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Langerhans cells protect from allergic contact dermatitis in mice by tolerizing CD8+ T cells and activating Foxp3+ regulatory T cells

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Allergic contact dermatitis is the most frequent occupational disease in industrialized countries. It is caused by CD8+ T cell–mediated contact hypersensitivity (CHS) reactions triggered at the site of contact by a variety of chemicals, also known as weak haptens, present in fragrances, dyes, metals, preservatives, and drugs. Despite the myriad of potentially allergenic substances that can penetrate the skin, sensitization is relatively rare and immune tolerance to the substance is often induced by as yet poorly understood mechanisms. Here we show, using the innocuous chemical 2,4-dinitrothiocyanobenzene (DNTB), that cutaneous immune tolerance in mice critically depends on epidermal Langerhans cells (LCs), which capture DNTB and migrate to lymph nodes for direct presentation to CD8+ T cells. Depletion and adoptive transfer experiments revealed that LCs conferred protection from development of CHS by a mechanism involving both anergy and deletion of allergen-specific CD8+ T cells and activation of a population of T cells identified as ICOS+CD4+Foxp3+ Tregs. Our findings highlight the critical role of LCs in tolerance induction in mice to the prototype innocuous hapten DNTB and suggest that strategies targeting LCs might be valuable for prevention of cutaneous allergy.

Introduction

Allergic contact dermatitis (ACD) is a common eczematous skin disease of high socioeconomic impact, as it is the most prevalent chronic occupational disease (1), with life-long persistence due to the absence of curative treatments. Skin inflammation results from a T cell–mediated contact hypersensitivity (CHS) reaction, which occurs in sensitized individuals at the site of contact with a variety of chemicals, also known as haptens, present in fragrances, dyes, metals, preservatives, and drugs (2). Most of our knowledge of the mechanisms that control ACD comes from mouse models of CHS to experimental haptens with strong sensitizing properties such as 2,4-dinitrofluorobenzene (DNFB) or oxazolone. Allergic sensitization during the asymptomatic phase of the disease leads to the priming of specific cytotoxic CD8+ T cells (3) after capture and presentation of the allergen by skin DCs to T cells in skin-draining LNs. During the symptomatic phase of CHS, elicited by reexposure to the hapten, activated effector CD8+ T cells are recruited into the skin and initiate the inflammatory cascade by inducing apoptosis of keratinocytes (4), leading to skin edema. Many studies have highlighted a central role of CD4+CD25+Foxp3+ Tregs in the control of CHS through their capacity to suppress specific CD8+ T cell effectors during both sensitization (5–7) and the resolution of skin inflammation (8–10). In contrast to strong experimental haptens such as DNFB, which sensitizes mice after a single contact, most human contact allergens fall into the category of weak haptens because they are immunogenic only after repeated exposure in a fraction of individuals and do not induce CD8+ T cell–mediated CHS responses in normal mice. The idea that immune tolerance rather than ignorance explains the general innocuity of weak haptens is supported both in mouse and human. Indeed, we showed that normal mice do not mount CHS responses to common chemical allergens of fragrance unless they are acutely depleted of Tregs (11). Moreover, studies of nickel allergy best illustrated that a weak sensitizing allergen can activate Tregs, as most healthy control individuals harbor functional allergen-specific and suppressive CD4+CD25+ Tregs (12). The mechanisms through which APCs prevent skin sensitization of normal individuals to these weak allergens remain to be elucidated.

Despite our growing knowledge of the immunobiology of skin DCs, identification of those that account for natural tolerance to weak sensitizing haptens is still lacking. Langerhans cells (LCs), which constitute the only DCs present in the epidermis at steady state, express the C-type lectin Langerin (CD207) responsible for the formation of Birbeck granules and the adhesion molecule EpCAM, and renew by local proliferation of radio-resistant precursors (13). The dermis contains CD207− dermal DCs (dDCs) and CD207+ dDCs, which derive from radiosensitive BM precursors (14–16) and can be further subcategorized based on CD103 and CD11b expression (14, 17). Recently, the use of BM chimeric mice and several Langerin knockin and transgenic mouse lines allowing for constitutive or acute depletion of CD207+ DCs has challenged the old view that LCs constitute the most critical APCs for initiation of skin immunity. Indeed, in most experimental settings, LCs are dispensable for initiation of CHS to strong haptens (18, 19), which may require CD207+ dDCs (14) and/or newly recruited monocyte-derived DCs (20, 21). Some of these studies in fact

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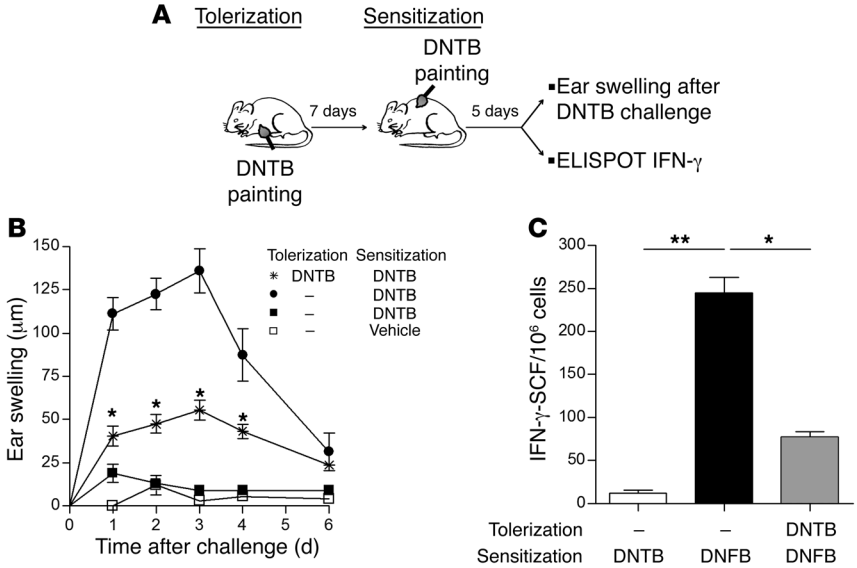

Figure 1

DNTB pretreatment suppresses priming of CD8+ T cells and CHS to DNFB. (**A**) Model of tolerance to DNTB. Mice are tolerized by epicutaneous painting of 100 μl of DNTB on the abdomen at day 0 and sensitized on the back with DNFB at day +7. Five days later, mice were either ear challenged with DNFB to follow the ear-swelling response or sacrificed to determine the frequency of IFN-γ+–producing CD8+ T cells by an ELISPOT assay. (**B**) The mean ear-swelling response (μm ± SD) at various times after DNFB challenge was determined. Mice sensitized with vehicle and challenged with DNFB were used as controls for nonspecific skin inflammation. (**C**) Frequency of DNP-specific IFN-γ SFCs in pooled cutaneous total LN cells 5 days after skin sensitization (mean ± SD). No spots were detected in CD8-depleted cells or without hapten restimulation. One representative experiment out of 5 is shown (5 mice per group). $^{*}P < 0.05$; $^{**}P < 0.01$.

suggest that LCs may have an important role in dampening skin immune response. Indeed, at steady state, LCs migrate continuously to draining LNs, presumably to induce or maintain tolerance to self or innocuous environment antigens (Ag) (22, 23). In line with this hypothesis, LCs were recently shown to act as negative regulators of the anti-Leishmania response (24), to contribute to UV-induced suppression of CHS (25, 26), and to dampen CHS response by a process involving IL-10 and cognate interactions with CD4+ T cells (27). Yet whether and how LCs may prevent initiation of ACD upon skin contact with weak allergens is unknown.

An appropriate antigenic candidate to address this issue is the hapten 2,4-dinitrothiocyanobenzene (DNTB). Although DNTB generates dinitrophenyl–self peptide (DNP–self peptide) motifs similar to those of the strong contact sensitizer DNFB (28, 29), it is unable to activate the inflammasome (30) and to initiate CHS responses in normal individuals (29). DNTB can nevertheless be considered as a prototype of weak contact allergen, as it can sensitize mice and induce a CHS of moderate intensity only under certain experimental conditions, such as repeated cutaneous exposures (ref. 31 and data not shown). Yet under conventional experimental conditions, DNTB behaves as a tolerogen and renders mice tolerant to a subsequent sensitization with DNFB (29). We thus used DNTB to investigate the dynamics and functions of skin DC subsets under tolerogenic conditions and to get insights into mechanisms preventing ACD. Using knockin mice expressing the human diphtheria toxin (DT) receptor (DTR) (Lang-DTR) or EGFP (Lang-EGFP) under the control of the *langerin* gene, and allowing the tracking and conditional depletion of LCs (19), we show here that LCs are the critical skin APCs that mediate induction of skin tolerance to this innocuous hapten and that protect from allergic skin inflammation by inducing anergy/deletion of allergen-specific T cells and by activating CD4+Foxp3+ Tregs.

