and SDLNs in the weeks following brief treatment with FTY720 (Figures S4B–S4D). This indicates that FTY720 treatment did not permanently impair the migration ability of Treg cells and that the reduced percentages of antigen-specific Treg cells seen in FTY720-treated animals were a consequence of exposure to antigen during this neonatal period of time. Consistent with these results, in specific-pathogen-free (SPF) mice treated neonatally with FTY720, without addition of Epi-2W, we observed increased frequency of myeloid cells in adult skin (data not shown). This indicates that our observations with the 2W antigen also extended to antigens expressed by native commensal microbes. Collectively, these findings suggest that transiently blocking Treg cell entry into neonatal skin significantly reduced the commensal-specific Treg:Teff cell ratio and thus resulted in a failure to establish and/or maintain tolerance to these microbes.

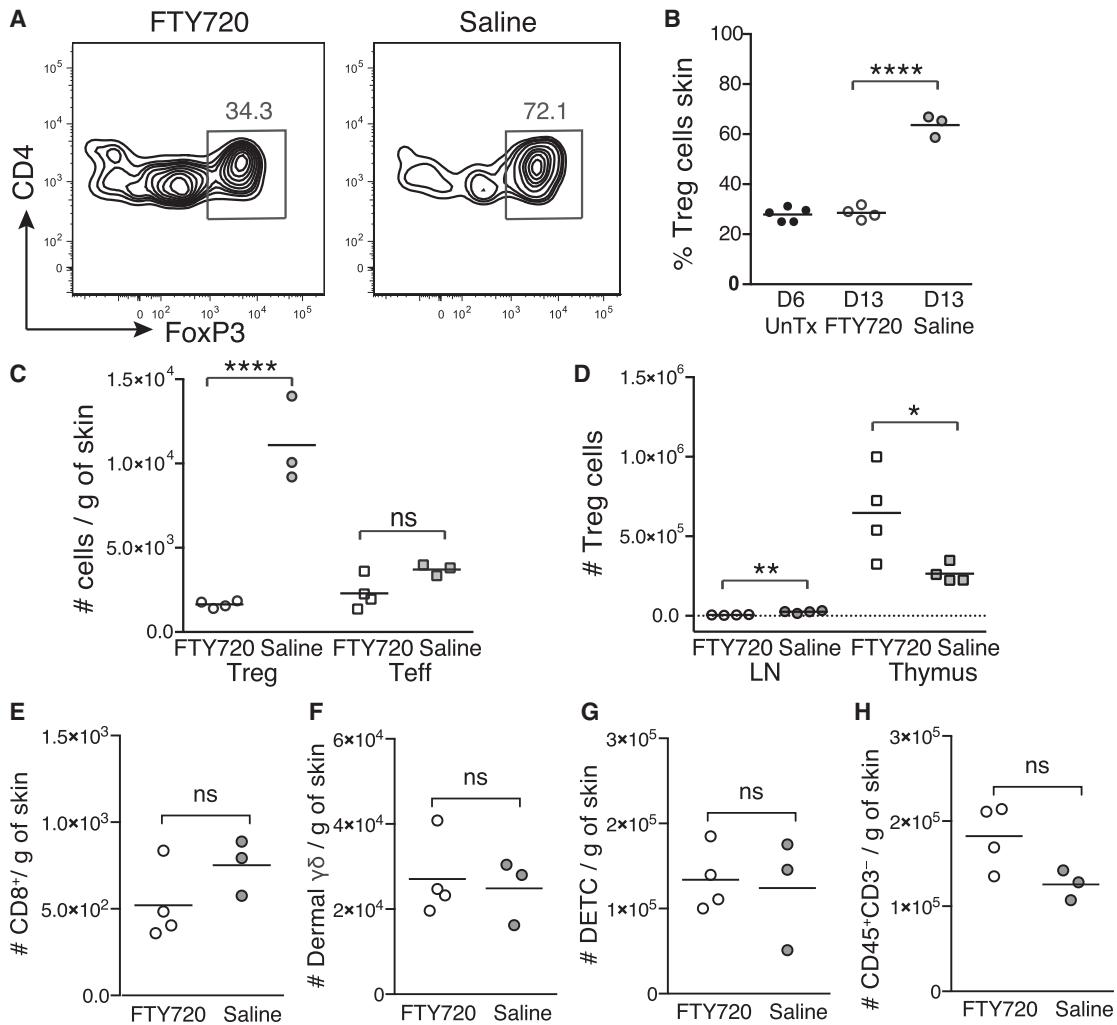


Figure 5. FTY720 Treatment Preferentially Blocks Migration of Treg Cells into Neonatal Skin

FTY720 or saline was administered every 48 hr between postnatal days 5 and 11, and skin, thymus, and SDLNs were harvested on day 13.

