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Skin-Resident Murine Dendritic Cell Subsets Promote Distinct and Opposing Antigen-Specific T Helper Cell Responses

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SUMMARY

Skin-resident dendritic cells (DCs) are well positioned to encounter cutaneous pathogens and are required for the initiation of adaptive immune responses. There are at least three subsets of skin DC— Langerhans cells (LC), Langerin$^+$ dermal DCs (dDCs), and classic dDCs. Whether these subsets have distinct or redundant function in vivo is poorly understood. Using a *Candida albicans* skin infection model, we have shown that direct presentation of antigen by LC is necessary and sufficient for the generation of antigen-specific T helper-17 (Th17) cells but not for the generation of cytotoxic lymphocytes (CTLs). In contrast, Langerin$^+$ dDCs are required for the generation of antigen specific CTL and Th1 cells. Langerin$^+$ dDCs also inhibited the ability of LCs and classic DCs to promote Th17 cell responses. This work demonstrates that skin-resident DC subsets promote distinct and opposing antigen-specific responses.

INTRODUCTION

Dendritic cells (DCs) in the periphery are specialized tissue-resident antigen-presenting cells that acquire exogenous antigen via multiple routes (Banchereau et al., 2000). In response to pathogen products, they become activated and migrate to regional lymph nodes where they present processed antigen acquired in the periphery to CD4$^+$ T helper (Th) cells and CD8$^+$ cytotoxic T lymphocytes (CTLs), thereby initiating adaptive immune responses (Banchereau et al., 2000; Itano et al., 2003). Recently, it has been demonstrated that only certain DC subsets have the ability to cross-present exogenous antigen to CTLs (Bedoui et al., 2009; Dudziak et al., 2007; Henri et al., 2010; Hildner et al., 2008). In contrast, most DC subsets appear to be capable of presenting antigen to CD4$^+$ T cells, and the Th cell phenotype that develops is largely determined by the adjuvant to which the DC is exposed (Joffre et al., 2010; Manicassamy and Pulendran, 2009; Reis e Sousa, 2004).

In uninflamed skin, three subsets of resident DCs can be distinguished on the basis of anatomical location and cell surface expression of the proteins Langerin and CD103 (Kaplan, 2010). Langerhans cells (Langerin$^+$ CD103$^-$) reside in the epidermis where they form a self-renewing population that has an ontogeny distinct from other DCs (Chorro and Geissmann, 2010; Ginhoux and Merad, 2010). In the dermis, DC subsets can be segregated into the well-defined Langerin$^+$ CD103$^+$ dermal DC (dDC) subset and a more heterogeneous Langerin$^-$ CD103$^-$ dDC subset that can be further subdivided with additional markers (Henri et al., 2010).

Contact hypersensitivity (CHS) to epicutaneous application of haptens is the classic assay to evaluate adaptive cutaneous immune responses. Examining CHS responses in mice lacking DC subsets is a commonly employed technique to determine the functional significance of skin DC subsets but the results have been mixed (Kaplan, 2010). Murine Langerin-diptheria toxin receptor (MuLangerin-DTR) mice lack LCs and Langerin$^+$ dDCs shortly after administration of diphtheria toxin (DT). Langerin$^+$ dDCs repopulate the skin more quickly than LCs so that 7–14 days after DT administration Langerin$^+$ dDC are largely intact while LCs remain absent (Bursch et al., 2007). CHS is reduced in mice sensitized shortly after DT administration but returns to normal when sensitization is delayed so that only LCs are absent. This suggests that Langerin$^+$ dDCs but not LCs are required for CHS. However, other studies using the same system with low doses of hapten or chimeric mice concluded that LC and Langerin$^+$ dDC have redundant functions (Honda et al., 2010; Noordegraaf et al., 2010). In contrast, CHS responses to multiple haptens are increased in transgenic mice that use the human *langerin* promoter to express either attenuated *Diphtheria* toxin subunit A (huLangerin-DTA) or the primate Diptheria toxin receptor (huLangerin-DTR) to specifically ablate LCs constitutively or inducibly (Bobr et al., 2010; Igyarto et al., 2009; Kaplan et al., 2005). Finally, *Batf3*$^{-/-}$ mice that lack Langerin$^+$ dDCs but not LCs develop normal CHS responses (Edelson et al., 2010).

LC-like cells derived in vitro from bone marrow precursors can efficiently cross-present soluble antigen to CD8$^+$ CTLs (Klechevsky et al., 2008). Similarly, LCs isolated ex vivo from human and mouse skin can also cross-present antigen when cultured in vitro

(Klechevsky et al., 2008; Stoitzner et al., 2006). In contrast, Langerin$^+$ dDCs isolated ex vivo from lymph nodes of mice after herpes simplex skin infection or from mice engineered to express ovalbumin in the epidermis are the only DC subset capable of efficiently cross-presenting antigen (Bedoui et al., 2009; Henri et al., 2010). Unfortunately, studies examining cross-presentation in vivo have relied on muLangerin-DTR mice or delivery of antigen to DC with antibody-antigen conjugates that cannot distinguish Langerin$^+$ dDCs from LCs (Idoyaga et al., 2008; Stoitzner et al., 2006). Hence, it is unresolved which DC subset(s) cross-presents skin-derived antigen in vivo. In addition, the contribution made to CD4$^+$ Th cell differentiation by individual skin-resident DC subsets has not been explored. Thus, the basic question of whether skin resident DCs have unique or redundant functions in vivo remains unresolved.

A major obstacle that has hampered the detailed analysis of skin DC function in vivo is the absence of a model in which antigen can be delivered to the skin that employs a physiologic adjuvant, allows for the examination of antigen-specific responses, but does not bypass the epidermis. Antigen-specific responses are difficult to examine in CHS (Igyarto et al., 2009). In addition, haptens have poorly defined adjuvant properties and small differences in technique lead to variable results (Kaplan, 2010). Epicutaneous protein immunizations also have poorly defined adjuvants and variable results depending on the anatomic site of immunization (Wang et al., 2008). Finally, established skin infection models require either dermal injection of pathogens (e.g., *Leishmania*) (Ritter et al., 2004) or severe skin wounding (e.g., *Staphylococcus*) (Miller et al., 2006) or can infect and kill LCs (e.g., HSV) (Cunningham et al., 2010).

Candida albicans is a commensal organism of mucosal sites in humans and also a pathogen that infects the vagina, oropharynx, and skin. Opportunistic systemic infections in immunocompromised patients is associated with a high mortality (Gudlaugsson et al., 2003; Wisplinghoff et al., 2004). In humans, Th17 CD4$^+$ T cells are an important component of the anti-*Candida* immune response. *C. albicans*-specific memory T cells are largely Th17 cells (Acosta-Rodriguez et al., 2007). In addition, patients with hyper IgE syndrome and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome develop severe mucocutaneous candidiasis and have defects that inhibit Th17 cell responses (Kisand et al., 2010; Ma et al., 2008). Meanwhile in mice, the secretion of interleukin-17 (IL-17) and Th17 cells are essential for host defense against *Candida* (Conti et al., 2009; Kagami et al., 2010; Lin et al., 2009).

In this study, we have engineered recombinant *C. albicans* to express multiple well-defined T cell antigens and developed a model of *C. albicans* skin infection that does not bypass the epidermis. By combining these tools with mice lacking specific skin DC subsets and examining antigen-specific responses in vivo, we have been able to delineate nonredundant functions for skin-resident DC subsets.

RESULTS

Cutaneous Candidiasis Model

We adapted the techniques described by (Sohnle and Hahn (1989) and Gaspari et al. (1998) to generate a protocol to reliably infect the skin of mice with *C. albicans.* Back hair was removed by shaving and chemical depilation followed by disruption of the stratum corneum with sandpaper. The procedure was calibrated to remove ~50% of the stratum corneum while leaving the epidermis intact as assayed by hematoxylin stained sections (unpublished data). *C. albicans* blastoconidia of the standard laboratory strain SC5314 (henceforth termed Calb-WT) was then inoculated onto the site. Within 48 hr, there was clear evidence of infection on the basis of the presence of erosions, erythema, and adherent crust (unpublished data). Histologic evaluation of the skin at this time point revealed numerous foci of yeast adherent the outer epidermis with filamentous forms (indicated by arrowheads) invading through the epidermis and into the dermis (Figure 1A). Importantly, filaments were seen penetrating through intact epidermis. The lesions began to resolve by day +4 and neither yeast or filaments could be found by histologic examination of the skin by day +7 (unpublished data).

