Langerhans cells are negative regulators of the anti-*Leishmania* response

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Migratory skin dendritic cells (DCs) are thought to play an important role in priming T cell immune responses against *Leishmania major*, but DC subtypes responsible for the induction of protective immunity against this pathogen are still controversial. In this study, we analyzed the role of Langerin+ skin–derived DCs in the *Leishmania* model using inducible in vivo cell ablation. After physiologically relevant low–dose infection with *L. major* (1,000 parasites), mice depleted of all Langerin+ DCs developed significantly smaller ear lesions with decreased parasite loads and a reduced number of CD4+ Foxp3+ regulatory T cells (T reg cells) as compared with controls. This was accompanied by increased interferon γ production in lymph nodes in the absence of Langerin+ DCs. Moreover, selective depletion of Langerhans cells (LCs) demonstrated that the absence of LCs, and not Langerin+ dermal DC, was responsible for the reduced T reg cell immigration and the enhanced Th1 response, resulting in attenuated disease. Our data reveal a unique and novel suppressive role for epidermal LCs in *L. major* infection by driving the expansion of T reg cells. A better understanding of the various roles of different DC subsets in cutaneous leishmaniasis will improve the development of a potent therapeutic/prophylactic vaccine.

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Abbreviations used: dDC, dermal DC; DT, diphtheria toxin; DTR, DT receptor; EC, epidermal cell; LC, Langerhans cell; SLA, soluble Leishmania antigen; T eff cell, effector T cell; T reg cell, regulatory T cell.

Langerhans cells (LCs) represent a unique DC subset in the epidermis. Langerin (CD207) is a C-type lectin predominantly expressed by LCs but also some murine CD8α^+ LN DCs (Takahara et al., 2002; Valladeau et al., 2002; Douillard et al., 2005; Kissenpfennig et al., 2005). New subsets of Langerin+ dermal DCs (dDCs), independent from epidermal LCs in transit, have been identified (Bursch et al., 2007; Ginhoux et al., 2007; Poulin et al., 2007). The dermis contains two more subsets of Langerin+ dDCs (distinguished by differential CD103 expression) and two subsets of Langerin^{neg} dDCs that differ in CD11b expression (Henri et al., 2010). Both Langerin+ dDC subsets constitute $\sim$3% of all dDCs, whereas Langerin^{neg} CD11b+ dDCs represent $\sim$66% of all dDCs, and Langerin^{neg} CD11b^{neg} dDCs are less frequent ($\sim$16%). Thus, murine skin contains at least five phenotypically distinct DC populations, i.e., epidermal LCs and two Langerin+ and two Langerin^{neg} dDC subsets, which may also differ in function, e.g., in their ability to (cross-) present

antigen (Kaplan et al., 2008; Nagao et al., 2009; Henri et al., 2010).

In experimental cutaneous leishmaniasis, parasite-infected DCs mediate the induction of protective immunity by producing IL-12 (von Stebut et al., 1998) and migratory skin DCs are considered to play an important role in priming T cell responses against *Leishmania major*. Originally, LCs had been thought to be essential for the control of cutaneous leishmaniasis because antigen-pulsed LCs can establish protective immunity (Moll et al., 1993). However, more recent studies demonstrated that other DC subtypes, including CD11b+ LN-resident DCs and CD8α^{neg} Langerin^{neg} dDCs, can be infected and act as principal antigen-presenting cells in leishmaniasis (Misslitz et al., 2004; Ritter et al., 2004; Iezzi et al., 2006). Furthermore, during *Leishmania* infection, monocyte-derived DCs form at the infection site, which controls the induction of a protective Th1 response (León et al., 2007).

B.E. Clausen and E. von Stebut contributed equally to this paper.

Because an effective *Leishmania* vaccine does not exist and (skin) DCs are critical regulators of the anti–*Leishmania* immune response, DCs are attractive targets for immunotherapeutic approaches. Thus, it is essential to understand the precise role of a particular DC subset in leishmaniasis. The development of knock-in mice expressing a diphtheria toxin (DT) receptor (DTR) cDNA under control of the *langerin* promoter allows us to unravel the in vivo dynamics and function of Langerin[+] DCs in general and LCs in particular (Bennett et al., 2005; Kissenpfennig et al., 2005; Kaplan et al., 2008). Application of DT to Langerin-DTR mice rapidly eliminates all Langerin^{+} cells from epidermis, dermis, and skin–draining LN.