Results

Induction of tolerance to the innocuous hapten DNTB is associated with migration of both LCs and CD207– dDCs to skin-draining LNs. In order to identify the mechanisms responsible for induction of cutaneous tolerance toward weak contact allergens, we used the innocuous hapten DNTB, which shares the DNP antigenic motif with the strong contact sensitizer DNFB (28). C57BL/6 (B6) mice were skin painted with either DNTB or DNFB and ear challenged 5 days later with DNFB, as depicted in Figure 1A. While DNFB-sensitized mice developed a robust CHS response, peaking at 48–72 hours after challenge, DNTB-painted mice did not develop skin inflammation (Figure 1B), in line with the inability of DNTB to prime hapten-specific IFN-γ–producing CHS effector T cells in skin-draining LNs (Figure 1C). In addition, painting with DNTB 1 week prior to DNFB sensitization strongly inhibited the priming of IFN-γ–producing CD8+ effector T cells (Figure 1C) and significantly reduced ear swelling (Figure 1B), in accordance with the previously reported tolerizing property of DNTB (29).

To analyze the migration of cutaneous DCs to draining LNs during induction of tolerance, we used Lang-EGFP mice (19), in which EGFP expression in skin is confined to epidermal LCs and to a minor population of dDCs that express the Langerin molecule (14–16). We applied the fluorescent dye tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC) a few minutes after DNTB, but without the irritant molecule dibutyl-phthalate (DBP), which is most often used in migration experiments (19). In control mice that received TRITC and only the vehicle used for DNTB, few TRITC+ cells were detected among LN DCs at 48 hours (Figure 2A), most likely reflecting steady state skin DC migration. Surprisingly, DNTB, similarly to the skin irritant DBP used as a positive control, strongly increased the frequency of EGFP+TRITC+ and EGFP–TRITC+ cells among LN DCs (Figure 2, A and B), indicating that DNTB induced migration of both CD207+ skin DCs and CD207– dDCs. To determine whether TRITC-carrying CD207+ DCs (Figure 2C) corresponded to skin-derived LCs or CD207+ dDCs (14–16), we generated BM chimeric mice using either Lang-EGFP or congenic CD45.1 B6 Ly5a donors. Eight weeks after reconstitution of lethally irradiated mice with BM cells, nearly all LCs remained of host origin due to their radio resistance (13), while the vast majority of CD207+ dDCs were replaced by donor BM–derived DCs (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI59725DS1). Importantly, 48 hours after DNTB painting, TRITC-carrying DCs

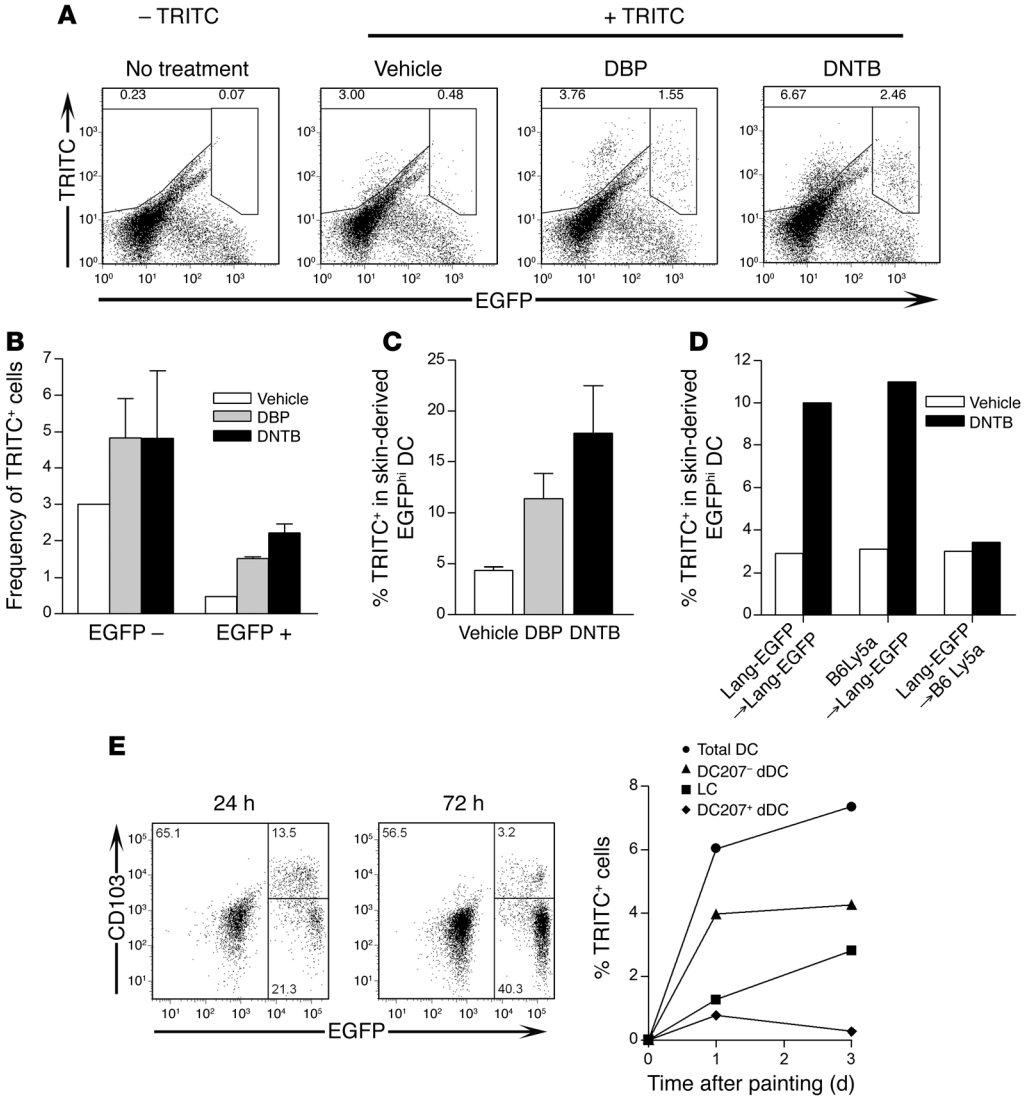

Figure 2

Both LCs and CD207⁻ dDCs migrate to LNs upon induction of cutaneous tolerance. To track DC migration from skin to draining LNs, Lang-EGFP mice (**A**–**C**), various types of BM chimeric mice (**D**), and B6 mice (**E**) were ear painted with either vehicle, DBP, or DNTB, followed by application of TRITC on the same site. At different time points after ear painting, CD11c⁺ cells were enriched from cervical LNs and the frequency of TRITC⁺ cells was determined by flow cytometry. (**A**) Representative dot plot of EGFP versus TRITC expression by LN DCs (gated as viable CD11c⁺ cells) at 48 hours. The numbers indicate the percentages of TRITC⁺EGFP^{hi} (LCs and CD207⁺ dDCs) and TRITC⁺EGFP⁻ (CD207⁻ dDCs) cells among LN DCs. (**B**) Percentages of TRITC⁺EGFP^{hi} and TRITC⁺EGFP⁻ cells in a pool of 3 independent experiments (mean ± SD). (**C**) Frequency of TRITC⁺ cells among LN EGFP^{hi} DCs in Lang-EGFP mice (mean ± SD) and (**D**) in various BM chimeric mice at 48 hours after painting. DC chimerism in both the epidermis and dermis is shown in Supplemental Figure 1, and results correspond to the mean of 2 independent experiments. (**E**) Representative profile of EGFP versus CD103 expression in skin-emigrated DCs (gated as viable CD11c⁺MHC-II^{hi}TRITC⁺ cells) at 24 hours and 72 hours after skin painting with DNTB showing the percentages of CD207⁻ dDCs, CD207⁺CD103⁺ dDCs, and CD207⁻CD103⁻ LCs (left panels). Absolute frequency of each TRITC⁺ DC subset among LN skin-derived CD11c⁺MHC-II^{hi} DCs (right panel).

were detected among EGFP⁺ DCs in LNs when EGFP expression was restricted to epidermal LCs (B6 Ly5a→Lang-EGFP chimeric mice) but not when only CD207⁺ dDCs were EGFP⁺ (Lang-EGFP→B6 Ly5a chimeric mice) (Figure 2D). In addition, analysis in normal nontransgenic mice of migratory skin TRITC⁺MHC-II^{hi} DCs (19) according to CD207 and CD103 expression (Figure 2E) revealed that CD207⁻ dDCs and LCs (CD207⁺CD103⁻) accounted for the majority of the TRITC⁺ DCs present in LNs 72 hours after skin painting with DNTB, while CD207⁺ dDCs were barely detected (Figure 2E). LCs arrived in LNs at 24 hours after skin painting and increased at 72 hours, while migration of CD207⁻ dDCs was already maximal at 24 hours. Together, these data demonstrate that DNTB painting induces emigration of bona fide radio-resistant LCs as well as CD207⁻ dDCs to LNs.