Wild-type (WT) mice and mice with a constitutive absence of LC (huLangerin-DTA, see Table 1) developed lesions with a similar appearance and histology. We compared the number of invasive *C. albicans* in each strain by culturing skin from infected mice that had been cleansed with povidone-iodine to remove noninvasive *C. albicans*. Both strains of mice harbored similar numbers of invasive *C. albicans* that peaked on day +2 after infection and became undetectable on day +7 (Figure 1B). Thus, superficial infection with *C. albicans* produced a transient infection that was not altered by the absence of LC.

To determine whether an adaptive immune response against *Candida* developed after infection and whether LCs participated, we infected WT, *Rag1*$^{-/-}$ and huLangerin-DTA mice. Specific delayed type hypersensitivity (DTH) responses in footpads challenged with heat killed *C. albicans* were examined 7 days later (Figure 1C). WT mice developed a robust DTH response that was significantly greater than *Rag1*$^{-/-}$ mice in which the response was barely detectable. Langerin-DTA mice, however, developed a greatly exaggerated DTH response that was two to three times greater than WT mice. Importantly, dermal injection of *C. albicans* that bypassed the epidermis did not produce exaggerated responses in huLangerin-DTA mice. Thus, a superficial skin infection with *C. albicans* generated a specific adaptive immune response that was exaggerated in the absence of LC. This was completely consistent with our prior work with CHS and demonstrated that this was an ideal system with which to compare the function of LC and dermal DC.

Generation of Recombinant *C. albicans*

We constructed a recombinant *C. albicans* strain by modifying an existing plasmid (pMG2120) (Selmecki et al., 2008), which encodes green fluorescent protein (GFP), the *ADH1ter* transcription termination sequence, and the *Nat1* selectable marker. We then fused the *Eno1* promoter and complete *Eno1* coding sequence, in frame, to the N-terminus of GFP. Next, we inserted synthetic oligonucleotides encoding the well-characterized immunodominant T cell epitopes, $OVA_{323-339}$, $OVA_{257-264}$, I-Eα_{50-66}, and 2W1S (Moon et al., 2007) in frame with the C terminus of GFP (Figure 2A) so that the *ADH1ter* transcription termination sequence and the *Nat1* selectable marker are 3′ to the T cell epitopes (Shen et al., 2005). The fusion construct was transformed into *C. albicans* wild-type strain SC5314.

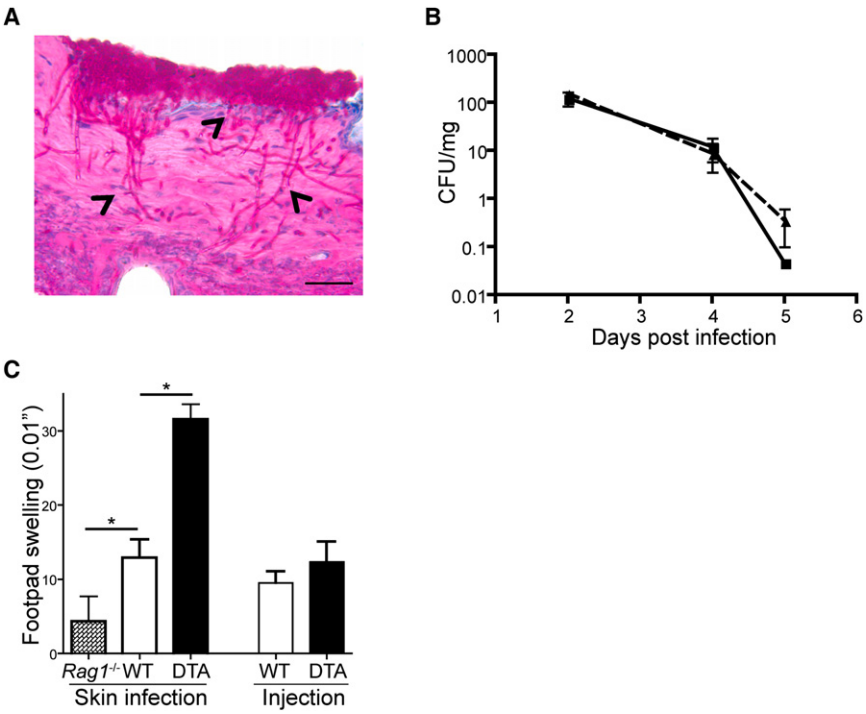

Figure 1. *C. albicans* Skin Infection Model

The skin of wild-type mice was infected with 2×10^8 blastoconidia of the SC5314 strain of *C. albicans*.

(A) Skin from infected mice harvested on day +2 after infection was sectioned and stained with PAS to highlight fungi. Numerous yeast forms are evident on the external surface of the skin. Filamentous forms (arrow heads) can be observed penetrating through the epidermis into the dermis. Representative images are shown. The scale bar represents 50μm.

(B) Skin samples from cohorts of WT (solid line, n = 8) and huLangerin-DTA (broken line, n = 8) mice were cleaned with provine-iodine prior to being harvested at the indicated time after infection. *C. albicans* CFU is expressed as colonies per mg of tissue.

(C) WT, *Rag1*$^{-/-}$, and huLangerin-DTA mice were infected on the skin or by intradermal injection of 10^7 blastoconidia. DTH responses on day +7 were measured by specific footpad swelling 24 hr after injection of 10^7 heat killed *C. albicans*. Data is representative of 3 independent experiments with cohorts of at least 6 mice per group. *p < 0.05.

A nourseothericin resistant clone, YJB11522 (Calb-Ag), was identified by GFP fluorescence (Figures 2B and 2C). Successful homologous recombination at the *Eno1* locus was verified by PCR (unpublished data).

To confirm successful expression and availability of the T cell epitopes, we cultured Calb-WT (shaded line) and Calb-Ag (solid line) with antigen presenting cells (APC) and carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled $CD8^+$ T cells from OT-I mice specific for $OVA_{257-264}$ and $CD4^+$ T cells from OT-II mice specific for $OVA_{323-339}$ (Figures 2D and 2E). The parental Calb-WT strain did not promote proliferation, whereas Calb-Ag induced robust OT-I and OT-II proliferation. Thus, the GFP-antigen fusion protein is highly expressed and available for processing and presentation to both $CD4^+$ and $CD8^+$ T cells.

Cross-presentation of Antigen In Vivo

To determine whether LC participate in cross-presentation of antigen to CTL in vivo, we adoptively transferred into WT and Langerin-DTA mice CFSE-labeled CD90.1 congenic, $CD44^{low}$ naive, OT-I cells isolated from OT-I TCR transgenic mice. The mice were then infected on their skin with either Calb-WT or Calb-Ag and the degree of proliferation was assessed in skin-draining lymph nodes 4 days later. As expected, OT-I did not proliferate in mice infected with Calb-WT (Neg) but did in mice infected with Calb-Ag (Figures 3A and 3B). There was, however, no difference in the degree of proliferation or total number of OT-I cells between WT and huLangerin-DTA mice. In addition, expression of granzyme B and IFN-γ, markers of CTL activation, were similar in both strains of mice (Figure S1). Thus, LCs are not required for cross-presentation of antigen in vivo.

Batf3$^{-/-}$ mice and muLangerin-DTR mice treated with DT lack Langerin$^+$ dDC (Table 1). As expected, OT-I proliferation in both lines was significantly reduced compared with WT mice, although a small amount of OT-I proliferation, ~2-fold greater than uninfected mice, was observed (Figures 3A and 3B). Although $CD8^+$ DCs are also absent from *Batf3*$^{-/-}$ and reduced in muLangerin-DTR mice, these cells did not acquire antigen in our skin-Infection model and are unlikely to participate in the response (unpublished data). Thus, Langerin$^+$ dDC are required for optimal cross-presentation in vivo, but another cell type can inefficiently cross-present cutaneous antigen in the absence of this DC subset.