In a previous study, subcutaneous high-dose infections of DT–treated Langerin-DTR mice with 3×10^6 stationary phase *L. major* parasites into foot pads showed that depletion of LCs had no effect on disease outcome and parasite clearance (Brewig et al., 2009). In the present study, we extend these findings using physiological low-dose infections with only infectious stage parasites (1,000 metacyclic promastigotes) and intradermal ear inoculation (Belkaid et al., 2000) to reveal for the first time that LCs have a regulatory function in an infectious disease model. Moreover, better parasite clearance was linked with enhanced Th1 (more IFN-γ), reduced lesional T reg cell numbers, and less IL-10 in the absence of LCs. With regard to vaccine development strategies, our results strongly suggest the use of approaches that aim to circumvent activation or targeting of LCs during anti–*Leishmania* immunization.

RESULTS AND DISCUSSION

Conditional ablation of Langerin[+] DCs leads to enhanced protective immunity against *L. major* infection

Langerin-DTR mice were injected i.p. with DT to deplete all Langerin[+] DCs, including epidermal LCs, Langerin[+] dDCs, and LN-resident Langerin[+] DCs (Fig. S1, A and B; Bennett et al., 2005, 2007). 2 d after DT treatment, mice were infected intradermally with 1,000 metacyclic *L. major* promastigotes and were subsequently treated weekly with DT to maintain depletion of all Langerin^{+} cells. DT treatment was well tolerated without any side effects as reported previously (Bennett et al., 2005; Bennett and Clausen, 2007). After infection, mice depleted of Langerin^{+} cells developed significantly smaller ear lesions as compared with PBS-treated controls (Fig. 1 A). Lesion sizes in the PBS control group were similar to those in C57BL/6 wild-type mice (Fig. S1 C). Decreased lesion sizes in Langerin-DTR + DT mice correlated with significantly lower parasite burdens in infected ears at week 6 after infection ($5.3 \pm 1.9 \times 10^4$ in DT–treated mice compared with 3.9 ± 1.10^5 in control mice, $n = 8$, $P = 0.008$; Fig. 1 B). Spleen parasite burdens were very low in both groups without significant differences.

We next assessed the cytokine profile 6 wk after infection. IFN-γ, IL-12, and IL-10 levels were determined after 48 h of restimulation of skin-draining LN cells with soluble *Leishmania* antigen (SLA). In *Leishmania* infections, Th1-inducing
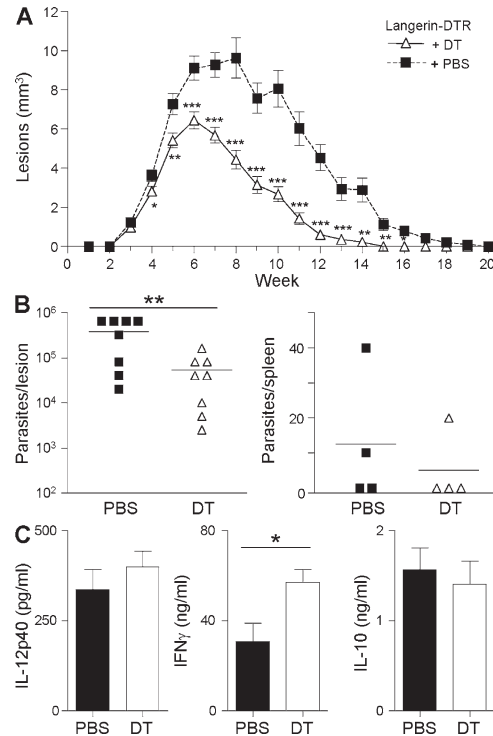

Figure 1. Depletion of Langerin[+] DC improves disease outcome in mice infected with *L. major*. (A) Groups of three to five animals of DT- or PBS-treated Langerin-DTR mice were infected intradermally with 10^3 metacyclic *L. major* promastigotes. DT or PBS treatment was performed 48 h before infection and repeated weekly. Lesion development was assessed (mean ± SEM), and pooled data from five or more independent experiments are shown. (B) Numbers of parasites in ear lesions (left) and spleens (right) were quantified using limiting dilution assays at 6 wk after infection. Mean parasite loads are shown as bars, and individual parasite counts/ear or spleen as squares (PBS) and triangles (DT). Results are representative of three independent experiments. (C) Antigen-specific cytokine release by LN cells from 6-wk-infected mice was determined by ELISA after restimulation with SLA. Pooled data from two independent experiments are shown. Values are mean ± SEM of 9–10 mice per time point (*, $P \leq 0.05$; **, $P \leq 0.02$; ***, $P \leq 0.002$, difference to PBS control mice).

IL-12 is primarily released by infected DCs (von Stebut et al., 1998; Ahuja et al., 1999; Sacks and Noben-Trauth, 2002) and is critical to mediate protection (Berberich et al., 2003). In this study, Langerin[+] DCs did not contribute to this IL-12 release (Fig. 1 C). Importantly, IFN-γ production was significantly increased in DT–treated Langerin-DTR mice as compared with controls (57 ± 6 vs. 31 ± 8 ng/ml, $P = 0.021$). No difference was observed in IL-10 production in week 6 after infection (Fig. 1 C). IL-4 levels in LN supernatants were low as expected (C57BL/6 *Leishmania*-resistant background) without differences between treatment groups.