The tolerogen DNTB is presented to CD8⁺ T cells primarily by skin-emigrating LCs. We next determined the nature of the LN DC subsets involved in the presentation of the tolerogen to CD8⁺ T cells, which are the major effector cell type initiating CHS responses (3). To

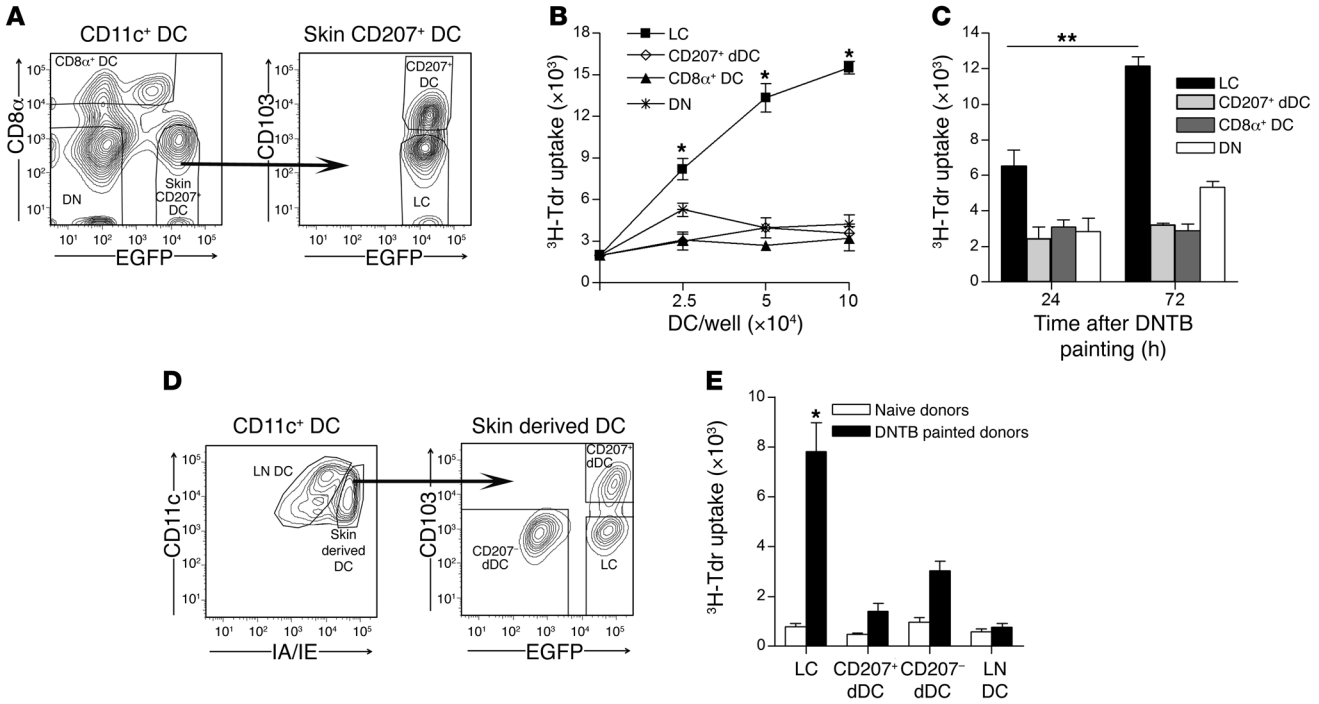

Figure 3

Presentation of DNTB-modified peptides in LNs is mostly performed by CD207+CD103− LCs. CD11c+ cells were enriched from pooled cutaneous LNs of Lang-EGFP mice 24 hours (**C**) or 72 hours (**B**, **C**, and **E**) after abdominal skin painting with DNTB and further FACS sorted into various DC subsets based on 2 different strategies. (**A**) DCs were separated into CD8α+EGFP−/lo DCs, CD8α−EGFP− DN DCs, CD8α−EGFPhiCD103− LCs, and CD8α−EGFPhiCD103+ DCs and cultured with 10^5 DNP-specific CD8+ effector T cells at serial dilutions (**B**) or at a fixed number (2.5×10^4) (**C**). (**D**) DCs were separated into MHC-IIint LN-resident DCs, MHC-IIhiEGFPhiCD103+ DCs (CD207+ dDCs), MHC-IIhiEGFPhiCD103− DCs (LCs), and MHC-IIhiEGFP−CD103− DCs (CD207− dDCs), and 2.5×10^4 cells of each DC subset were cultured with DNP-specific CD8+ effector T cells (white bars: DCs from control naive mice, black bars: DCs from DNTB-painted mice). Proliferation of CD8+ T cells was determined after 3 days by thymidine uptake and is expressed by mean cpm ± SD of triplicate wells. Data are representative of 1 out of 2 (**C** and **E**) to 4 (**B**) independent experiments. *$P < 0.05$; **$P < 0.01$.

this end, we used an ex vivo presentation assay using DNP-specific CD8+ CHS effectors from day 5 DNFB-sensitized mice as indicator cells, based on the fact that DNTB and DNFB painting generates similar antigenic DNP motifs (28) recognized by T cells. Total LN CD11c+ DCs isolated 72 hours after skin painting with DNFB or DNTB similarly induced the proliferation of DNP-specific CD8+ T cells (Supplemental Figure 2A), indicating that DNP motifs are presented to T cells in LNs during both sensitization and tolerization. To determine whether presentation of the tolerogen in LNs requires transport of the hapten by skin DCs, we analyzed the consequences of skin dissection as a way to prevent migration of cutaneous DCs to draining LNs (32). When skin was excised 5 hours after DNTB delivery, the hapten-presenting capacity of LN DCs was strongly reduced (Supplemental Figure 2B). We interpret the remaining Ag-presenting activity as a consequence of incomplete removal of the hapten delivery site, although it is possible that this results from the rapid migration of a minor proportion of skin DCs. These data suggest that presentation of DNTB to CD8+ T cells requires migration of skin DCs to LNs.

Isolation of various LN DC subsets from Lang-EGFP mice on the basis of CD8α and CD207 expression (Figure 3A) indicated that DNTB was presented primarily by skin-derived CD207+ (EGFPhi) DCs (Supplemental Figure 2C), with little if any contribution of LN resident CD8α+ DCs and CD8α−EGFP− double-negative (DN) DCs. Further subsorting of EGFPhi DCs according to CD103 expression (14, 17) revealed that CD207+CD103− LCs, but not CD207+CD103+ dDCs, induced DNP-specific CD8+ T cell proliferation when isolated either at 24 hours or 72 hours after DNTB skin delivery/application (Figure 3, B and C). Importantly, all DC subsets were able to induce proliferation of specific CD8+ T cells, albeit with different efficacy, when pulsed ex vivo with DNBS (the soluble form of the hapten) or the OVA SIINFEKL class I peptide (Supplemental Figure 3), indicating that the sorting strategy had not altered their Ag-presenting functions.

We then confirmed that Ag presentation was confined to bona fide LCs and was not due to the minor fraction of CD207+ dDCs present in LN CD207+CD103− DCs (17) by using DCs from DNTB-painted BM chimeras. Indeed, proliferation of DNP-specific CD8+ T cells was observed when EGFPhi DCs consisted of radio-resistant host-derived LCs (B6 Ly5a→Lang-EGFP mice), but not when they only contained donor-derived CD207+ dDCs (Lang-EGFP→B6 Ly5a mice) (Supplemental Figure 4). DN DCs contained several subsets, including CD11b+ LN DCs and CD207− dDCs. To directly assess the Ag-presenting function of the latter cells, which migrate to LN during DNTB tolerance (Figure 2, B and E), we used a different sorting strategy (Figure 3D) to isolate total LN DCs (MHC-IIint DCs) and the 3 subsets of skin-emigrating DCs (MHC-IIhi DCs) (19). Stimulation of DNP-specific CD8+ T cells was primarily observed with LCs and, to some extent, with CD207− dDCs, but neither with CD207+ dDCs nor LN DCs (Figure 3E).

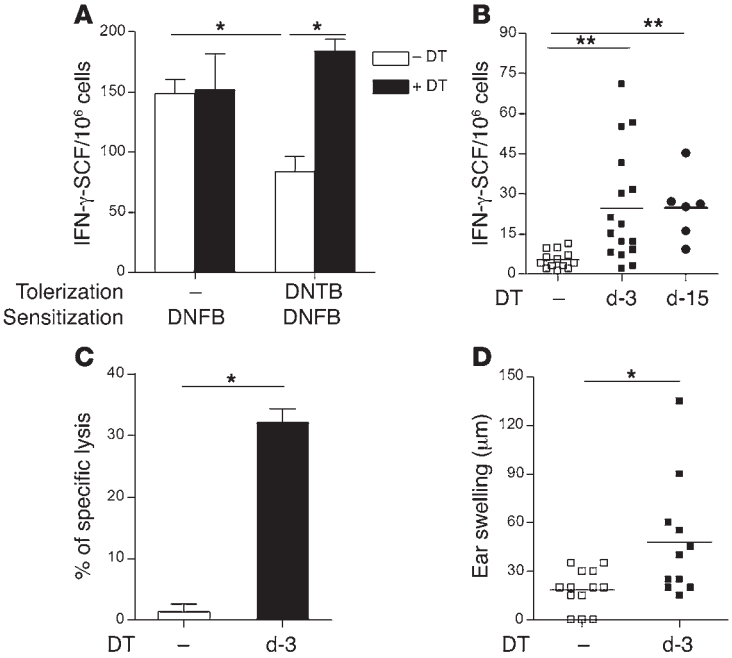

Figure 4

In vivo depletion of CD207⁺ DCs abrogates skin tolerance to DNTB and allows for priming of cytotoxic CD8⁺ T cells and CHS responses. (**A**) Lang-DTR mice injected (black bars) or not (white bars) with DT at day –3 were tolerized with DNTB at day 0 or left untreated, and were sensitized with DNFB at day +7. The frequency of hapten-specific IFN-γ SFCs in LNs was determined by ELISpot in individual mice and is expressed as mean ± SD SFCs per 10^6 total LN cells. (**B**–**D**) Lang-DTR mice injected with DT at day –3 or day –15 were painted at day 0 with DNTB. On day +5, the frequency of hapten-specific IFN-γ–producing cells in LNs was determined (mean ± SD) (**B**). In vivo hapten-specific cytotoxic activity was calculated after injection of DNP-loaded target cells (mean ± SD) (**C**), and the ear-swelling response was determined 24 hours after ear challenge with DNTB (**D**). Each symbol corresponds to an individual mouse, and horizontal bars indicate the mean. Results correspond to 1 representative experiment out of 3, with 3 mice per group in each (**A** and **C**) and to pooled data from 2 (**B**, day –15; **D**) to 4 (**B**, day –3) independent experiments. Statistical analysis was performed using (**A** and **B**) the Kruskal-Wallis test with Dunn's post-test and (**C** and **D**) the Mann-Whitney test. $^*P < 0.05$; $^{**}P < 0.01$. Data are shown as mean ± SD.