Antigen Presentation by LC Is Necessary for Th17 Cell Development

To examine whether LC can present antigen to $CD4^+$ T cells, we adoptively transferred CFSE-labeled, CD90.1 congenic $CD4^+$ T cells isolated from TEα transgenic mice that had been maintained on a *Rag1*$^{-/-}$ background into WT and huLangerin-DTA mice. Mice were infected and the degree of proliferation was assessed. We consistently observed slightly more CFSE dilution and greater total numbers of TEα cells in WT mice (13-fold expansion) than huLangerin-DTA mice (8-fold expansion) (Figures 4A and 4B). Experiments using $CD4^+$ T cells isolated from OT-II mice produced similar results (unpublished data).

Table 1. Comparison of the Skin DC Subsets Ablated in DC-Deficient Mice

Mice	LCs	Langerin$^+$ dDCs	Langerin$^-$ dDCs
WT	+	+	+
huLangerin-DTA	–	+	+
Batf3$^{-/-}$	+	–	+
muLangerin-DTR	–	–	+

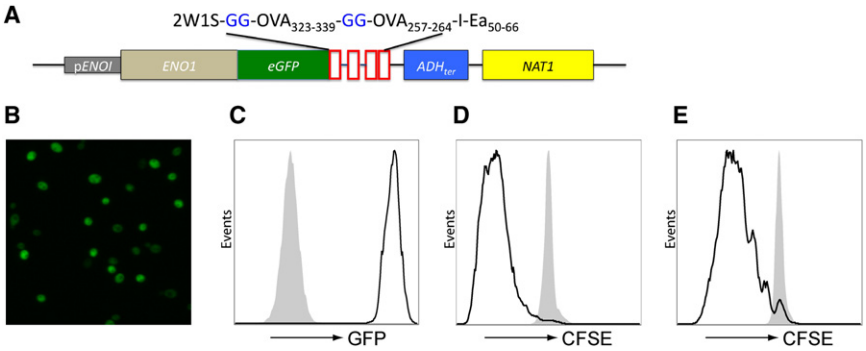

Figure 2. Generation of Recombinant *C. albicans*

In (A), EGFP and the model T cell antigens, 2W1S, $OVA_{323-339}$, $OVA_{257-264}$, and I-Eα_{50-66} were inserted in frame into the genome of *C. albicans* (Calb-Ag) (SC5314) at the C terminus of the enolase gene. Expression GFP was confirmed by (B) direct visualization fluorescence of Calb-Ag blastoconidia and by (C) flow cytometry comparing Calb-Ag (solid line) with Calb-WT (shaded). For confirming expression and availability of the T cell epitopes, Calb-WT (shaded) and Calb-Ag (solid line) were cultured with irradiated spleen cells and with CFSE-labeled CD90.1 congenic OT-I (D) or OT-II (E) cells. The amount of CFSE on CD90.1 gated cells T cells is shown after 3 days in culture. Data are representative of three independent experiments.

We next examined the cytokine profile of TEα cells that expanded during infection. Calb-Ag infection produced a mix of Th1 cells producing IFN-γ and Th17 cells producing IL-17A, IL-17F, and IL-22 in WT mice (Figures 4C and 4D). All Th17 cells coexpressed IL-22 but not IFN-γ (Figure S2). Surprisingly, the number of cells producing IL-17A, IL-17F, and IL-22 was dramatically reduced in the absence of LC (Figure 4D). In contrast, the number of cells producing IFN-γ was only slightly reduced in huLangerin-DTA mice and did not achieve statistical significance. We did not observe significant expression of IL-4, IL-10, IL-13, or Foxp3 (unpublished data). Thus, the absence of LC has a profound effect on the development of Th17 cells.

To determine whether direct presentation by LC is required for Th17 cell development, we performed similar experiments in huLangerin-Cre x I-Aβ-flox mice. Cre recombinase is constitutively expressed by LC in Langerin-Cre mice (Kaplan et al., 2007). Like the huLangerin-DTA mice, cells other than LCs are not affected. Thus, huLangerin-Cre on an I-Aβ-flox background have a constitutive and durable absence of major histocompatibility complex class II (MHC-II) limited to LCs (Igyarto et al., 2009). As was observed with huLangerin-DTA mice, the absence of MHC-II on LCs led to reduced proliferation of TEα cells and a near-complete absence of Th17 cells (Figure 5). Thus, direct presentation of antigen by LC is required for the development of a Th17 but not a Th1 cell response.

Antigen Presentation by LC Is Sufficient for Th17 Cell Development

In order to limit antigen presentation to LCs, we sought to target antigen to LCs by using Langerin antibodies that have been associated with antigen. Published efforts using this technique have used antibodies to mouse Langerin that target several DC subsets in addition to LCs (Idoyaga et al., 2008). To overcome this problem, we generated a monoclonal antibody (2G3) that is specific for the ectodomain of human Langerin and that does not cross react with murine Langerin (Figure S3). Thus, introduction of 2G3-antigen complexes into transgenic mice expressing huLangerin will selectively target antigen to LCs.

HuLangerin-DTR transgenic mice express human Langerin and the diphtheria toxin receptor exclusively on LCs (Bobr et al., 2010). HuLangerin-DTR mice that had not been treated with DT were injected intraperitoneally (i.p.) with anti-huLangerin (2G3) conjugated to Alexa-647. As expected, LCs isolated from the epidermis had efficiently acquired the antibody (Figure 6A). Keratinocytes, dendritic epidermal T cells (DETCs), and LCs from WT mice did not acquire the antibody. Similarly, in lymph node, 2G3 could only be detected in LCs from huLangerin-DTR mice and not in LCs from WT mice, Langerin$^+$ dDCs, or Langerin$^-$ dDCs (Figure 6C). All other cells, including B cells and macrophages, in both lymph node and spleen did not acquire 2G3 (unpublished data). Immunofluorescence of LC isolated from the epidermis showed that 2G3 (red) colocalized with the endogenous mouse Langerin (green) (Figure 6B). Thus, 2G3 efficiently binds to LCs in vivo and localizes to the same subcellular compartments as the endogenous muLangerin, suggesting that it should enter the endocytic processing pathway.

We generated recombinant 2G3 with the peptide sequences for $OVA_{257-264}$, I-Eα_{50-66} or 2W1S (for which an efficient MHC-II tetramer is available) fused to the C terminus (Moon et al., 2007). To test the ability of LC to cross-present antigen during infection with *C. albicans*, we injected OT-I adoptively transferred mice i.p. with 2G3-$OVA_{257-264}$. Mice were then infected with Calb-WT for LC activation. The only source of antigen was 2G3-$OVA_{257-264}$. OT-I did not proliferate in WT or huLangerin-DTR mice (Figure 6D). However, OT-I did proliferate in response to subcutaneous (s.c.) immunization with 2G3-$OVA_{257-264}$ in complete Freund's adjuvant (CFA), thereby demonstrating that $OVA_{257-264}$ was present. In contrast to OT-I, analogous experiments with TEα cells and 2G3-I-Eα_{50-66} showed a robust CD4$^+$ T cell expansion in *C. albicans*-infected mice (Figure 6e). Activation of LCs with either *C. albicans* or the common skin pathogen, *Staphylococcus aureus*, resulted in expression of IL-17A (Figure 6f). We also observed that endogenous 2W1S-specific CD4$^+$ T cells expanded and expressed IL-17A in *C. albicans*-infected huLangerin-DTR mice injected with 2G3-2W1S (Figure S4). Thus, antigen presentation by LC during skin infection with *C. albicans* or *S. aureus* is sufficient for driving proliferation of CD4 T cells and differentiation of Th17 cells but not for cross-priming of CTL.