Our findings demonstrate a novel and unexpected suppressive role of Langerin[+] DCs, which is only visible in the physiologically relevant low-dose infection model. When we used high-dose inocula (intradermal infection with 2×10^5 metacyclic promastigotes) in Langerin[+] DC-depleted mice, we did not observe comparable changes regarding lesion

development, parasite burdens, and cytokine profile (Fig. S2, A–C). This is consistent with data from Brewig et al. (2009), who used high doses of parasites (3×10^6) and subcutaneous inoculation, which masques any regulatory role of LCs in experimental leishmaniasis. Thus, other differences between that prior study and ours (other mouse strain, different route of parasite inoculation, and distinct parasite strains) are not responsible for the incongruent results.

LCs, not Langerin+ dDCs, suppress the anti–*Leishmania* response

As both types of skin DC, i.e., epidermal LCs and Langerin+ dDCs, are depleted by continuous DT treatment in our experiments, it was not clear which cell type was responsible for the suppressive effects shown in Fig. 1. To analyze the role of LCs in more detail, we performed timed toxin treatments. After conditional ablation of Langerin+ DCs in vivo using a single injection of DT, Langerin+ dDCs started reappearing already at days 3–5 and were largely restored by day 14 (Fig. S3 A; Noordegraaf et al., 2010), whereas this one-time DT administration depleted LCs for at least 2–4 wk with, occasionally, very few cells detectable at 2 wk and only partial reconstitution as late as 10 wk after DT (Fig. S3 B; Bennett et al., 2005). We next took advantage of this differential repopulation kinetics of LCs and Langerin+ dDCs to discriminate between the two DC subsets. Specifically, Langerin-DTR mice were injected once with DT 2 wk before *Leishmania* inoculation such that essentially all LCs were still depleted (Bennett et al., 2005), whereas the majority of Langerin+ dDCs had already reappeared (Fig. S3 A; Noordegraaf et al., 2010). Upon selective depletion of LCs alone, lesion sizes were significantly reduced as compared with controls, with the largest difference in week 7 (9.6 ± 1 and 5 ± 0.6 mm^3 in PBS and DT 1× groups, respectively, $P = 0.0001$; Fig. 2 A). No obvious difference between the two different DT treatment protocols was seen.

To confirm these results, we generated hematopoietic BM chimeras. C57BL/6 donor BM was used to reconstitute lethally irradiated (9 Gray) Langerin-DTR recipient mice. Epidermal LCs are radioresistant and remain of host (Langerin-DTR) origin after irradiation and BM transplantation. In contrast, all other DC subtypes are radiosensitive and replaced by donor-derived cells (C57BL/6). The resulting chimeric animals were treated once with DT 48 h before *L. major* infection, causing selective depletion of LCs (Fig. S3 C). Subsequently, mice were infected with low-dose inocula of *L. major* and lesion development was assessed weekly. As shown in Fig. 2 B, selective depletion of LCs resulted in significantly reduced lesion sizes. In contrast, in irradiated C57BL/6 mice reconstituted with Langerin-DTR BM, treatment with DT led to depletion of all Langerin+ dDCs, except LCs (Fig. S3 C). Interestingly, in these mice, lesion development was somewhat worsened as compared with controls, but the differences were not significant (Fig. 2 C). Moreover, these experiments ruled out an unspecific DT-induced phenomenon, as lesion sizes were only reduced after selective depletion of LCs but not after DT-mediated depletion of Langerin+ dDCs.
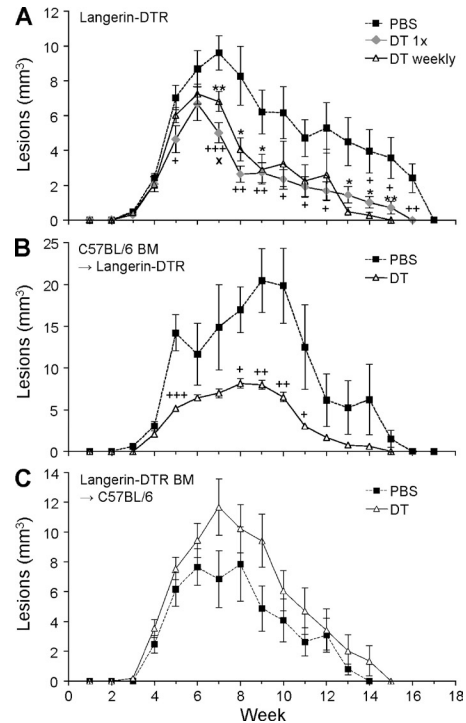