Together, these ex vivo functional assays demonstrate that during induction of tolerance with DNTB, LCs, and to a lesser extent CD207⁻ dDCs, transport and present the hapten to CD8⁺ T cells in LNs.

Depletion of LCs breaks tolerance to DNTB and allows for priming of cytotoxic CD8⁺ T cells mediating CHS responses. To determine the contribution of LCs to DNTB-induced tolerance, Lang-DTR mice were depleted of CD207⁺ cells by injection of DT 3 days before DNTB tolerization and were subsequently sensitized with DNFB. FACS analysis at the time of DNTB delivery confirmed that DT-injected mice were devoid of all skin CD207⁺ DCs, including both CD207⁺ (EGFP⁺EpCAM⁺) LCs residing in the epidermis or transiting in the dermis and CD207⁺ (EGFP⁺EpCAM⁻) dDCs (Supplemental Figure 5). Importantly, DNTB painting significantly reduced the priming of hapten-specific IFN-γ–producing T cells in response to DNFB sensitization in control Lang-DTR mice, but not in CD207⁺ DC–deficient mice (Figure 4A). In addition, while normal mice did not show any signs of CD8⁺ T cell priming after DNTB skin delivery, Lang-DTR mice depleted in all CD207⁺ DCs (DT injection at day –3) displayed DNP-specific IFN-γ⁺CD8⁺ T cells (Figure 4B) and in vivo cytotoxic CD8⁺ T cell activity (Figure 4C) in LNs and developed moderate but significant skin inflammation — as measured by ear swelling — after DNTB ear skin challenge (Figure 4D). Similar results were observed in mice solely deficient in LCs (Figure 4B), which were obtained by injecting Lang-DTR mice with DT (14–16), a protocol allowing recovery of CD207⁺ dDCs to nearly normal numbers at the time of DNTB painting (Supplemental Figure 5). Together, the above results demonstrate that LCs are responsible for DNTB-induced skin tolerance and protect from development of a CHS response to this weak and innocuous allergen.

Epidermal LCs can transfer tolerance to naive mice. To confirm the tolerogenic potential of LCs, we tested their capacity to confer DNTB-specific suppression upon in vivo transfer to naive B6 mice. LCs were purified by density gradient centrifugation from the epidermis of Lang-EGFP mice 4 hours after application of either DNTB or the vehicle as control, and 2.5×10^4 cells were transferred s.c. to naive B6 recipients 7 days prior to sensitization with DNP-loaded BM-derived DCs (BMDCs). Five days later, mice were either sacrificed to measure the DNP-specific CD8⁺ T cell response or ear challenged with DNFB to measure the CHS response (Figure 5A). LC suspensions (low density fraction) contained 70%–90% EGFP⁺ LCs and few contaminating keratinocytes, but no EGFP⁻CD3⁺ dendritic epidermal T cells (DETC), which were only present in the high-density fraction (Supplemental Figure 6). Similarly to DNTB painting, adoptive transfer of LCs from DNTB-painted mice inhibited the priming of DNP-specific CHS effectors (Figure 5, C–E), resulting in nearly complete prevention of the ear swelling in response to DNFB challenge (Figure 5B). To exclude a contribution of contaminating keratinocytes to immune suppression, we used Lang-DTR donor mice, in which LCs were depleted by DT injection. In contrast to LCs from DNTB-painted and DT-untreated Lang-DTR mice, the low-density fraction from DNTB-painted and DT-treated animals (which contained mostly keratinocytes; Supplemental Figure 6) was unable to suppress the priming of DNP-specific IFN-γ⁺CD8⁺ T cells (Figure 5E), demonstrating that LCs but not keratinocytes were the tolerogenic cells. Although DETCs are (innate) γδ T lymphocytes reported to regulate inflammatory diseases (33), DETC-enriched cells from DNTB-painted mice (Supplemental Figure 6) were unable to suppress priming of CHS effector CD8⁺ T cells, even when they were purified from LC-deficient donors (Figure 5E). These data demonstrate that LCs constitute the only epidermal cell type able to induce DNTB tolerance.

Tolerance by LCs requires cognate interactions with CD8⁺ and CD4⁺ T cells. CHS is initiated by hapten-specific MHC-I–restricted cytotoxic CD8⁺ T cells (34) and controlled by MHC-II–restricted CD4⁺CD25⁺Foxp3⁺ Tregs (5, 8). Thus, we asked whether LCs induced tolerance by direct interaction with CD8⁺ T cells and/or indirectly via induction and/or activation of Tregs. In contrast to epidermal LCs from WT B6 donors, LCs from DNTB-painted *β2m*$^{-/-}$ or *H2-Aβ1*$^{-/-}$ donor mice (i.e., deficient in MHC-I or -II molecules, respectively) were unable to suppress DNP-specific CD8⁺ T cells and CHS responses upon transfer to naive B6 recipients (Figure 5, F and G). It is unlikely that MHC-I–deficient LCs failed to induce