Langerin$^+$ dDCs Promote Th1 and Inhibit Th17 Cell Differentiation

IL-1β, IL-6, and transforming growth factor β (TGF-β) participate in the initial development of Th17 cells, and IL-23 maintains their

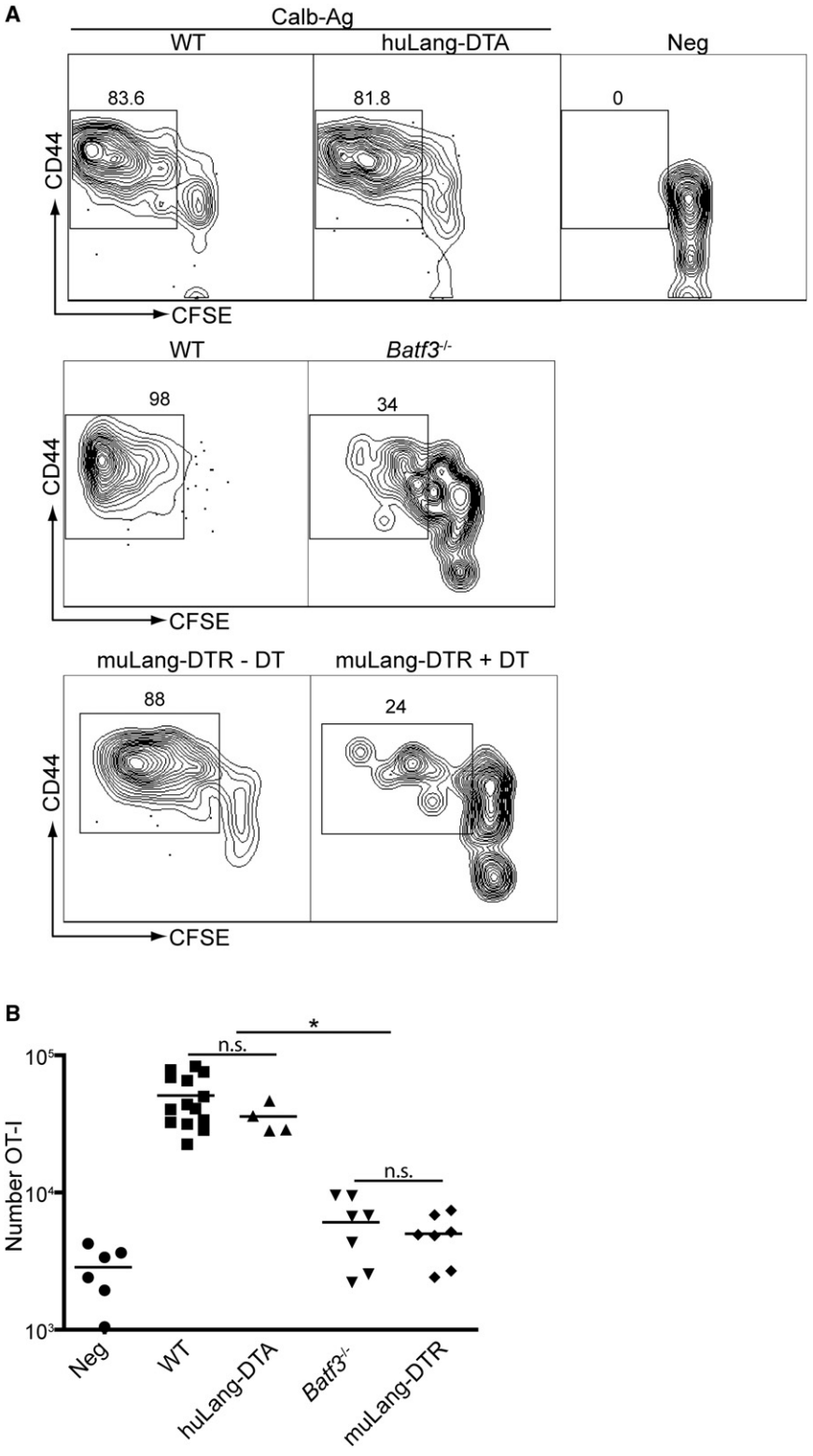

Figure 3. LCs Are Not Required for Antigen Cross-presentation In Vivo

(A) A total of 3 × 10^5 CFSE-labeled, CD90.1, $CD44^{lo}$ OT-I cells (CTL specific for $OVA_{257-264}$) were adoptively transferred into WT, huLangerin-DTA (top panel), *Batf3*$^{-/-}$ (middle panel), or muLangerin-DTR (bottom panel) mice. Mice were infected on their skin with either Calb-WT (Neg) or Calb-Ag. Skin-draining LNs were harvested 4 days later. CFSE and expression of CD44 are shown on $CD8^+$, $CD90.1^+$ gated cells. Numbers adjacent to outlined areas are the percentage of cells that have diluted CFSE.

(B) Total numbers of $CD90.1^+$, OT-I cells recovered from each strain are shown. Each symbol represents an individual animal. WT and huLangerin-DTA are not significantly different. *Batf3*$^{-/-}$ and DT-treated muLangerin-DTR mice are not significantly different from one another, but are significantly less than WT and huLangerin-DTA mice ($p < 0.0001$). n.s., not significant. See also Figure S1.

phenotype (Korn et al., 2009). To examine the mechanism of LC-mediated induction of Th17 cells, we compared cytokine expression in LC sorted from lymph nodes of naive and infected mice (Figure 7A and Figure S5A). LC increased expression of IL-1β (~6-fold) and IL-6 (~8-fold) in response to infection, but TGF-β and IL-23 remained unchanged. IL-12 could not be reliably detected. We next compared expression of IL-1β, IL-6, TGF-β, and the regulated chains of the IL-12 family members IL-12(p35), IL-23(p19), and IL-27(p28) between LC, $Langerin^+$ dDCs and Langerin- dDCs sorted from lymph node (LN) after skin infection with *C. albicans*. $Langerin^+$ dDCs produced significantly less IL-1β and IL-6 than LCs (Figure 7B and Figure S5A). We also observed that $Langerin^+$ dDCs but not LCs expressed message for IL-12. Interestingly, $Langerin^+$ dDCs expressed higher amounts of IL-27, which has been demonstrated to suppress Th17 cell development (Stumhofer et al., 2010). Similar patterns of expression were observed after skin infection with *S. aureus* (Figure S5B). This pattern of cytokine expression is certainly consistent with our observations that LCs promote Th17 cells. However, it also raises the possibility that $Langerin^+$ dDC could promote Th1 and simultaneously inhibit Th17 cell responses.

To test this hypothesis, we infected WT and *Batf3*$^{-/-}$ mice (Table 1) with Calb-Ag and analyzed the phenotype of TEα cells. The absence of $Langerin^+$ dDCs did not alter proliferation based on CFSE dilution (Figure 7C) or total numbers of TEα cells (Figure S6A). IFN-γ-producing cells did not develop in *Batf3*$^{-/-}$ mice and the numbers of cells expressing Th17-associated cytokines was increased ~2-fold (Figure 7D). Thus, $Langerin^+$ dDCs are required