Figure 2. Selective depletion of LC leads to reduced lesion volumes. (A) Groups of Langerin-DTR mice were injected i.p. with PBS or DT. 2 wk later, mice were infected with 10^3 *L. major* parasites per ear. A third group was injected i.p. with DT 48 h before *Leishmania* infection and subsequently treated with DT once per week for the entire course of infection. Pooled data from three independent experiments are shown ($n \geq 8$ mice/group; +, statistical differences between PBS and DT 1× treatment; *, statistical differences between PBS and DT weekly; x, statistical differences between DT 1× and DT weekly; *,+,x, $P \leq 0.05$; **,++, $P \leq 0.02$; ***,+++, $P \leq 0.002$). (B) Langerin-DTR mice were lethally irradiated with 9 Gray, adoptively transferred with C57BL/6 BM (5×10^6 cells/mouse i.v.), and rested for 6 wk. Mice were treated once with DT or PBS 48 h before infection for the selective depletion of LC and infected with physiologically relevant low-dose inocula of *L. major* (10^3 metacyclic promastigotes). (C) C57BL/6 (recipient) mice were lethally irradiated with 9 Gray and adoptively transferred with Langerin-DTR (donor) BM for the depletion of Langerin+ dDC. DT- or PBS-treated chimeras were infected with 10^3 metacyclic *L. major* promastigotes. DT and PBS treatment was repeated weekly. In B and C, pooled data from two independent experiments are depicted. Lesion development was calculated as ellipsoid (mean ± SEM; +, $P \leq 0.05$; ++, $P \leq 0.02$; +++, $P \leq 0.002$).

Collectively, using two different experimental approaches, our results demonstrate that depletion of LCs alone is sufficient to significantly reduce lesion sizes, indicating that LCs are the relevant DC subset suppressing efficient effector T cell (T eff cell) responses in this skin infection model.

Depletion of Langerin+ DCs reduces the number of regulatory T cells in *L. major*–infected ears

To elucidate the mechanism underlying the observed phenotype, we analyzed the CD4+ and CD8+ T cell populations in skin draining LN and infected ears in the presence or absence of Langerin+ cells biweekly after infection. No difference was

observed in the total numbers of CD4^{+} and CD8^{+} T cells during the course of disease in DT- and PBS-treated groups, neither in the skin-draining LN nor in the infected ears (unpublished data).

Next, we asked if improved disease outcome in Langerin[+] DC–ablated mice was associated with altered numbers of regulatory T cells (T reg cells) infiltrating the infected ears. It was shown previously that T reg cells are essential for the development and maintenance of chronic cutaneous infection in *L. major*–resistant C57BL/6 mice (Belkaid et al., 2002; Mendez et al., 2004; Yurchenko et al., 2006). T reg cells accumulate at sites of infection, where they suppress antipathogen CD4^{+} T cell responses and consequently control concomitant immunity (Mendez et al., 2004). Using IL-10^{−/−} mice or administration of anti–IL-10 antibody led to complete eradication of the parasite in these mice, whereas an increase in the number of T reg cells promoted development of a new systemic infection (Belkaid et al., 2001, 2002). Interestingly, the important contribution of T reg cells in *L. major* infections was revealed only by using the physiological low-dose infection model (Belkaid et al., 2002; Yurchenko et al., 2006).

In this study, infiltrating ear cells of DT- and PBS-treated mice were isolated at the indicated time points after infection and analyzed for their CD4 and Foxp3 expression (Fig. 3 A). In the absence of Langerin^{+} DCs, the percentage of CD4^{+} Foxp3^{+} T reg cells in infected ears was significantly reduced in weeks 5 and 7 after *Leishmania* infection as compared with control mice (8.2 ± 1.7% vs. 14.1 ± 1.7%, *n* = 8 mice/group in week 5, P = 0.027; and 6.6 ± 1.4% vs. 11.3 ± 1.1%, *n* ≥ 10 mice/group in week 7, P = 0.015, in DT- and PBS-treated mice, respectively). Similar results were obtained when total numbers of CD4^{+} Foxp3^{+} T reg cells infiltrating infected ears of DT- and PBS-treated mice were compared. In this case, the T reg cell number was significantly reduced from, for example, 11.0 ± 1.9 × 10^{3} in the PBS group to 3.3 ± 1.0 × 10^{3} in DT-treated mice at week 7 (*n* ≥ 10, P = 0.0029; unpublished data).