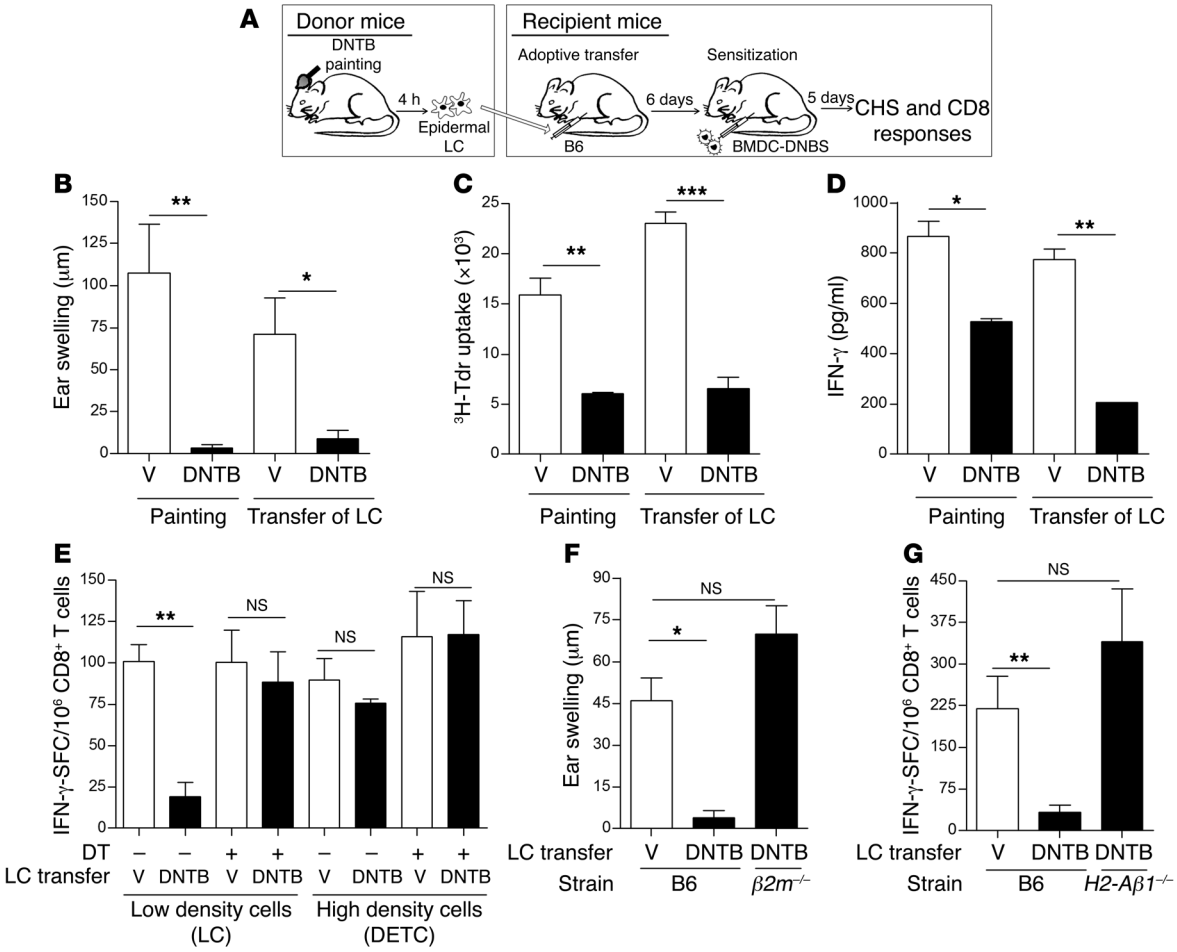

Figure 5

Epidermal LCs can transfer tolerance to DNTB. (**A**) Assessment of LC function by adoptive transfer. Ear epithelial sheets were prepared 4 hours after vehicle or DNTB painting and used to enrich for LCs by centrifugation over an OptiPrep gradient (low-density fraction). Naive B6 mice were transferred s.c. with 2.5×10^4 enriched LCs and were immunized 6 days later by s.c. injection of 10^5 DNBS-loaded BMDCs. The effect of LC transfer was determined by measuring the following: (**B** and **F**) the ear swelling that developed 24 hours after DNFB challenge (mean μm ± SD of 6 individual mice), (**C** and **D**) the capacity of CD8⁺ T cells isolated from LN 5 days after immunization to proliferate and to produce IFN-γ in vitro after stimulation with DNBS-loaded APCs (mean ± SD of triplicate wells), and (**E** and **G**) the frequency of DNP-specific IFN-γ–producing CD8⁺ T cells in LNs (mean SFCs per 10^6 CD8⁺ T cells ± SD of pooled mice, using 3–4 serial dilutions performed in duplicate). (**B**–**D**) Comparisons of the suppressive effect of LC transfer versus direct DNTB painting. (**E**) Lang-DTR mice, injected or not with DT 3 days before DNTB or vehicle ear painting, were used to prepare LCs (low-density fraction) or DETCs (high-density fraction) using an OptiPrep gradient (Supplemental Figure 5). (**F** and **G**) Comparison of the suppressive potential of LCs from B6, β*2m*⁻/⁻ (**F**), and *H2-Aβ1*⁻/⁻ (**G**) donor mice. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

tolerance due to their elimination by host NK cells because WT and β*2m*⁻/⁻ DCs survived equally well in recipient LNs (data not shown). This suggests that LCs need to interact directly with CD8⁺ T cells and with CD4⁺ T cells to induce DNTB tolerance.

Direct DNTB presentation by LCs to CD8⁺ T cells in skin-draining LNs induces their deletion or anergy in vivo. The fact that LCs present DNTB to specific CD8⁺ T cells ex vivo and require MHC-I expression for their tolerogenic function suggested that cognate interactions with LCs might impair the capacity of DNTB-specific CD8⁺ T cells to subsequently differentiate into CHS effectors, as has been previously documented for T cells interacting with plasmacytoid DCs during oral tolerance (35). To test this hypothesis, we used our previously described CD8⁺ T cell transfer model of CHS in T cell–deficient *Cd3*ε⁻/⁻ mice (Figure 6A and ref. 35) to analyze the outcome of DNTB skin painting on the CHS effector function of skin-draining LN CD8⁺ T cells. CD8⁺ T cells from control vehicle–painted or DNFB-sensitized B6 mice differentiated into IFN-γ⁺ effector cells in *Cd3*ε⁻/⁻ recipients, while those from DNTB-tolerized mice were virtually nonresponsive to skin sensitization with DNFB (Figure 6B). Alternatively, CD8⁺ T cells from Lang-DTR donor mice that were treated with DT prior to DNTB painting exhibited a normal ability to respond to DNFB sensitization (Figure 6C), suggesting that LCs induced DNP-specific CD8⁺ T cell unresponsiveness in lymphoid organs.

To further determine whether direct cognate interaction between LCs and CD8⁺ T cells was sufficient to initiate DNP-specific CD8⁺ T cell hyporesponsiveness, CD8⁺ T cells from naive B6 mice were precultured for 3 days with epidermal LCs from either vehicle- or DNTB-treated animals and transferred into *Cd3*ε⁻/⁻ recipients (Figure 6A). CD8⁺ T cells cocultured with LCs from DNTB-tolerized

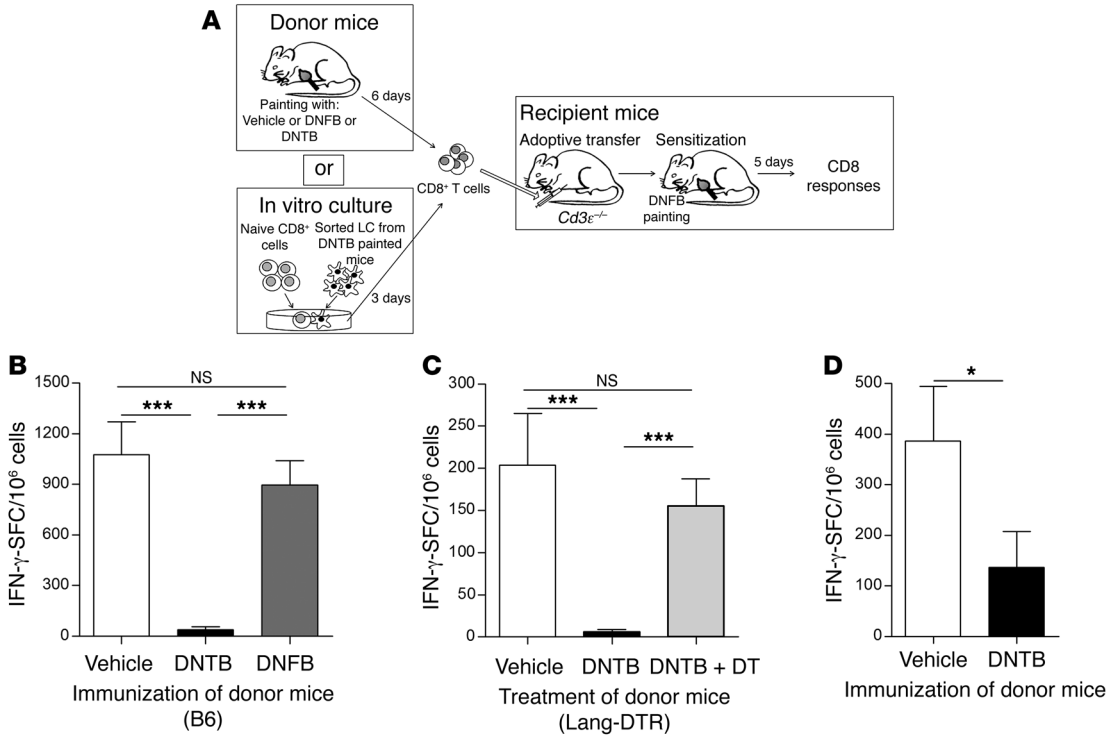

Figure 6

LCs renders hapten-specific CD8$^+$ T cells hyporesponsive to subsequent skin sensitization. (**A**) In vivo assay to study CD8$^+$ T cell functions. *Cd3*ε$^{-/-}$ T cell–deficient mice were injected i.v. with 5 × 10^6 (**B**) or 1 × 10^6 (**C** and **D**) purified CD8$^+$ T cells and were skin sensitized with DNFB 1 day (**B**) or 4 weeks later (**C** and **D**). Five days after sensitization, recipient mice were sacrificed, and the frequency of the DNP-specific IFN-γ–producing cells in cutaneous LNs was determined by ELISpot. (**B**) Functions of CD8$^+$ T cells purified from cutaneous LNs of B6 mice 6 days after abdominal skin painting with vehicle (white bars), DNTB (black bars), or DNFB (gray bars). (**C**) CD8$^+$ T cells were isolated from Lang-DTR donor mice that were injected or not with DT 3 days prior to DNTB painting. (**D**) CD8$^+$ T cells from naive B6 mice were cultured in vitro with LCs (1 LC for 20 CD8$^+$ T cells) isolated from the epidermis 4 hours after vehicle (white bar) or DNTB (black bar) painting, were sorted to high purity after 3 days to remove LCs, and were injected into *Cd3*ε$^{-/-}$ mice. Results are expressed as the mean value ± SD of IFN-γ SFCs for 3 individual mice and are representative of 2 (**B**) and 3 (**C** and **D**) independent experiments. *$P < 0.05$; ***$P < 0.001$.

mice generated roughly 3 times less DNP-specific IFN-γ–producing CD8$^+$ effector T cells as compared with CD8$^+$ T cells cultured with control LCs (Figure 6D). These data demonstrate that presentation of DNTB by LCs to naive CD8$^+$ T cells abrogates their ability to differentiate into IFN-γ$^+$ CHS effectors.