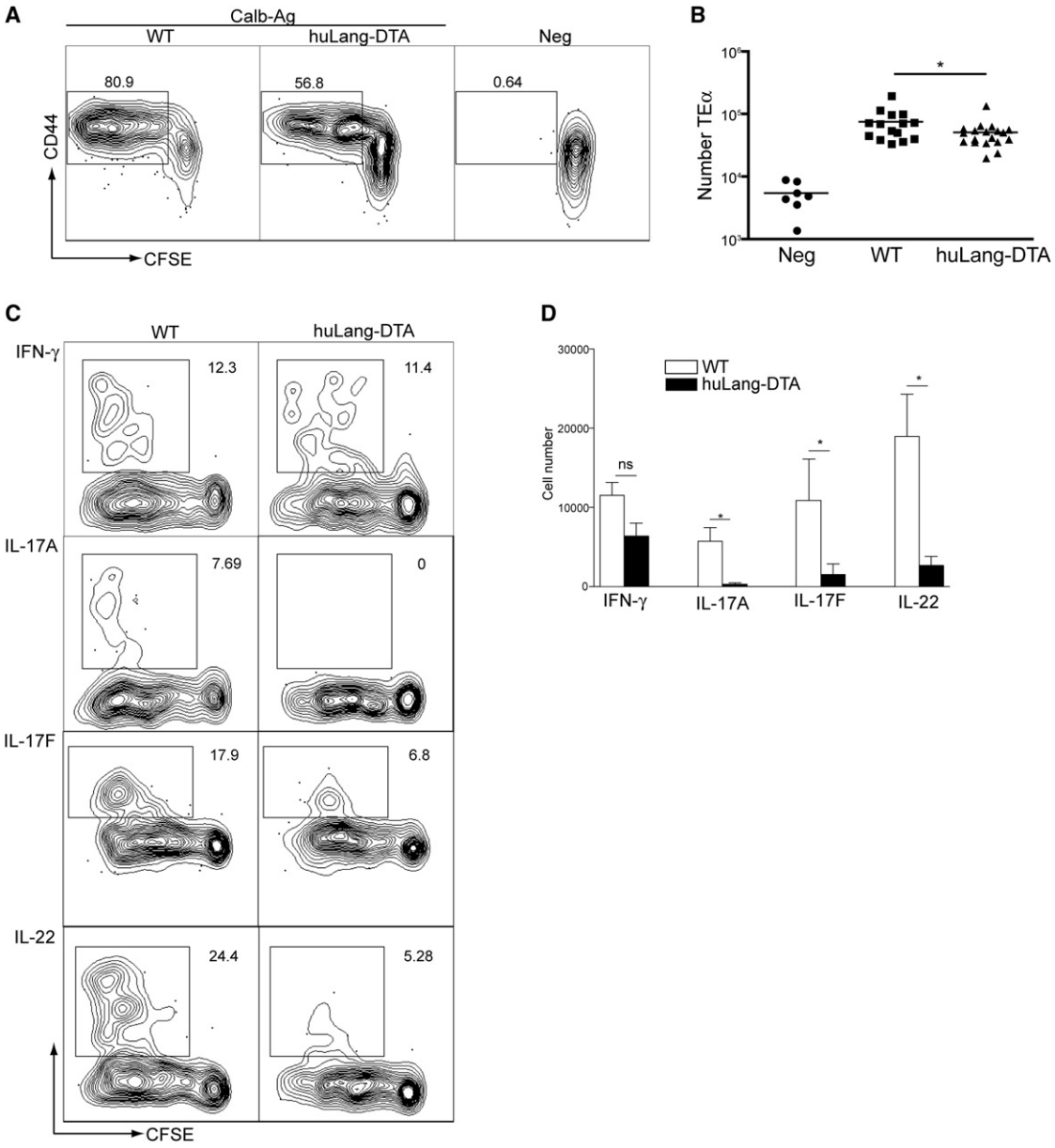

Figure 4. LCs Are Necessary for Th17 Cell Development

(A) A total of 3×10^5 CFSE-labeled, CD90.1 TEα cells ($CD4^+$ T cells specific for I-$E\alpha_{50\text{-}66}$) were transferred into WT and huLangerin-DTA mice. Mice were infected on their skin with either Calb-WT (Neg) or Calb-Ag. Skin-draining LNs were harvested 4 days later. CFSE and expression of CD44 are shown on $CD4^+$, $CD90.1^+$ gated cells.

(B) Total numbers of $CD90.1^+$, TEα cells recovered from each strain are shown. Each symbol represents an individual animal.

(C) As in (A), except that cells were restimulated in vitro with PMA-ionomycin prior to intracellular staining with the indicated cytokine antibody. Representative plots are shown.

(D) Total numbers of cytokine producing TEα cells from each strain is shown. Data have been pooled from three independent experiments (ns, not significant; *$p < 0.05$). See also Figure S2.

for the development of Th1 cells and inhibit the development of Th17 cells.

We repeated these experiments in muLangerin-DTR mice that lack both LCs and $Langerin^+$ dDCs (Table 1). As in the *Batf3*$^{-/-}$ mice, TEα cells in muLangerin-DTR mice showed normal proliferation and a near-complete absence of IFN-γ-producing cells (Figures 7E and 7F and Figure S6B). Unexpectedly, the absence of both LCs and $Langerin^+$ dDCs resulted in normal numbers of Th17 cells (Figure 7F), indicating that Langerin- dDCs were sufficient for Th17 cell induction. Interestingly, the cytokines expressed by Langerin- dDCs were similar to LCs (Figure 7B and Figure S5B). Thus, like LCs, Langerin- dDCs can promote Th17 cell differentiation but only in the absence of inhibition from $Langerin^+$ dDCs.

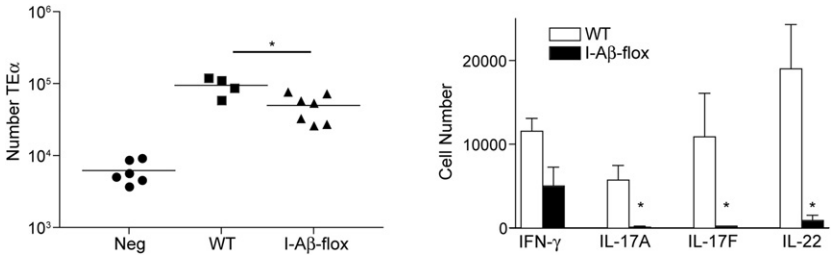

Figure 5. Direct Antigen Presentation by LCs Is Required for Th17 Cell Development

As in Figure 4, TEα cells were adoptively transferred into WT and huLangerin-Cre × I-Aβ-flox mice prior to skin infection.

(A) The expansion of TEα cells in Calb-WT (neg) infected WT mice Calb-Ag infected WT and huLangerin-Cre × I-Aβ-flox is shown. Each symbol represents an individual animal.

(B) Total numbers of cytokine producing TEα cells from each strain is shown. Data have been pooled from two independent experiments (ns, not significant; *p < 0.05).

To confirm that altered cytokine expression in TEα cells correlated with functional outcome, we next examined whether exaggerated Th17 and reduced Th1 cell responses in *Batf3*$^{-/-}$ mice affected rechallenge with *C. albicans.* To this end, we challenged naive and previously infected WT and *Batf3*$^{-/-}$ mice by intradermal injection of 5 × 10^6 *C. albicans* blastoconidia. Three days later, the skin was harvested and colony forming units (CFU) determined. As expected, WT mice harbored significantly fewer *C. albicans* than naive ("Neg") mice (Figure 7G). Importantly, the number of CFU in *Batf3*$^{-/-}$ mice was reduced by ~20-fold compared to WT. Thus, the Th cell skewing observed in *Batf3*$^{-/-}$ mice in response to skin infection with *C. albicans* was associated with an increased resistance to *C. albicans* infection.

DISCUSSION

Herein, we have demonstrated that LCs are neither necessary nor sufficient for cross-presentation of *C. albicans* antigen to CD8$^+$ CTLs in vivo. Instead, dermal Langerin$^+$ DCs were required for efficient cross-presentation. We also found that LCs were necessary and sufficient for the development of Th17 cell responses. In contrast, Langerin$^+$ dDCs were required for the generation of Th1 cell responses. Moreover, the absence of Langerin$^+$ dDC enhanced Th17 cell responses, suggesting that these cells also inhibited the development of Th17 cells. Thus, skin-resident DC subsets have specific, nonredundant functions and promote distinct and opposing antigen-specific responses in vivo.