We also assessed antigen-specific IFN-γ production over time after infection of PBS- and DT-treated mice. In parallel with the time points of maximal differences in T reg cell numbers, IFN-γ production of LN cells was significantly increased in DT-treated Langerin-DTR mice as compared with controls in weeks 5 and 7 (60 ± 15 vs. 21 ± 6 ng/ml in week 5, P = 0.031; and 83 ± 8 vs. 53 ± 10 ng/ml in week 7, P = 0.036, respectively; Fig. 3 B). Studies from Belkaid et al. (2000) revealed two distinct phases in the pathogenesis of cutaneous leishmaniasis (low dose infection). The initial silent phase, lasting 4–5 wk, favored dermal parasite amplification without lesion formation (Belkaid et al., 2000). The second phase corresponded to the development of lesions associated with an acute infiltration of inflammatory cells into the dermis and an expansion of T cells capable of producing IFN-γ in the draining LN, resulting in parasite killing (Belkaid et al., 2000). Accordingly, in our study the strongest difference in lesion sizes was detected between weeks 7 and 12 after infection after depletion of Langerin[+] DCs, together with significant
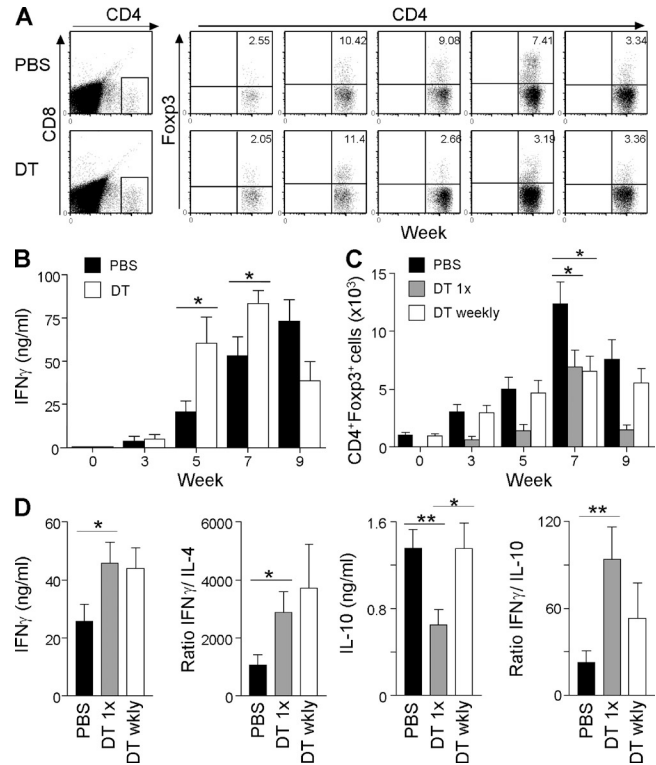

Figure 3. Decreased numbers of CD4^{+} Foxp3^{+} T reg cell in *L. major*–infected ears of DT-treated Langerin-DTR mice. Langerin-DTR mice were injected i.p. with DT or PBS. After 48 h, mice were infected with 10[3] *L. major* parasites/ear and treated weekly with DT or PBS. Ear-infiltrating cells were isolated at the indicated time points. After pregating on CD4^{+} T cells, the percentage of Foxp3^{+} cells in individual ears was calculated. (A) Representative CD4^{+} Foxp3^{+} FACS profiles and percentages are shown. (B) Antigen-specific IFN-γ release by LN cells from infected mice was determined by ELISA after restimulation with SLA. (C) Mice were treated once with DT (DT 1×) 2 wk before *Leishmania* infection for selective depletion of LC or weekly (DT wkly) for depletion of both LC and Langerin^{+} dDC. Total numbers of CD4^{+} Foxp3^{+} cells in infected ears are shown. (D) In week 7, antigen-specific cytokine release of LN cells was determined by ELISA. In B–D, pooled data from two or more independent experiments are shown (mean ± SEM, *n* ≥ 5 mice/group; *, P ≤ 0.05; **, P ≤ 0.02; ***, P ≤ 0.002).

differences in IFN-γ production between weeks 5 and 7 (and not in the silent phase of infection).

In a separate set of experiments, we assessed if selective depletion of LCs leads to similar results. As indicated in Fig. 3 C, significantly reduced numbers of CD4^{+} Foxp3^{+} T reg cells were observed in the ears of both DT-treated groups (e.g., week 7: PBS, 12.4 ± 1.9 × 10^{3}; DT 1×, 6.9 ± 1.5 × 10^{3}; and DT weekly, 6.5 ± 1.3 × 10^{3}, P = 0.049 and P = 0.016, respectively, *n* ≥ 14), indicating that LCs alone are sufficient for T reg cell expansion in vivo. In addition, similar to continuous DT treatment, the IFN-γ production and IFN-γ/IL-4 ratio were significantly increased in the DT 1× treatment group in comparison with PBS controls in week 7 (Fig. 3 D).