(A and B) Flow cytometry (A) and percentage of Treg cells in day 13 (D13) skin or untreated day 6 (D6) neonates (B). Plots are gated on live CD45⁺CD3⁺CD4⁺ cells.

(C) Absolute numbers of Treg cells and CD4⁺Foxp3⁻ (Teff) cells in skin on day 13.

(D) Absolute numbers of Treg cells in the thymus and SDLNs on day 13.

(E–H) Absolute numbers of CD8⁺ T cells (E), dermal γδ T cells (F), dendritic epidermal T cells (DETC) (G), and CD45⁺CD3⁻ myeloid cells (H) in skin on D13. Data represent two independent experiments with at least three mice per group.

Treg cells in the thymus) suggests that the skin might rely primarily on a thymus-derived population during this neonatal window; however, the origin of these cells remains to be definitively determined. Regardless of their ontogeny, our findings highlight that Treg cell accumulation appears unique to skin in this neonatal window and plays an essential role in establishing tolerance to commensals. The specific molecular mechanisms by which skin and intestinal Treg cells mediate tolerance to commensals might also be a point of divergence for these two barrier sites. Whereas Treg cell production of interleukin-10 is critical for preventing colitis provoked by enteric antigens (Kühn et al., 1993), and interleukin-10 is a key immunoregulatory cytokine produced by Treg cells generated in response to *B. fragilis* (Round and Mazmanian, 2010), interleukin-10 deficiency has minimal impact on skin immune homeostasis (Rubtsov et al., 2008). The molec-

ular mechanisms that skin Treg cells use to promote tolerance to either self- or commensal antigens remain poorly defined and are an area of active investigation.

We observed a distinct wave of Treg cells entering neonatal skin. We did not observe similar enrichment of highly activated Treg cells in the neonatal intestine. Although a transient microbiota-dependent increase in neonatal lung Treg cells was recently reported to be protective in an allergy model, these constitute no more than 15% of lung CD4⁺ T cells at their peak on postnatal day 8 and have not been shown to mediate tolerance to commensals (Gollwitzer et al., 2014). This suggests that the abrupt accumulation of Treg cells in neonatal skin by day 13 is tissue specific and not a consequence of a global or systemic increase in thymic efflux. Postnatal hair-follicle morphogenesis occurs during the same time frame as this