By examining *C. albicans*-infected mice in which antigen cannot be presented by LCs or can be presented only by LCs, we have demonstrated that LCs are necessary and sufficient for Th17 cell development. We observed that in response to infection with *C. albicans* or *S. aureus*, LC produced elevated amounts of IL-6, IL-1β, and IL-23, which both promote and stabilize Th17 cell differentiation (Korn et al., 2009). Thus, we propose that presentation of cutaneous antigen to naive CD4$^+$ T cells in the presence of these cytokines is responsible for the ability of LCs to promote Th17 cells, and such a finding is consistent with in vitro data (Aliahmadi et al., 2009; Mathers et al., 2009). Activated LCs expressed low amounts of IL-27 and do not express IL-12; IL-27 and IL-12 are both key cytokines for Th1 cell differentiation (Szabo et al., 2003). In addition, *C. albicans*-infected Langerin-DTA mice had an intact Th1 cell response, which suggested that LCs did not participate in Th1 cell development. *Batf3*$^{-/-}$ mice lack Langerin$^+$ dDCs and did not develop Th1 cells. Langerin$^+$ dDC expressed higher amounts of IL-12 and IL-27 than LCs, which probably explains the requirement of this DC subset for Th1 cell development. Langerin$^+$ dDCs also had lower IL-1β and IL-6 expression as well as the absence of IL-23, which makes them poor promoters of Th17 cell differentiation. Interestingly, IL-12 and IL-27 as well as IFN-γ from Th1 cells have all been reported to inhibit Th17 cell formation and proliferation (McGeachy and Cua, 2008; Stumhofer et al., 2006; Stumhofer et al., 2010). The absence of these inhibitory cytokines in *Batf3*$^{-/-}$ mice is likely to explain the observed exaggerated Th17 cell responses. Thus, presentation of the same antigen by LCs and Langerin$^+$ dDCs promotes opposing effects that together allowed for the development of both Th1 cell and appropriately balanced Th17 cell responses.

Cross-presentation by LCs has been clearly demonstrated with cells derived in vitro from human bone marrow precursors or isolated ex vivo from human skin explants when cultured with soluble antigen in vitro (Klechevsky et al., 2008). LCs isolated ex vivo from murine skin behave similarly (Flacher et al., 2010; Stoitzner et al., 2006). In contrast, LCs isolated ex vivo from LNs of mice in which antigen is only available to LCs in vivo either during steady-state or via herpes virus infection cannot cross-present antigen (Bedoui et al., 2009; Henri et al., 2010). Our data from *C. albicans*-infected mice clearly demonstrate that LCs are not necessary for cross-presentation. Similarly, presentation by LCs was not sufficient to activate OT-I when antigen was selectively targeted to LCs with anti-huLangerin-Ag complexes. Thus, it appears that in vivo, LCs do not have the capacity for cross-presentation. The ability of LCs isolated from skin to cross-present in vitro may be specific to LCs while in the epidermis or could be acquired during in vitro culture. Our experiments with *Batf3*$^{-/-}$ and muLangerin-DTR mice demonstrated that Langerin$^+$ dDCs were required for efficient cross-presentation of skin-derived antigen. This is consistent with the data examining DC populations in vivo (Wang et al., 2008) and those isolated from LN ex vivo (Bedoui et al., 2009; Henri et al., 2010). Targeting antigen to LCs in vivo with muLangerin antibodies (clone L31) has been reported to result in efficient cross-presentation (Idoyaga et al., 2008). Given that both Langerin$^+$ dDCs and LCs express muLangerin, we suggest that Langerin$^+$ dDCs, not LCs, are responsible for the observed effect.

Throughout this report, we have divided DCs into LCs, Langerin$^+$ dDCs, and Langerin- dDCs for the sake of expediency. Although the first two are well defined and can be manipulated in vivo, Langerin$^-$ dDCs represent a heterogeneous population that include "classic" CD11b$^+$ dDCs as well as a more recently

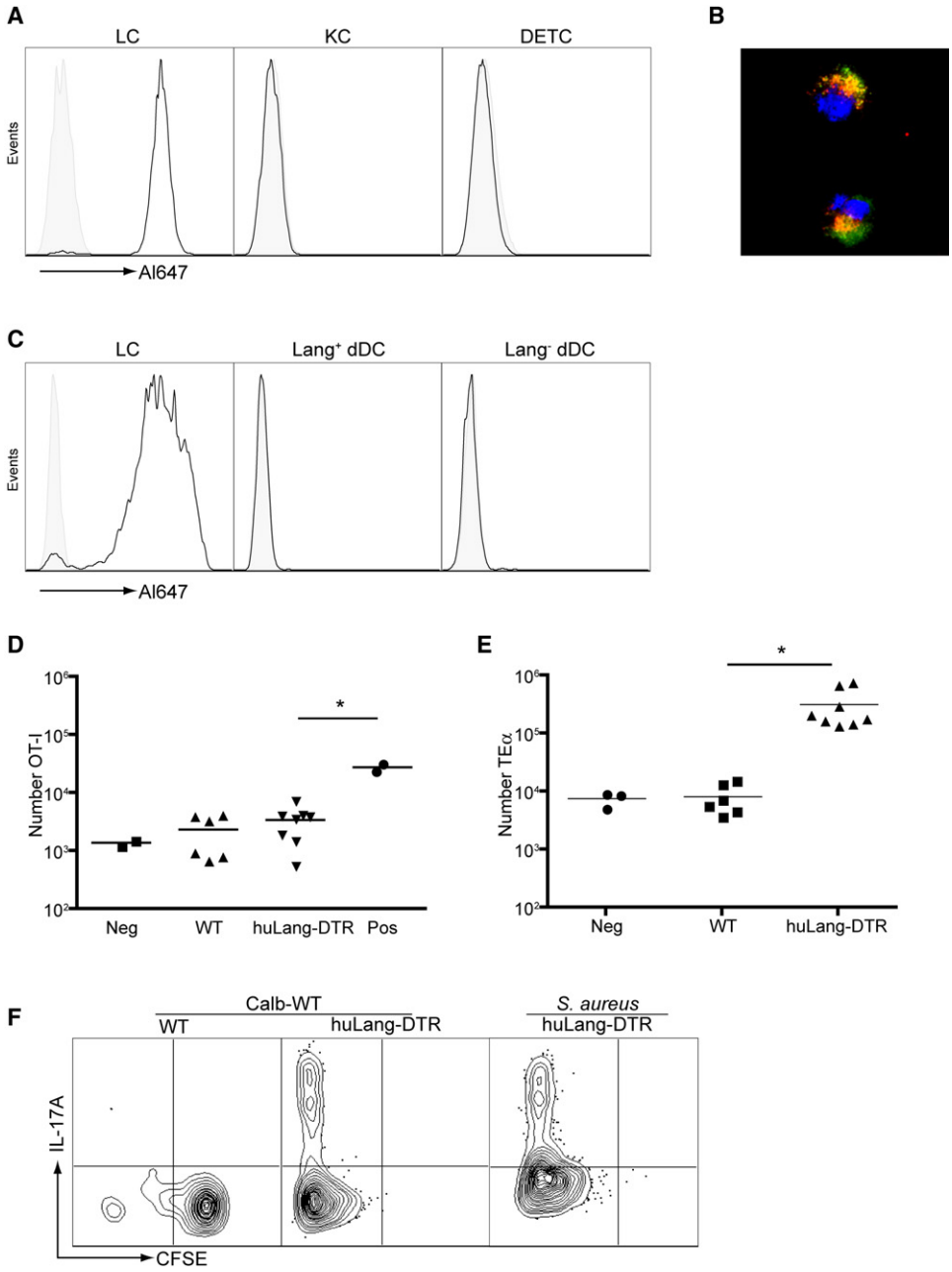

Figure 6. Antigen Presentation by LCs Is Sufficient for Th17 Cell Development

(A) WT (shaded) and huLangerin-DTR (solid line) mice were injected i.p. with 10 μg anti-huLangerin (2G3) conjugated to Alexa-647. The amount of Alexa-647 16 hr after injection is shown in epidermal cells gated on LCs ($CD45^+$, $MHC\text{-}II^+$), keratinocytes (KC; CD45-, MHC-II-), and dendritic epidermal T cells (DETC; $CD45^+$, $MHC\text{-}II^-$).

(B) As in (A) except that LCs isolated from the epidermis were identified by expression of endogenous mouse Langerin (green). The cellular location of 2G3-Alexa-647 (red) and colocalization with muLangerin (yellow) was visualized by immunofluorescence.