Preexisting Foxp3$^+$ Tregs are activated by LCs and are essential for LC-mediated tolerance. CD4$^+$Foxp3$^+$ Tregs are critical regulators of CHS responses (5, 8), and our recent data highlighted the unique suppressive potential of activated ICOS$^+$CD4$^+$Foxp3$^+$ Tregs in CHS to DNFB (7). Although DNTB painting did not affect the overall frequency of CD4$^+$Foxp3$^+$ Tregs in skin-draining LNs (data not shown), the proportion of activated ICOS$^+$ cells among CD4$^+$Foxp3$^+$ Tregs increased by 2- to 3-fold 5 days after tolerance induction (Figure 7A), indicating that an innocuous and tolerogenic hapten can activate Tregs. Importantly, transfer of LCs from DNTB-painted mice similarly resulted in a dramatic increase in the frequency and number of ICOS$^+$CD4$^+$Foxp3$^+$ Tregs, and this required MHC-II expression by LCs (Figure 7A). To determine whether preexisting Tregs are instrumental for LC-induced tolerance, we tested the outcome of LC transfer into DEREG recipient mice (Figure 7B), which allowed for conditional ablation of CD4$^+$Foxp3$^+$ Tregs by injection of DT (8). In DT-treated DEREG mice, CD4$^+$Foxp3$^+$ Tregs were almost completely absent (>95% depletion) at the time of LC transfer, but had recovered by more than 70% at the time of DNP-BMDC immunization (Supplemental Figure 7). As expected, transfer of LCs from DNTB-tolerized mice strongly suppressed the priming of DNP-specific IFN-γ$^+$CD8$^+$ T cells in DT-injected WT mice. In contrast, suppression of T cell priming was no longer achieved in DT-treated DEREG mice (Figure 7B). These data demonstrate that preexisting CD4$^+$Foxp3$^+$ Tregs are required for LC-mediated tolerance and suggest that the tolerogenic potential of LCs is linked to their efficacy in triggering their activation and expansion.

Discussion

ACD results from a breakdown of skin tolerance that allows for the priming of pathogenic CD8$^+$ T cells to normally innocuous weak haptens. Yet the nature of skin DCs and mechanisms responsible for induction of tolerance to such common allergens remains poorly understood. Our study, using DNTB as a prototype of such a weak contact allergen, demonstrates that epidermal LCs are the only skin DCs that protect from ACD by orchestrating 2 complementary mechanisms mediated by in vivo presentation of the allergen by MHC-I and -II molecules, respectively. First, LCs induce anergy or deletion of a large fraction of allergen-responsive CD8$^+$ T cells, and second, they expand ICOS$^+$CD4$^+$Foxp3$^+$ Tregs from a preexisting pool to achieve efficient suppression of ACD.

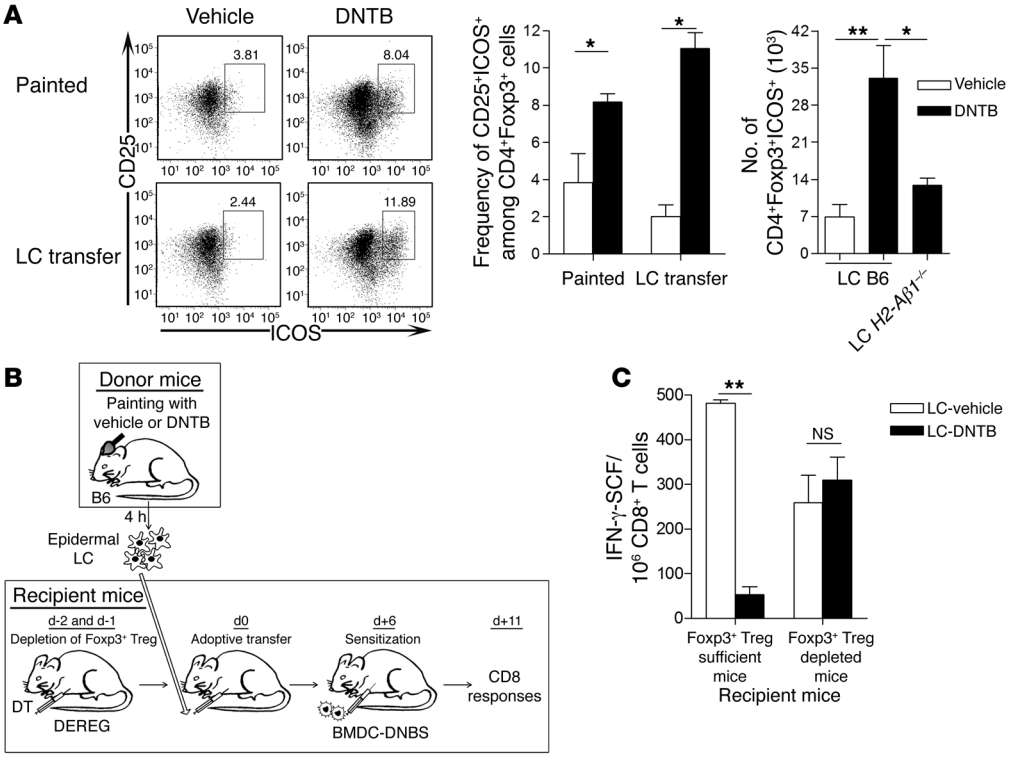

Figure 7

LCs require CD4+Foxp3+ Tregs to induce tolerance. (**A**) Expression of ICOS and CD25 on LN CD4+Foxp3+ Tregs from B6 mice 5 days after skin painting with vehicle or DNTB, or after s.c. transfer of LCs isolated from either vehicle- or DNTB-painted B6 or *H2-Aβ1*$^{-/-}$ mice. Representative dot plots showing the percentages of ICOS+CD25+ cells in gated CD4+Foxp3+ cells are shown (left panel). The mean frequency of ICOS+CD25+ cells among CD4+Foxp3+ Tregs and the absolute number of ICOS+CD4+Foxp3+ Tregs observed in 4 independent mice (mean ± SD) are also shown (right panels). (**B**) Foxp3-DTR (DEREG) mice and WT littermates were injected twice with 1 μg of DT at day –2 and day –1 and received at day 0 LCs from ear epidermis of vehicle-painted (white bars) or DNTB-painted (black bars) mice. (**C**) Suppressive function of LCs was assessed as depicted in Figure 6A by measuring the frequency of DNP-specific IFN-γ+ T cells in LNs (mean ± SD) after immunization with DNBS-loaded BMDCs. The data are representative of 2 independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

The first important finding relates to the unexpected observation that a weak contact allergen elicits the emigration of LCs from epidermis to draining LNs. It is generally believed that only strong haptens such as DNFB, which are able to activate the inflammasome (30), trigger LC emigration from the skin (18). Indeed, based on undetectable changes of LC density in epidermal sheets (ref. 36 and data not shown), it was concluded that weak haptens such as DNTB induce very little if any migration of skin DCs. In order to follow skin DC migration under more physiologic conditions, we employed a modified version of the commonly used migration assay with the fluorescent dye TRITC, circumventing the untoward irritancy of DBP by the use of acetone to dilute TRITC. By analyzing the phenotype of skin-emigrated DCs and by using BM chimeric mice (14–16), we were able to demonstrate emigration of bona fide epidermal LCs and other skin DC subsets to draining LNs after DNTB skin exposure. As soon as 24 hours after DNTB skin exposure, all subsets of skin DCs were detected in LNs, with CD207^{-} dDCs and LCs contributing to 65% and 21% of skin-emigrated DCs, respectively. While migration of CD207^{-} dDCs was already maximal at 24 hours, migration of LCs increased at later time points. The fact that migration of CD207^{+} dDCs was barely detected may be due to the insufficient sensitivity threshold of our assay for this quantitatively minor skin DC subset (14–16) or to insufficient innate signals induced by DNTB or possibly to the presence of inhibitory signals, as was suggested elsewhere for UV-induced immune suppression (25, 26).

The second original finding pertains to the unanticipated rapid kinetics of LC presentation of DNTB to CD8^{+} T cells in LNs. FACS sorting of the various LN DC subsets from DNTB-painted Lang-EGFP transgenic mice revealed that (a) only LCs, and to a lesser extent CD207^{-} dDCs, but not CD207^{+} dDCs or LN CD8α^{+} DCs, were able to present the allergen to CD8^{+} T cells in LNs and (b) Ag presentation by LCs could be detected as early as 24 hours after skin exposure to the tolerogen. This demonstrates that LCs do not simply ferry the Ag for presentation by LN-resident CD8α^{+} DCs, which are endowed with the capacity to cross-present Ag from incoming DCs (37). In addition, our observations that LCs reached LNs and presented the hapten as soon as 24 hours after skin delivery of DNTB contrast with the general view that LCs reach LNs far later than dDCs after skin exposure to Ag (18, 19) and may thus preferentially regulate the late phases of T cell activation. It should be emphasized, however, that this dogma was mostly raised from studies of DC migration under inflammatory settings associated with the irritancy of DBP used to dilute the fluorescent tracers TRITC or FITC. At variance with our modified TRITC-painting protocol, these models cause early and massive mobilization of

CD207⁻ dDCs to LNs (19, 38), which may overwhelm the more limited migration of LCs. In addition, in all these studies, the differential emigration of DC subsets has never been compared with their capacity to present cutaneous Ag. In this context, we found that although LCs accounted for only 20% of skin-emigrated DCs at day 1 after DNTB skin delivery, they induced a far better proliferation of DNTB-specific CD8⁺ T cells as compared with CD207⁻ dDCs, suggesting that LCs may transport much higher amounts of the hapten than do dDCs.