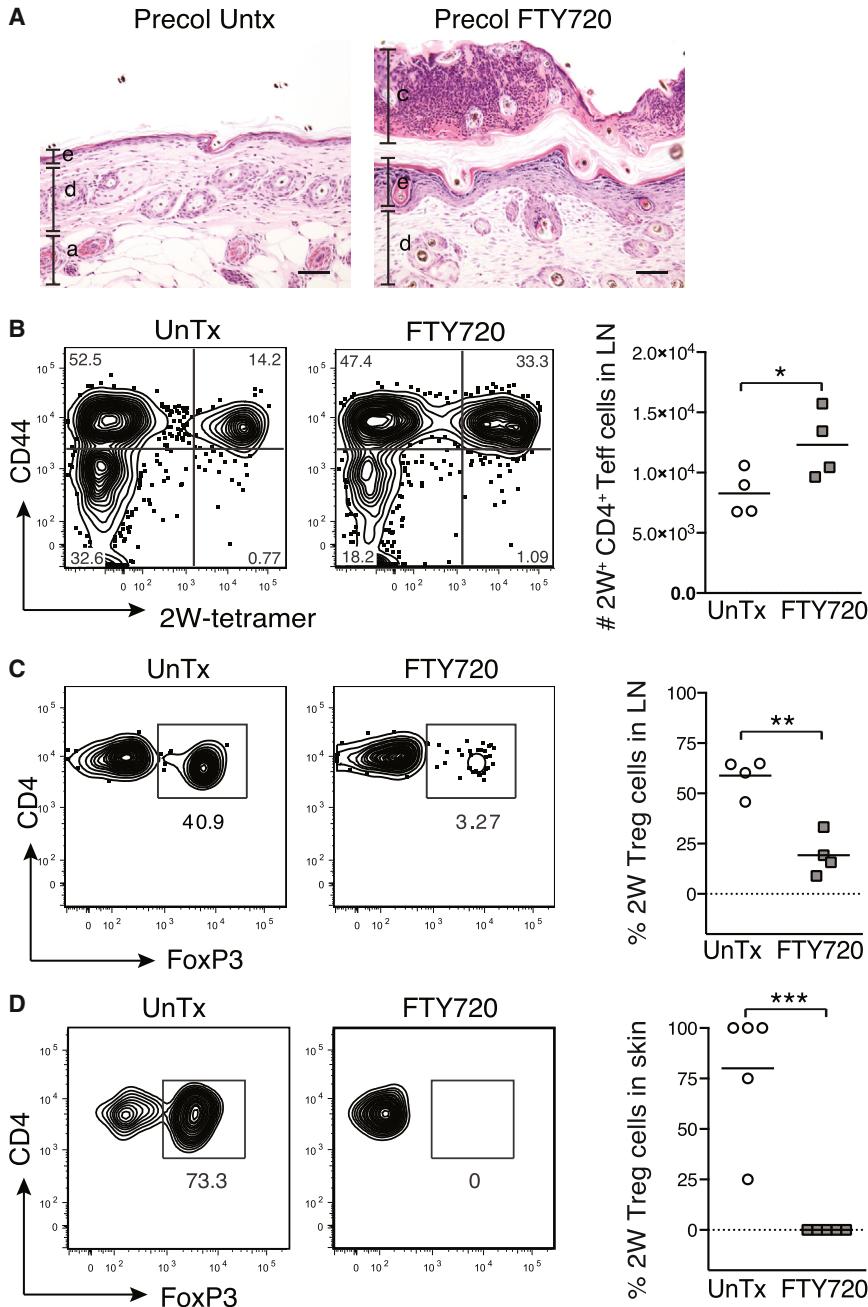


Figure 6. Inhibiting Treg Cell Migration to the Skin in Neonatal Life Prevents Tolerance to Commensal Bacteria

Neonatal mice were colonized with Epi-2W for 1 week beginning on day 7, and FTY720 or saline (UnTx) was administered on postnatal days 5 and 7. Mice were then challenged with Epi-2W and superficial skin abrasion 3–4 weeks later.

(A) Representative histology of skin 10 days after challenge. Scale bars represent 50 μ m. Abbreviations are as follows: e, epidermis; d, dermis; a, adipose; c, crust.

(B) Flow cytometry and absolute numbers of CD44 $^+$ CD4 $^+$ 2W $^+$ Foxp3 $^-$ cells in SDLNs. Plots are gated on a live D lo CD45 $^+$ CD3 $^+$ CD4 $^+$ Foxp3 $^-$ population in a tetramer-enriched fraction.

(C and D) Flow cytometry and percentage of 2W-specific Treg cells in SDLNs (C) and skin (D). Plots are gated on a live D lo CD45 $^+$ CD3 $^+$ CD4 $^+$ CD44 $^+$ 2W $^+$ population in a tetramer-enriched fraction for SDLNs and a total unenriched fraction for skin. Each point represents data pooled from two mice.

Data represent three independent experiments with at least six mice per group. See also Figure S4.

whether they are the same for human skin remains to be determined.

The observation that a defined period of colonization is required for promoting antigen-specific tolerance to skin commensals suggests that timing might be critical for inducing tolerance at other barrier sites. Neonatal life also plays a formative role in shaping the host-commensal relationship in the intestine, given that altering the intestinal flora during this window can permanently influence host metabolism (Cox et al., 2014), as well as the function of a subset of intestinal natural killer T cells (Cox et al., 2014; Gollwitzer et al., 2014; Olszak et al., 2012). Unique features of the neonatal immune system, specifically a CD71 $^+$ erythroid population, have been shown to broadly dampen defense to bacterial infections in this period (Elahi et al., 2013). However,

wave of Treg cells migrates into neonatal skin (Paus et al., 1999). Given that Treg cells in both mouse and human skin preferentially localize to hair follicles (Gratz et al., 2013; Sanchez Rodriguez et al., 2014), it is interesting to speculate that a hair-follicle-related chemokine is directing them into the tissue, as has been shown for Langerhans cells (Nagao et al., 2012). Given that a significant proportion of skin commensal bacteria reside in hair follicles (Lange-Asschenfeldt et al., 2011; Leeming et al., 1984), such a mechanism would have clear evolutionary advantages for establishing and maintaining host-commensal tolerance. The molecular mechanisms that drive Treg cell accumulation in neonatal skin are not yet understood, and

studies examining mechanisms of immune tolerance to gut microbes have not considered the role of timing in exposure to commensal antigens. Scurvy mice succumb to disease early in life and display a pronounced skin phenotype, highlighting a critical role for tissue Treg cells in this developmental window (Lyon et al., 1990). Recent work has suggested that Treg cells generated in neonatal life might be endowed with a unique potential to promote self-tolerance (Yang et al., 2015). Our data suggest that this principle might extend more broadly to include tolerance to commensal antigens. Although the phenomenon of a wave of Treg cells into neonatal tissue appears to be unique to skin, the functional characteristics and relative contribution of neonatal