(C) As in (A), cells were isolated from skin-draining LNs. The amount of 2G3-Alexa647 on LC ($MHC\text{-}II^{hi}$, $CD11c^+$, $CD8^-$, $Langerin^+$, $CD103^-$), $Langerin^+$ dDCs ($MHC\text{-}II^{hi}$, $CD11c^+$, $CD8^-$, $Langerin^+$, $CD103^+$), and $Langerin^-$ dDCs ($MHC\text{-}II^{hi}$, $CD11c^+$, $CD8^-$ $Langerin^-$) is shown.

(D) WT or huLangerin-DTR mice were adoptively transferred with 3×10^5 naive OT-I cells 24 hr prior to i.p. injection of 1.0 ug 2G3-$OVA_{257\text{-}264}$ or isotype control (neg). Mice were then infected with Calb-WT on their skin. As a positive control, WT mice were immunized with 20 μg 2G3-$OVA_{257\text{-}264}$ in CFA (pos). Skin-draining LNs were harvested on day +4 and the total number of OT-I cells is shown.

(E) As in (D), except that TEα cells were transferred and mice were immunized with 2G3-Eα.

(F) CFSE and expression of IL-17A from TEα isolated from WT and huLangerin-DTR mice infected with *Calb-WT* or *S. aureus* are shown. Data are representative of three individual experiments. See also Figures S3 and S4.

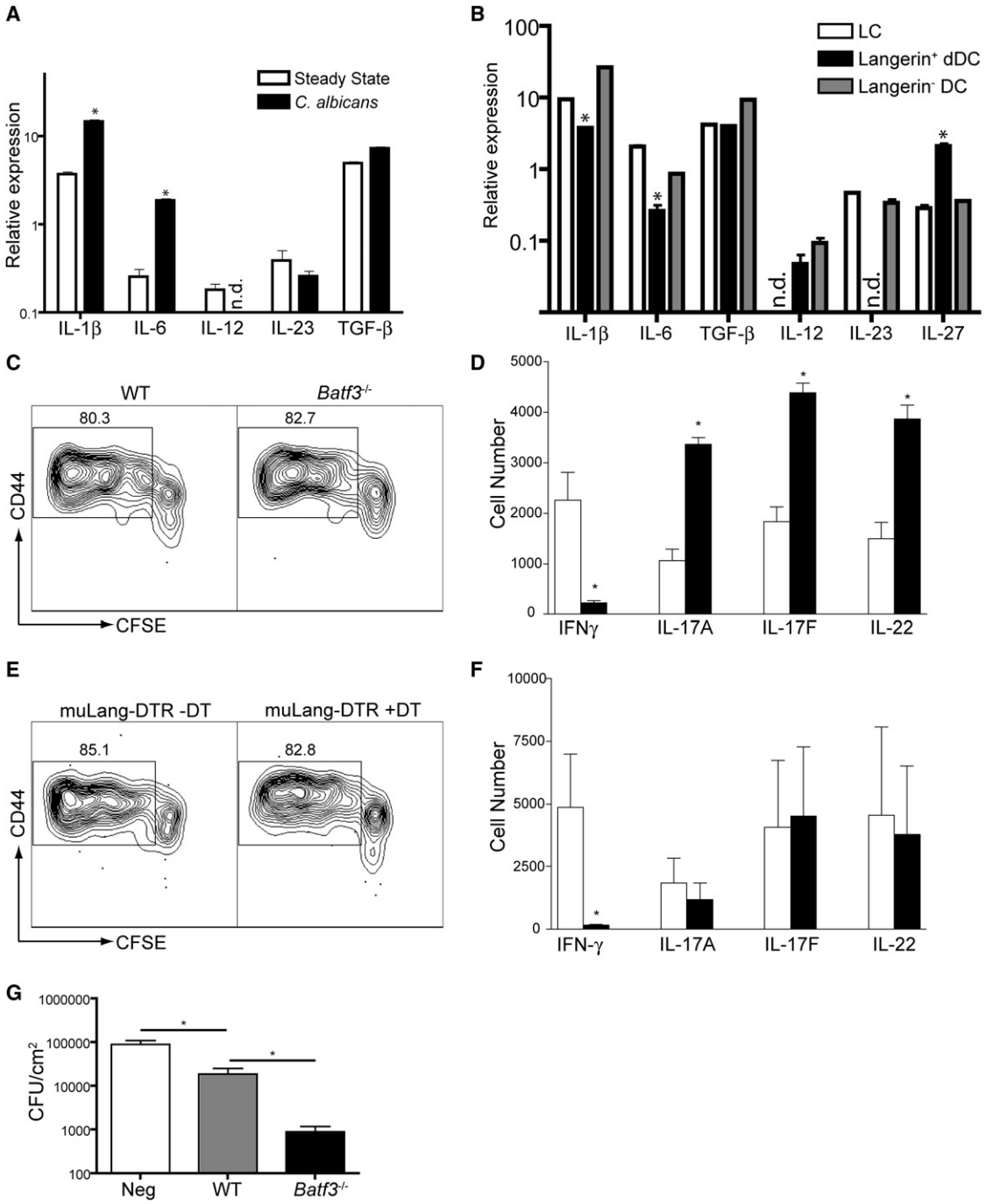

Figure 7. Langerin$^+$ dDCs Promote Th1 and Inhibit Th17 Cell Differentiation

(A) LCs were sorted by flow cytometry on day +4 from skin-draining LNs from sham (open bars) or Calb-WT-infected (black bars) muLangerin-EGFP reporter mice. Relative expression of mRNA of the indicated cytokines are shown.

(B) LCs (open bars), Langerin$^+$ dDCs (black bars), and Langerin$^-$ dDCs (gray bars) were sorted by flow cytometry from Calb-WT-infected muLangerin-EGFP mice. Relative expression of mRNA of the indicated cytokines are shown.

(C and D) As in Figure 4, WT (open bars) and *Batf3*$^{-/-}$ (black bars) mice were infected with Calb-Ag. Proliferation (C) and cytokine expression (D) are shown.

(E and F) As in (C), except that muLangerin-DTR mice that had been injected with DT (black bars) or PBS (open bars) were used. Data are representative of three individual experiments.

(G) Cohorts of four to eight WT and *Batf3*$^{-/-}$ mice were skin infected with Calb-WT or sham ("Neg"). Mice were then reinfected by intradermal injection of 5×10^6 Calb-WT on day +9. Three days later, skin was harvested and CFU obtained. (*$p < 0.05$). Data are pooled from two independent experiments. See also Figures S5 and S6. n.d., not detected.

defined CD11b$^-$ population (Henri et al., 2010). The function of these DCs has been poorly explored, though, intriguingly, CD103$^-$ dDCs have the unique ability to express retinoic acid and may be important for the generation of Treg cell during steady state (Guilliams et al., 2010). We cannot directly manipulate these Langerin$^-$ dermal DC subsets with our present tools, but they are the only skin-resident subset that remains in DT-treated muLangerin-DTR mice. In the absence of LCs and Langerin$^+$ dDCs, we observed a modest amount of cross-presentation of *C. albicans*-derived antigen. Thus, at least one of the subsets contained within the Langerin$^-$ dDC population has the capacity for cross-presentation. Moreover, this population has a cytokine profile similar to LCs (high IL1β, IL-6, and IL-23 and low IL-12 and IL-27) and is sufficient to promote Th17 cell differentiation when inhibition from Langerin$^+$ dDCs is absent (Figure 7). Recently, yet another DC subset derived from monocytes in response to LPS via TLR4 stimulation has been reported (Cheong et al., 2010). Although LPS is absent from our system, we cannot exclude that a similar inflammatory-type DC was present within the Langerin$^-$ dDC subset.

In conclusion, we have demonstrated in vivo, that skin-resident DC subsets can promote unique and opposite T cell responses directed against the same antigen. This has important implications for vaccination strategies that selectively target DC populations (Palucka et al., 2010). In addition, the requirement for LCs in the development of Th17 cells suggests these cells may participate in the early pathogenesis of Th17 cell-mediated skin diseases such as psoriasis. At present, we have explored the response to *C. albicans* and *S. aureus*, which both promote Th17 cell-type responses. An important question for future investigation is whether Langerin$^+$ dDCs and LCs also promote opposite T cell responses during the steady state and in response to pathogens or adjuvants that do not induce Th17 cell-type responses.