Because T reg cells exert their suppressive function largely by secretion of IL-10 (Belkaid et al., 2002), we asked whether
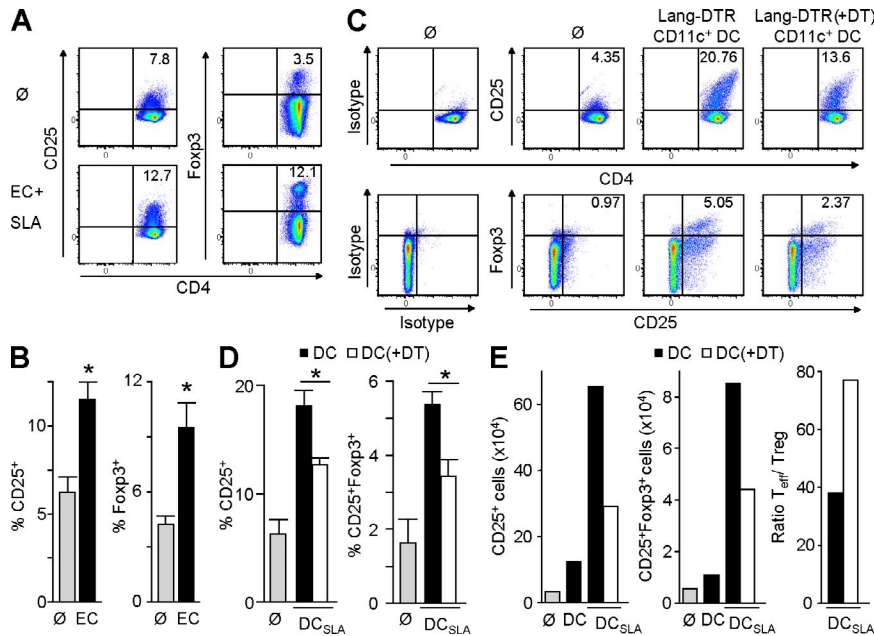
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Figure 4. SLA–loaded Langerin+ DCs expand CD4+ Foxp3+ T reg cell. (A) 10^5 ECs were pulsed with SLA and co-cultured with 5×10^5 CD4+ T cells for 24 h. Representative FACS staining for CD4+ CD25+ and CD4+ Foxp3+ T cells are depicted. (B) Pooled percentages of CD25 and Foxp3 expression on CD4+ cells from three independent experiments are shown (mean ± SEM; *, P ≤ 0.05). (C) Enriched CD11c+ DCs from LN of DT- or PBS-treated Langerin-DTR mice (10^5) were pulsed with SLA, and co-cultured with 5×10^5 CD4+ T cells for 24 h. Representative FACS staining for CD4+ CD25+ and CD4+ CD25+ Foxp3+ T cells are presented. (D) Pooled data from three independent experiments are shown (mean ± SEM; *, P ≤ 0.05). (E) DCs alone or DCs pulsed with SLA (10^5) were co-cultured with 5×10^5 CD4+ CD25neg T cells for 3 d. Induction of CD25 and Foxp3 on CD4 cells was assessed by FACS and cell numbers were calculated. For calculation of the T eff/T reg cell ratio, numbers of total CD4 T cells (minus T reg cells) were divided by total numbers of CD4+ CD25+ Foxp3+ T reg cells. One representative of three experiments is shown. In B, D, and E, Ø = T cells without APC. In D and E, black bars indicate DCs containing Langerin+ cells and white bars indicate DCs prepared from DT-treated Langerin-DTR mice.

there was a shift in the balance between CD4+ T eff cells producing IFN-γ and T reg cells producing IL-10 when LCs were absent. As shown in Fig. 3 D, IL-10 production was significantly reduced in LC-depleted mice as compared with PBS controls and mice depleted of both LCs and Langerin+ dDCs (0.7 ± 0.1, 1.4 ± 0.2, and 1.4 ± 0.2 ng/ml in DT 1×, PBS, and DT weekly treatment groups, respectively, P = 0.007 and P = 0.02). Consequently, we detected an increase in the IFN-γ/IL-10 ratio, indicating that there were more CD4+ T eff cells producing IFN-γ and fewer CD4+ Foxp3+ T reg cells producing IL-10 present in the skin draining LN of LC-depleted as compared with LC-competent mice (94 ± 22 vs. 23 ± 8 in DT 1× as compared with PBS groups, P = 0.0068). In conclusion, conditional ablation of LCs resulted in enhanced protective immunity against physiological low-dose infection with *L. major* associated with an increased Th1 and an attenuated T reg cell immune response.