Whether LCs exert an inductive or suppressive role during CHS to strong haptens such as DNFB or oxazolone remains so far controversial (19, 39, 40). Importantly, our present study clearly demonstrates that LCs confer natural protection from development of ACD to a prototype weak/tolerogenic hapten. Indeed, we found that acute depletion of LCs reversed DNTB-induced tolerance and allowed for in vivo priming of DNP-specific IFN-γ–producing cytotoxic CD8⁺ T cells and development of a CHS response upon subsequent DNTB challenge. Interestingly, while CHS to strong experimental haptens seems to be initiated by newly recruited monocyte-derived skin DCs (21) and CD207⁺ dDCs (14), CHS to weak/tolerogenic hapten that develops in the absence of LCs might be initiated by CD207⁻ dDCs. This is supported by the fact that DNTB is unable to recruit monocyte-derived inflammatory DCs in the skin (M. Gomez de Agüero and B. Dubois, unpublished observations) and that CD207⁻ dDCs are the only LN cells responsible for hapten presentation in LC-depleted mice (Supplemental Figure 8). The tolerogenic potential of LCs is also corroborated by adoptive transfer experiments, which have not been conducted in previous studies. We clearly document that even low numbers of LCs from epidermis of DNTB-exposed mice transferred tolerance to naive B6 mice, while neither keratinocytes nor innate DETCs from these mice could do so.

The finding that expression of both MHC-I and -II by LCs was mandatory for their ability to transfer DNTB tolerance highlighted 2 complementary mechanisms of tolerance dictated by allergen presentation to both CD8⁺ and CD4⁺ T lymphocytes, which respectively act as initiators (3, 4) and regulators (5, 8) of ACD. Presentation of DNTB by LCs to CD8⁺ T cells in skin-draining LNs resulted in a state of functional hyporesponsiveness by anergy and/or deletion. Indeed, when CD8⁺ T cells from DNTB-painted mice were transferred into *Cd3*ε$^{-/-}$ recipients, they were unable to subsequently differentiate into IFN-γ⁺ CHS effector cells in response to DNFB skin sensitization. Importantly, CD8⁺ T cell unresponsiveness was abrogated by LC depletion of DNTB-painted donor mice and could be reproduced in vitro by coculture of naive CD8⁺ T cells with DNTB-presenting epidermal LCs. Because chronic exposure to the Ag is required to maintain anergy in T cells (41), the fact that LC-stimulated CD8⁺ T cells remain unresponsive in T cell–deficient mice even after a resting period of 4 weeks suggests that a significant proportion of allergen-specific CD8⁺ T cells are deleted after cognate interaction with LCs in LNs. Deletional CD8⁺ T cell tolerance has been previously demonstrated in mice expressing a transmembrane form of OVA as a model self Ag in keratinocytes, but seems to be induced by the specialized Ag cross-presenting CD103⁺CD207⁺ dDCs rather than epidermal LCs (17, 42). This suggests that CD8⁺ T cell tolerance to self Ag expressed in keratinocytes versus exogenous Ag penetrating through intact skin may involve different DC subsets. Alternatively, that haptens are efficiently presented by LCs, which are so far considered as poor cross-presenting cells, may rely on the particular properties of haptens as compared with proteins. Indeed, it is noteworthy that haptens can directly bind to peptides already present in the groove of MHC-I molecules on the surface of APC (43) and, thanks to their lipid-soluble properties, can also enter the cell and conjugate with intracellular endogenous proteins (44), thus bypassing the need for cross-presentation.

Because efficient induction of tolerance by the sole deletion of CHS effector T cells would require that most circulating allergen-reactive CD8⁺ T cells interact with LCs in LNs, it is likely that complementary mechanisms operate systemically to prevent priming of the residual hapten-reactive CD8⁺ T cells. Increasing evidence indicates that CD4⁺CD25⁺Foxp3⁺ Tregs are essential to controlling the development and severity of skin allergy in both mouse models (5, 8) and in humans (12). Indeed, allergen-specific Tregs can be detected in allergen-exposed but nonallergic individuals (12) and are likely mandatory for prevention of sensitization, as depletion of Tregs in mouse models allows for development of CHS to otherwise nonsensitizing weak haptens (11). Yet an understanding of how allergen-specific Tregs are generated is still elusive. Along with the need for MHC-II expression by LCs to transfer tolerance, we documented that both DNTB painting and adoptive transfer of LCs from tolerized mice induced the appearance of a subset of CD4⁺Foxp3⁺ Tregs expressing ICOS. Importantly, LCs failed to transfer DNTB-specific tolerance in DEREG recipient mice previously depleted in CD4⁺Foxp3⁺ Tregs by DT injection. Together with the fact that, conversely to dDCs (45, 46), LCs could not induce CD4⁺Foxp3⁺ Treg conversion from CD4⁺CD25⁻ conventional T cells (data not shown), this suggests that MHC-II–dependent hapten presentation by LCs induces activation and expansion of preexisting CD4⁺Foxp3⁺ Tregs. These findings are consistent with the fact that Tregs (a) regulate CHS to strong haptens by controlling the priming of CD8⁺ effector T cells in skin-draining LNs (5, 8) and (b) that ICOS identifies a subset of highly suppressive CD4⁺Foxp3⁺ Tregs (47), which we recently showed arose from allergen activation of preexisting CD4⁺Foxp3⁺ Tregs in the LNs and displayed the most robust suppressive potential in CHS (7). Interestingly, our results showing that transfer of LCs in CD4⁺Foxp3⁺ Treg-deficient animals did not at all affect the priming of CD8⁺ T cells suggest that LCs need to coordinate with Tregs to induce CD8⁺ T cell hyporesponsiveness.

Our study thus assigns a critical role to epidermal LCs in protection from development of T cell–mediated cutaneous allergies to the weak sensitizer DNTB and identifies 2 complementary pathways of skin tolerance that are reminiscent of those described for orally induced tolerance (35, 48). The exact molecular mechanisms by which LCs induce anergy/deletion of allergen-specific CD8⁺ T cells and activation/expansion of ICOS⁺ Tregs remain to be characterized and will require further in-depth investigations. Obvious potential candidates, to name a few molecules, are 2,3-indolamine-deoxygenase (IDO) (49), TGF-β, IL-10 (27), PD1-L, and FasL (27). Whether such LCs' suppressive functions extend to other types of allergens/Ag and may help in understanding the pathophysiology of certain human cutaneous inflammatory diseases remains to be explored. One may speculate that a deficiency in LC numbers or migration, or reversion of their natural suppressive function — for example, by local inflammation or microbial colonization — may contribute to disease pathogenesis. In this respect, impaired LC migration has been reported in psoriasis (50) and could be responsible for deregulated immune response to self Ag expressed in the skin. In addition, several currently used treatments for inflamma-

tory diseases might act at least partly through LCs. These include the calcineurin inhibitor tacrolimus, whose beneficial effect in atopic dermatitis correlates with an increase of epidermal LCs harboring a resting phenotype (51), and glucocorticosteroids, which are efficient in nickel-allergic patients and were recently reported to endow LCs with Treg-promoting properties (52). In addition, in mouse models of sublingual immunotherapy, the allergen is captured within the oral mucosa by LC-like DCs producing IL-10 and TGF-β, and upregulating IDO, suggesting that such cells contribute to the efficacy of these promising treatments (53). More recently, epicutaneous delivery of a protein Ag through intact skin via an occlusive chamber was found to favor uptake by epidermal DCs reaching the superficial layers of the stratum corneum and to reduce allergen-specific responses in sensitized mice (54). In view of our data and the aforementioned observations, it may thus be anticipated that strategies that exploit, or even increase, the tolerogenic functions of epithelial LCs could be of invaluable interest in developing innovative treatments for human allergic diseases.

Methods

Mice. Lang-EGFP and Lang-DTR knockin mice (19) and Foxp3-DTR/EGFP transgenic DEREG mice (8) were previously described. These mice, together with *Cd3ε*$^{-/-}$, *H2-Aβ1*$^{-/-}$, *β2m*$^{-/-}$, OT-I, and CD45.1 mice, all on a B6 background, were bred at the Plateau de Biologie Expérimentale de la Souris (PBES) (Ecole Normale Supérieure de Lyon, Lyon, France) under specific pathogen–free (SPF) conditions. B6 mice were purchased from Charles River Laboratories. Mice were used between 6 and 11 weeks of age.

CHS to DNFB and DNTB tolerance. Mice were sensitized epicutaneously by painting the shaved abdominal skin with 25 μl of 0.5% DNFB (Sigma-Aldrich) in a vehicle consisting of acetone and olive oil (AOO) (4:1, v/v) and challenged 5 days later by topical application onto both sides of the right ear of 4 μl of 0.10% DNFB in AOO or 16 μl of 2% DNTB (Lancaster Synthesis) in AOO. CHS was determined by the mouse ear-swelling test as previously described (5). For induction of cutaneous tolerance (29), 100 μl of 1% DTNB in AOO was applied to the abdominal skin 7 days before sensitization with 0.5% DNFB on the back skin.

Assessment of hapten-specific T cell responses. For proliferation studies, 2×10^5 CD8$^+$ T cells, isolated using anti-CD8 Ab–coated microbeads (Miltenyi Biotec) from pooled cutaneous LNs (brachial, inguinal, and axillary) were cultured in the presence of DNBS-pulsed (Sigma-Aldrich) irradiated spleen cells for 3 days. Supernatants were collected for titration of IFN-γ by ELISA, and the proliferative response was determined after addition of 0.5 μCi ^{3}H-thymidine for the last 18 hours of culture as previously described (5). ^{3}H-thymidine incorporation was assessed using a TopCount counter (PerkinElmer), and results were expressed as cpm ± SD of triplicate wells.