EXPERIMENTAL PROCEDURES

Mice

HuLangerin-DTA (Kaplan et al., 2005), huLangerin-Cre I-Aβ-flox (Igyarto et al., 2009), huLangerin-DTR (Bobr et al., 2010), muLangerin-DTR (Kissenpfennig et al., 2005), and muLangerin-EGFP (Kissenpfennig et al., 2005) mice have been previously described. *Batf3*$^{-/-}$ mice (Hildner et al., 2008) that had been backcrossed ten generations onto the C57BL/6 background were a generous gift from K. Murphy (Washington University, St Louis, MO). B6 *Rag-1*$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-I.PL *Cd8* T cell receptor (TCR) transgenic specific for OVA$_{257-264}$ (Hogquist et al., 1994), OT-II *Rag1*$^{-/-}$ *Cd4* TCR transgenic recognizing OVA$_{323-339}$ (Barnden et al., 1998), and TEα *Rag1*$^{-/-}$ *Cd4* TCR-transgenic to I-Eα$_{50-66}$ (Grubin et al., 1997) mice on CD90.1 C57BL/6 backgrounds were also used. All experiments were performed with 6- to 10-week-old and sex-matched mice. Mice were housed in microisolator cages and fed irradiated food and acidified water. The University of Minnesota institutional care and use committee approved all mouse protocols.

DC Depletion with Diphtheria Toxin

MuLangerin-DTR mice were i.p. injected with 1 μg of diphtheria toxin (List Biological Laboratories, Campbell, CA) 2 days before infection, as previously described (Bobr et al., 2010; Kissenpfennig et al., 2005).

Antibodies

Fluorochrome-conjugated antibodies to CD4, CD8, CD11b, CD11c, CD90.1, CD103, MHC class II, IFN-γ, and IL-17A were purchased from Biolegend (San Diego, CA). Anti-mouse Langerin (L31), IL-17F, and IL-22 were acquired from eBioscience (San Diego, CA).

Adoptive T Cell Transfer

Skin-draining lymph nodes and mesenteric lymph node of OT-II and TEα mice were disrupted through a cell strainer and washed with sterile HBSS. Naive (CD44^{low}) OT-I T cells were purified from lymph nodes of OT-I.PL mice as described (Bursch et al., 2009). Cell purity (>95%) was determined by flow cytometry before adoptive transfer. The cells were labeled with CFSE (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions and resuspended in sterile PBS at a concentration of 1 × 10^6 cell/ml. Three hundred μl (3 × 10^5) cells were injected intravenously. Transferred cells were detected in recipient mice based on expression of congenic CD90.1.

Flow Cytometry

Single-cell lymph node suspensions were obtained and stained as previously described (Kaplan et al., 2005). For evaluating cytokine expression, cells were incubated for 5 hr in complete RPMI 1640 supplemented with PMA (50 ng/ml) and ionomycin (1.5 μM; Sigma-Aldrich, St. Louis, MO), with GolgiStop (BD PharMingen, San Jose, CA) added for the final 4 hr. The intracellular cytokine staining was performed with BD Bioscience Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) in accordance with the manufacturer's instructions. Samples were analyzed on LSR-II flow cytometers (BD Biosciences). Detection of 2W1S specific CD4 T cells was performed as published with an I-Ab-2W1S tetramer (a kind gift of M. Jenkins) (Moon et al., 2007). Data were analyzed with FlowJo software (TreeStar, Ashland, OR). Isolation of DC subsets by flow cytometric cell sorting and analysis of mRNA expression is described in Supplemental Experimental Procedures.

Skin Infection Model

Mice were first anesthetized with a mixture of ketamine and xylazine (100/10 μg/kg body weight), shaved on the back with electric clipper, and chemically depilated with Nair hair remover (Church & Dwight, Princeton, NJ) per the manufacturer's instructions. The stratum corneum was removed with 15 strokes with 220 grit sandpaper (3M, St Paul, MN). *C. albicans* (WT or recombinant) was grown in YPAD medium (Sherman, 1991) at 30°C until the OD$_{600}$ reached 1.5–2.0. After washing with sterile PBS, 2 × 10^8 *C. albicans* in 50 μl of sterile PBS was applied on to the skin. In some experiments, *Staphylococcus aureus* (ALC2906) grown in LB media was applied onto shaved mouse skin at a concentration of 2 × 10^8 bacteria in 50 μl of PBS.

Delayed-Type Hypersensitivity

Eight days after skin infection, mice were challenged with footpad injection of 10^7 heat killed (60°C for an hour) *Candida albicans* yeast cells. The specific DTH was determined on the basis of the degree of footpad swelling 24 hr after challenge in infected mice subtracted by the degree of swelling in sham infected control mice.

Mouse Skin CFU

The mice infected with *Candida albicans* were sacrificed prior to infection and on day 2, 4, 5, and 7 after infection. The infected area was cleansed with povidone-iodine, which was removed once dry, and a 2.0 cm^2 section of skin was homogenized in sterile PBS containing penicillin and streptomycin prior to plating on YPAD plates. In some experiments, previously infected mice were rechallenged by intradermal injection of 5 × 10^6 *C. albicans*. Three days later, 1.0 cm^2 of skin surrounding the injection site was harvested and homogenized in sterile PBS containing penicillin and streptomycin prior to plating on YPAD plates. Colony counts were obtained 24–48 hr later.

Construction of Recombinant *C. albicans*

All *Candida albicans* strains used in this study were derived from SC5314 (Calb-WT) (Fonzi and Irwin, 1993). Construction of recombination vectors and generation of recombinant *C. albicans* is described in the Supplemental Experimental Procedures.

In Vitro T Cell Stimulation

OT-I and OT-II cells were labeled with CFSE and used as responders. For stimulators, *Rag1*$^{-/-}$ CD90.1 spleen cells were harvested and irradiated with

cesium-irradiator with 25 Gy. A total of 4 × 10^5 responders were cocultured with 2 × 10^5 stimulators in 200 μl complete RPMI 1640 in CO_2 incubator for 3 days supplemented with 10^5 heat killed Calb-WT or Calb-Ag yeast. Cells were harvested and analyzed by flow cytometry.

Monoclonal Antibody Generation

The anti-human Langerin 2G3 hybridoma (ATCC PTA 9853) was derived from mice immunized with human Langerin ectodomain fused to human IgFc as previously described (Klechevsky et al., 2010; Ni et al., 2010). Recombinant antibody fusion protein production is described in supplementary methods.

LC Targeting with Anti-huLangerin

WT and huLangerin-DTR mice were injected with 10 μg of 2G3-AL647. The mice were sacrificed 16 hr later and the presence of the AL647 signal was analyzed in epidermal and lymph node cells by flow cytometry as described (Kaplan et al., 2005). In separate experiments, 3 × 10^5 TEα or OT-I cells were transferred into WT and huLangerin-DTR mice. A day later, the mice were immunized by intraperitoneal injection of 1.0 μg of 2G3-Eα, 2G3-$OVA_{257-264}$ or 2G3-2W1S in 100 μl of sterile PBS. Six hours later, the mice were infected with Calb-WT on the dorsal skin. The skin draining lymph nodes were harvested 4 days later and analyzed by flow cytometry.

Immunofluorescence Staining

Immunofluorescence was performed as previously described (Kaplan et al., 2005). Images were captured with a microscope (DM5500; Leica) with digital system and LAS AF software (version 1.5.1).

Histology

Skin samples were fixed overnight in 10% formalin, dehydrated, and embedded in paraffin. The 7 μm microtome sections were stained with Periodic Acid-Schiff stain in accordance with the manufacturer's instructions (Sigma-Aldrich).

Statistics

Significant differences were calculated with the Student's unpaired, two-tailed t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, and two tables and can be found with this article online at doi:10.1016/j.immuni.2011.06.005.

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