Antigen–loaded Langerin+ DCs, but not Langerinneg DCs, preferentially expand CD4+ Foxp3+ T reg cell

Our in vivo data strongly suggest that LCs contribute to the expansion of T reg cell at the site of infection. To confirm these results, we analyzed the effect of SLA-loaded Langerin+ DCs on regulatory CD4+ T cells ex vivo. Epidermal cells (ECs; including LCs) were isolated from murine ear skin and pulsed with SLA. Subsequently, these ECs were co-cultured with CD4+ T cells isolated from *Leishmania*-infected and healed C57BL/6 mice. After 24 h, T cells were analyzed for their CD25 and Foxp3 expression using flow cytometry (Fig. 4 A). A significant increase of the percentage of CD25+ T cells was found when antigen-loaded ECs were used for antigen presentation as compared with negative controls without ECs (11.5 ± 1.0 vs. 6.2 ± 0.9%, respectively, P = 0.017; Fig. 4 B). In addition,

this was also true for the frequency of Foxp3+ CD4+ cells (9.5 ± 1.4 vs. 4.2 ± 0.5%, P = 0.021; Fig. 4 B). These results strongly suggest that epidermal LCs can expand the T reg cell population.

In a subsequent experiment, DCs were isolated using anti-CD11c–coated MACS beads from LN of Langerin-DTR mice treated with either DT (Langerin+ DC-depleted) or PBS. As described in the previous paragraph, these DCs were loaded with SLA and co-cultured with CD4+ T cells (Fig. 4 C). After 24 h, expansion of CD25+ T cells (18.2 ± 1.4 vs. 12.7 ± 0.6%, P = 0.012) and CD25+ Foxp3+ T cells (5.4 ± 0.4 vs. 3.4 ± 0.5%, P = 0.011) was significantly increased when the antigen-presenting DCs comprised Langerin+ DCs as compared with those lacking Langerin+ DCs (Fig. 4 D). Thus, Langerin+ DCs, and not Langerinneg DCs, drive the expansion of antigen-specific T reg cell and, corroborating the in vivo data (Fig. 3, A and C), expansion of T reg cell was significantly reduced after Langerin+ DCs had been depleted.

Finally, to address the question of whether Langerin+ DCs promote T reg cell survival or whether they are able to induce proliferation of T reg cell, SLA-pulsed LN DCs (including/excluding Langerin+ DCs) were co-cultured with CD4+ CD25neg T cells, and induction of T reg cell was assessed after 3 d. Absolute numbers of CD4+ CD25+ Foxp3+ and, additionally, the ratio of T eff/T reg cell was strongly increased when Langerin+ DCs had been present in the co-culture (Fig. 4 E). DCs without antigen showed only a weak capacity to induce T reg cell.

Collectively, our results reveal a novel inhibitory role of LCs in orchestrating the anti-*Leishmania* response by suppressing efficient development of protective Th1 immunity.

In addition, LCs favor the recruitment of T reg cell to the site of infection, resulting in aggravated disease. Mice on a *Leishmania*–resistant C57BL/6 background exhibit attenuated disease in the absence of epidermal LCs, suggesting that the parasites may possess well adapted strategies to exploit the regulatory capacities of LCs to escape efficient immune surveillance. Nevertheless, LC-competent C57BL/6 mice still display a self-healing phenotype, arguing that the presence of LCs is insufficient to mediate disease susceptibility in this strain. Our data are also relevant for DC-based vaccination strategies, as infected LCs may induce *Leishmania*-specific immunosuppression rather than parasite control. Indeed, our findings suggest that efforts to selectively deplete LCs from the skin or avoid targeting LCs during vaccination may benefit patients suffering from (cutaneous) leishmaniasis.

MATERIALS AND METHODS

Mice. Langerin-DTR mice (C57BL/6) were bred as heterozygotes (Bennett et al., 2005) and housed under specific pathogen-free conditions in the animal care facility in Mainz. Animal experimentation was conducted in accordance with current federal guidelines and approved by the national authorities (Landesuntersuchungsamt, LUA 23 170–07/ G07-1-022).

Parasites and infection. Metacyclic promastigotes of *L. major* clone VI (MHOM/IL/80/Friedlin) were prepared as described previously (von Stebut et al., 2003). Groups of three to five mice were infected with low-dose (10^3) inocula by intradermal injection into ear skin. In some experiments, DT (Sigma-Aldrich) was diluted in sterile PBS, and ∼26 ng/g body weight was injected i.p. as indicated. Lesion volumes were measured weekly in three dimensions and are reported as ellipsoids: [(a/2 × b/2 × c/2) × 4/3π]. Parasites present in lesional tissue were enumerated using a limiting dilution assay as previously described (von Stebut et al., 2003).

Preparation of epidermal sheets. The dorsal and ventral halves of mouse ears were split mechanically and placed dermis down into Petri dishes containing 0.5 M NH$_4$SCN dissolved in 0.1 M Na-KHPO$_4$ buffer at 37°C for 20 min. Epidermis was removed, fixed with ice-cold acetone, and rehydrated in PBS. Epidermal sheets were stained with FITC anti–mouse I-A/I-E (2G9; BD) for 1.5 h at room temperature. After washing with PBS, the epidermal sheets were mounted on slides with Fluorescent Mounting Medium (Dako). Images were analyzed at room temperature using a fluorescent microscope (Axiovert S100; Carl Zeiss).