Treg cells in establishing tolerance to commensals should be carefully examined in other tissues. Further work is also required for clarifying whether tolerance to commensals established in this neonatal window persists indefinitely throughout life or whether distinct mechanisms are required for its maintenance.

To date, research examining the role of commensal microbes in human health has focused primarily on the intestinal tract. However, alterations in the epidermal protein filaggrin confer risk not only for atopic dermatitis but also for asthma (Rodríguez et al., 2009), indicating that skin-barrier function influences more than local immunity. We have shown that skin colonization results in commensal-specific T cells that are found both locally and systemically, suggesting that maintaining a healthy microbe-host immune dialog in skin might have implications for systemic and tissue-specific immune homeostasis. Recognizing that there is a defined developmental window for establishing tolerance to skin commensal bacteria provides mechanistic insights into how limiting microbial exposures early in life can contribute to allergic disease via a skin-specific mechanism (Ege et al., 2011). In this context, altering the composition of skin commensal microbiota in the neonatal period might limit the opportunity to establish tolerance to a wide array of microbial antigens, possibly resulting in chronic tissue inflammation. Indeed, many chronic inflammatory diseases of the skin have been postulated to result, at least in part, from abnormal anti-commensal immune responses (Belkaid and Segre, 2014; Nazary et al., 2011; Sanford and Gallo, 2013). Thus, the composition of the cutaneous microbiome in neonatal life could have formative effects on the adaptive immune response to commensals, and disrupting this could have enduring health implications.

EXPERIMENTAL PROCEDURES

Mice

Wild-type C57BL/6 mice were used for all experiments. Mice were originally purchased from Jackson Laboratories, but all experimental mice were born, bred, and maintained in the University of California, San Francisco (UCSF) SPF facility in accordance with the guidelines of the Laboratory Animal Resource Center and Institutional Animal Care and Use Committee of UCSF. Animals in experimental groups were matched for gender (and age where appropriate).

Bacterial Strains and Culture Conditions

S. epidermidis strain Tu3298 (Allgaier et al., 1986; Augustin and Götz, 1990; provided by M.O.) and *Staphylococcus aureus* (*S. aureus*) strain RN4220 (Nair et al., 2011) were used in this study and grown in tryptic soy broth at 37°C.

Generation of Epi-2W

Plasmid pJL71 (Gauger et al., 2012) was modified to include the 2W peptide sequence optimized for expression in *Staphylococcus* (DNA2.0, Menlo Park). Specifically, primers TS083 (5'-GGATCCGAATTCTTAGGAGGATGATTATT TATGAAAGCATGGGGAGCTTAGCAATTGGGCAGTTGATTCAAGCTGGTTC AGGTTCAAGGTTCAAGGTGTGAGCAAGGGCGAGGAGGATAATATGG-3') and TS084 (5'-GGATCCGGCGCGCCTACTTGTACAGCTCGTCCATGCCGCTG TAGAATGTC-3') were used with the pJL71 template for the generation of a construct encoding 2W at the 5' end of *gpmCherry* (Gram⁺ adapted mCherry) via a 7-mer glycine-serine linker sequence. This 2W-gpmCherry construct was cloned into plasmid pJL71 between the EcoRI and Ascl restriction sites downstream of the *agr* promoter (in place of *gpmCherry* alone), creating the new plasmid, pJL71-2W-gpmcherry (GenBank: KP065813). The restriction-deficient *S. aureus* strain RN4220 was transformed with pJL71-2W-gpmcherry via electroporation. The plasmid was then recovered via maxi prep and used

for transforming *S. epidermidis* Tu3298, via selection with erythromycin, for the generation of Epi-2W. See also Figure S1.