Cytokine profiles of infected mice. For measurement of antigen-specific cytokine production, retroauricular LNs were recovered and single cell suspensions were prepared. One million LN cells/200 μl complete RPMI 1640 (BioWhittaker) were added to 96-well plates in the presence of 25 μg/ml SLA. Supernatants were harvested 48 h after stimulation and assayed using ELISAs specific for IL-12p40 and IFN-γ (R&D Systems), as well as IL-4 and IL-10 (BD).

Cell isolation from infected mouse ears. Ears infected with *L. major* were excised, soaked in 70% ethanol, and washed with PBS. The ears were split into halves and placed in 0.5 mg/ml liberase (Sigma-Aldrich) diluted in RPMI 1640 with 5% penicillin/streptomycin for 1.5 h at 37°C. Liberase was inactivated by adding complete RPMI 1640 containing 5% FCS. The ears were put into 50 μM Medicon homogenizers (BD) with 1 ml of complete RPMI 1640 and homogenized in a Medimachine (BD) for 7 min. The homogenized ears were then passed through a 70-μm-pore size filter and centrifuged at 200 *g* for 8 min. Cells were resuspended in PBS and labeled with antibodies for flow cytometry.

DC and CD4$^+$ T cell isolation from LN cells. For DC isolation, LNs from DT- or PBS-treated Langerin-DTR mice were harvested and single cell suspensions were prepared. DC isolation was performed using MACS CD11c MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. For isolation of CD4$^+$ T cells, LN cells from *L. major*–infected and healed C57BL/6 were collected and enriched using MACS CD4$^+$ MicroBeads (Miltenyi Biotec). For isolation of CD4$^+$ CD25$^-$ T cells, LN cells from *L. major*–infected and healed C57BL/6 were collected and enriched using MACS CD4$^+$ CD25$^+$ regulatory T cell isolation kit (Miltenyi Biotec), finally using the CD4$^+$ CD25$^-$ cell fraction.

Isolation of EC and dermal cell suspensions. ECs were prepared from ear skin by trypsinization (0.5% trypsin [GE Healthcare] in HBSS [BioWhittaker] for 30 min at 37°C) and dissociation of epidermal sheets by gentle pipetting in HBSS/0.05% DNase/30% FCS. Cell clumps were removed by passing the cell suspension through a 70-μm nylon mesh.

Dermal cell suspensions were prepared from ear skin after removal of the epidermis. Dermal tissue was minced into small pieces and digested for 2 h at 37°C with a solution of HBSS, 0.5% Collagenase (Sigma-Aldrich), 0.05% DNase, and 2% FCS. Dermal Langerin$^+$ cells were identified by expression of CD11c, Langerin, and CD103.

Antibodies and flow cytometry. The following antibodies were purchased from BD: PerCP-Cy5.5 rat anti–mouse CD8α (53–6.7), PE rat anti–mouse CD4 (L3T4), and FITC rat anti–mouse CD25 (7D4). Intracellular APC anti–mouse Foxp3 (FJK-16s; eBioscience) staining was performed using eBioscience reagents according to the manufacturer's protocol. Single cell suspensions were stained first for surface markers, followed by a permeabilization step and staining for Foxp3. For analysis of skin and LN DCs the following antibodies were used: Pacific blue anti–mouse MHCII (M5/114.15.2; BioLegend), PerCPCy5.5 anti–mouse CD103 (2E7; BioLegend), APC anti–mouse CD11c (HL3; BD), PE anti–mouse CD11c (N418; eBioscience), and Alexa Fluor 488 Langerin (929F3.01; Dendritics) for intracellular staining. Cells were analyzed using a FACSCalibur or LSRII cytometer (BD) and FlowJo software (Tree Star).

BM chimeras. Langerin DTR and C57BL/6 donor BM was used to reconstitute lethally irradiated (9 Gray) recipient mice (C57BL/6 and Langerin-DTR, respectively). The resulting chimeric mice were allowed to reconstitute their hematopoietic compartment for at least 6 wk before analysis. Subsequently, mice were infected with physiologically relevant low dose inocula of *L. major* and lesion development was assessed weekly.

Statistical analysis. Statistical analysis was performed using StatView software and Student's *t* test.

Online supplemental material. Fig. S1 shows the depletion of Langerin$^+$ DCs in the epidermis, dermis, and LN of Langerin-DTR mice 48 h after administration of DT. Fig. S2 illustrates the course of disease in high-dose–infected Langerin-DTR mice with and without DT treatment. Fig. S3 depicts the selective depletion/slow reconstitution of LCs 2 wk after DT administration and confirms specific depletion of LCs and Langerin$^+$ dDCs in different BM chimeras. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20102318/DC1.

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