Epi-2W Skin Colonization and Skin-Abrasions Model

Epi-2W was cultured for 48 hr at 250 rpm in the presence of erythromycin to achieve stationary phase growth that demonstrated consistent and peak mCherry expression by flow cytometry. Cells were washed and re-suspended in PBS, and 10⁸–10⁹ colony-forming units (CFUs) of Epi-2W were applied with a pipette and sterile PBS-soaked cotton-tipped swab to the back skin of mice. This procedure was repeated every 3 days for a total of three applications to constitute one round of colonization. When mice were in the active stage of hair growth, back hair was shortened with clippers 24 hr prior to colonization for facilitating application of bacteria. For replicating physiologic exposure to commensal skin bacteria in the context of skin abrasion, clippers and depilatory cream were first used to remove back hair. The upper layers of epidermis were then removed via repeated application and removal of adhesive tape (Shurtape HP-500), and 10⁸–10⁹ CFUs of Epi-2W were applied as above. This procedure was repeated every 3 days for a total of three times to constitute one round of challenge. Back skin and back SDLNs (axillary, brachial, and inguinal) were harvested 10 days after initiation of the challenge.

Neonatal Administration of FTY720

FTY720 (Selleck Chemicals) was dissolved in normal saline and administered to neonates via intraperitoneal injection at a dose of 10 mg/kg. For experiments depicted in Figure 5, FTY720 was administered every 48 hr on postnatal days 5–11. For experiments depicted in Figures 6 and S4, migration was blocked more transiently with FTY720 treatment only on postnatal days 5 and 7. Control mice (age-matched litters or littermates) were treated with equal volumes of normal saline according to the same schedule.

Tissue Processing and Histopathology

Isolation of cells from skin and intestinal lamina propria for flow cytometry was performed as previously reported (Broadhurst et al., 2012; Gratz et al., 2014) and is further described in the *Supplemental Experimental Procedures*. For histopathology, skin tissue was fixed in 10% formalin and embedded in paraffin, sectioned, and stained with H&E by the UCSF Mouse Pathology Core. Images were acquired on a Leica microscope with a DS-Ri1 camera and NIS-Elements software (Nikon).

Tetramer Staining and Enrichment

Phycoerythrin-conjugated MHC class II i-A^b 2W1S_{55–68} tetramer (provided by J.M.) and anti-PE conjugated magnetic beads (Miltenyi Biotec) for enrichment from lymph nodes and enumeration in tissues have been previously reported (Moon et al., 2007). The procedure was adapted for skin as described in the *Supplemental Experimental Procedures*.

Flow-Cytometry Staining

After isolation from tissues, cells were labeled with surface antibodies (see *Supplemental Experimental Procedures*) in PBS or, for experiments incorporating the 2W-tetramer, in blocking solution with anti-Fc_γR antibody (24G2 hybridoma supernatant), rat serum (StemCell Technologies), and normal mouse serum (Jackson ImmunoResearch). For intracellular staining, cells were fixed and permeabilized with the Foxp3 Staining Buffer Kit (BD Biosciences). Samples were run on a Fortessa (BD Biosciences) in the UCSF Flow Cytometry Core. For longitudinal experiments comparing mice across time or ages, voltages were standardized with SPHERO Rainbow Calibration Particles (BD Biosciences). AccuCheck Counting Beads (Invitrogen) were used for calculating absolute numbers of cells. Flow-cytometry data were analyzed with FlowJo software (FreeStar).

Statistics

The number of mice per group used in an experiment is annotated in the corresponding figure legend. Although no prior sample-size estimation was performed, we used as many mice per group as possible. Data followed a Gaussian distribution, and variation was similar between groups for each condition analyzed. Significance was assessed with the unpaired (separate groups of mice) Student's t test. In all figures, the mean value is visually depicted. p values correlate with significance symbols as follows: ns, p > 0.05;

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. Mice were allocated into experimental groups according to age and gender. Investigators remained unblinded to group assignments throughout the experiment. No animals were excluded from analysis. Statistical analysis was performed with GraphPad Prism software.

ACCESSION NUMBERS

The accession number for the complete pJL71-2W-gpmcherry plasmid sequence reported in this paper is GenBank: KP065813.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.10.016>.

AUTHOR CONTRIBUTIONS

T.C.S. designed the studies, performed the experiments, and analyzed the data. T.C.S. and M.D.R. wrote the manuscript. K.S.V. assisted in mouse experiments, mouse husbandry, and data collection and analysis. H.A.T., M.L.P., S.G., and A.N. assisted with mouse experiments. M.O. and J.L. assisted in generation of transgenic *S. epidermidis*. I.K.G. and J.J.M. assisted in study design. M.D.R. oversaw all study design and data analysis. M.A.F. and A.K.A. were involved in study design and data analysis. All authors discussed results and commented on the manuscript.

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