The frequency of hapten-specific IFN-γ–producing T cells was determined using an ELISpot assay as described (21). Serial numbers of either purified CD8$^+$ T cells supplemented with spleen cells from naive B6 mice or total LN cells were cultured in the presence of 0.4 mM DNBS or medium alone. The number of IFN-γ spot-forming cells (SFCs) was determined, and the results were expressed as the number of IFN-γ SFCs per 10^6 cells (either total LN cells or purified CD8$^+$ T cells).

The in vivo DNP-specific CD8$^+$ T cells cytotoxic activity was analyzed 5 days after DNTB application. Briefly, mice were injected i.v. with a mixture of DNBS-pulsed (1.6 mM) and unpulsed spleen cells (10^7 each) that were stained with a low (0.5 mM) or high (5 mM) dose of CFSE (Molecular Probes), respectively. Mice were sacrificed 24 hours later, and 10,000 CFSE$^+$ cells from cutaneous LNs were analyzed by flow cytometry (FACS). In vivo cytotoxicity was calculated by determining the ratio of control targets to hapten-loaded targets in immunized versus control naive mice.

Antibodies. Anti-CD3ε (145-2C11), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD11c (HL3), anti-CD25 (PC-61), anti-CD45 (30-F11), anti-CD45.2 (clone 104), anti-CD103 (M290), and anti-ICOS (E7.17G9) Abs and matching isotype controls were all purchased from BD Biosciences — Pharmingen. Anti-CD45.1 (A20), anti-EpCAM (G8.8), and anti–I-A/I-E (M5/114.15.2) Abs were from BioLegend, and anti-Foxp3 (FJK-16s) Abs were from eBioscience. Anti-CD207 (929F3.01) Abs were purchased from Dendritics. FACs analysis was performed using a FACSCanto system or LRSII (BD Biosciences), and analysis was performed using FlowJo software (Tree Star Inc.).

Tracking of skin DC migration. B6, Lang-EGFP transgenic, and BM chimeric mice were painted with either vehicle (16 μl), 1% DNTB (16 μl), or DBP (20 μl, 1 v/v) on the dorsal sides of both ears. Then, 20 μl TRITC (Molecular Probes) diluted at 0.1 mg/ml in 10% DMSO (Sigma-Aldrich) and 90% acetone was applied. At different time points, CD11c$^+$ cells were enriched from cervical LNs using anti-CD11c mAb–coated microbeads (Miltenyi Biotec) and were analyzed by FACS for EGFP and TRITC expression or for I-A/I-E, CD207, and CD103 expression.

Generation of BM chimeric mice. Eight- to ten-week-old B6 Ly5a (CD45.1$^+$) or Lang-EGFP (CD45.2$^+$) mice were lethally irradiated with 2 doses of 5 Gy each, 4 hours apart, and then injected i.v. with 10^7 whole BM cells from either B6 Ly5a or Lang-EGFP mice. Chimeras were kept on antibiotic-containing water (0.2% Bactrim; Roche) during the whole experiment. Eight to ten weeks after reconstitution, chimerism was determined in blood by FACS, using anti-CD45.1 and anti-CD45.2 Abs, and at that time, more than 99% of B cells were of donor origin.

Sorting of DCs and ex vivo presentation assay. Cutaneous LNs from mice previously immunized with 1% DNTB on the abdominal skin were cut into small pieces and incubated with collagenase IA (Sigma-Aldrich) and DNAse I. CD11c$^+$ cells were first enriched using CD11c-coated microbeads and then stained with either anti-CD103, anti-CD8α, and anti-CD11c Abs or with anti-CD11c, anti-IA/IE (MHC-II), and anti-CD103 Abs. DC subsets were subsequently sorted on a FACSAria system (BD). The purity of DC subsets was routinely greater than 99%. Serial numbers of each DC subset were cultured with 2×10^5 CD8$^+$ T cells isolated from B6 mice at day 5 after DNFB immunization. After 3 days of culture, presentation of DNP peptides by sorted DC subsets was revealed by measuring the proliferation of CD8$^+$ T cells by thymidine uptake.

In vivo depletion experiments. To deplete CD207$^+$ DCs or LCs, 1 μg of DT (List Biological Laboratories) was injected i.p. in Lang-DTR mice at day –3 or –15, respectively. DEREG mice were depleted of CD4$^+$Foxp3$^+$ Tregs (8) by i.p. injection of 1 μg DT at days –2 or –1 with respect to day 0 of LC transfer.

Isolation of skin cells and LC transfer experiments. Mouse ears were split into halves and incubated for 2 hours in PBS containing dispase II (Roche Diagnostics). Small pieces of separated epidermal and dermal sheets were incubated with collagenase IV (Worthington) and DNase I (Roche) to obtain homogeneous cell suspensions. For enrichment of LCs, epidermal cell suspensions were overlaid on an OptiPrep (Axis-Shield) gradient consisting of 15% and 11.5% iodixanol, according to the manufacturer's instructions. After 20 minutes of centrifugation, the low-density fraction was collected on top of 11.5% iodixanol and contained 70%–90% LCs. For some experiments, the high-density fraction was also collected at the interface between 11.5% and 15% iodixanol and contained 30%–50% DETCs. From 2 to 5×10^4 enriched LCs or DETCs isolated from ears 4 hours after painting with 1% DNTB (16 μl per ear side) were transferred s.c. into the abdominal region of naive B6 mice. Six days later, recipient mice were immunized by s.c. injection in the same area of 10^5 DCs that were generated by culture of BM progenitors with GM-CSF (BMDCs) and pulsed in vitro with DNBS (1.6 mM), as previously described (5).

Statistics. The Mann-Whitney nonparametric test was used for experiments involving only 2 groups. To compare 3 or more experimental groups, we used the nonparametric Kruskal-Wallis test with Dunn's multiple comparison test to generate *P* values for selected pairwise comparisons. GraphPad Prism 4.0 software was used for statistical analysis. Data are shown as mean ± SD. $P < 0.05$ was considered significant.

Study approval. All mouse studies were conducted with the approval of and in accordance with the guidelines for animal experiments of the local ethics committee (CECCAPP Lyon, registered by the French National Ethics Committee of Animal Experimentation [CNREEA] under no. 15).

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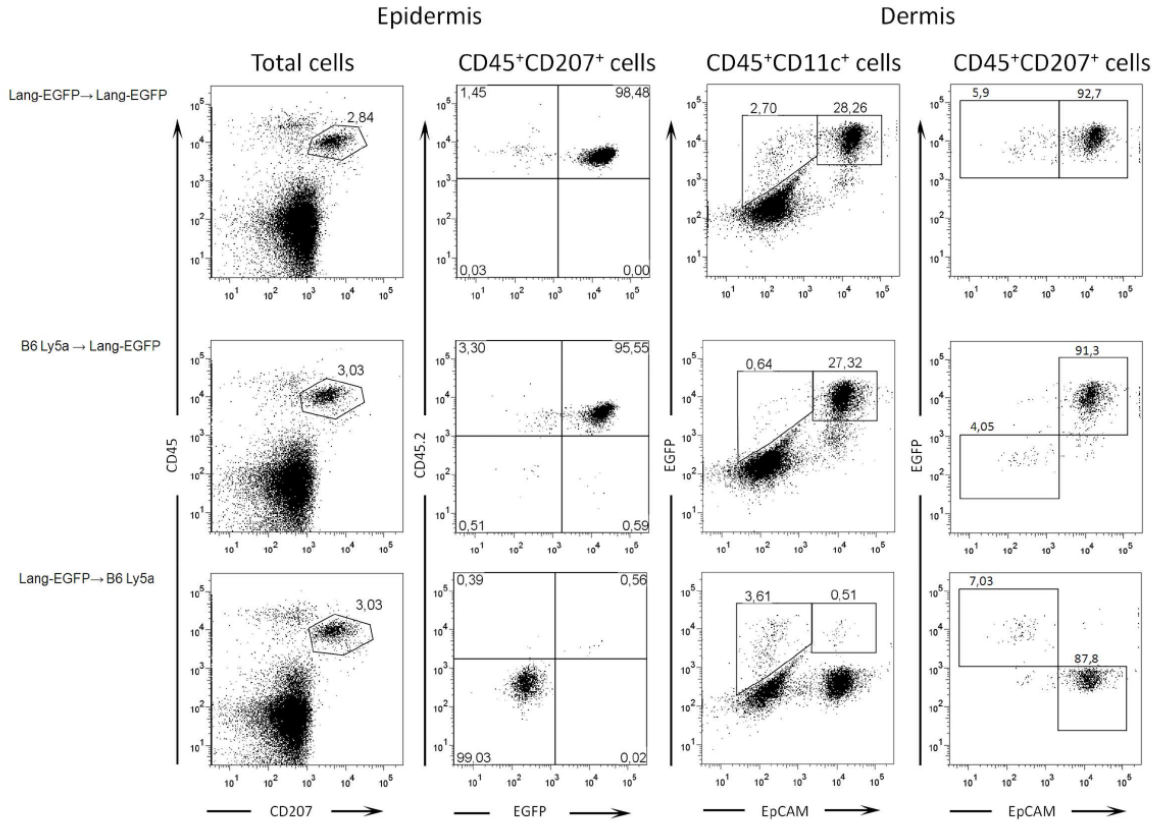
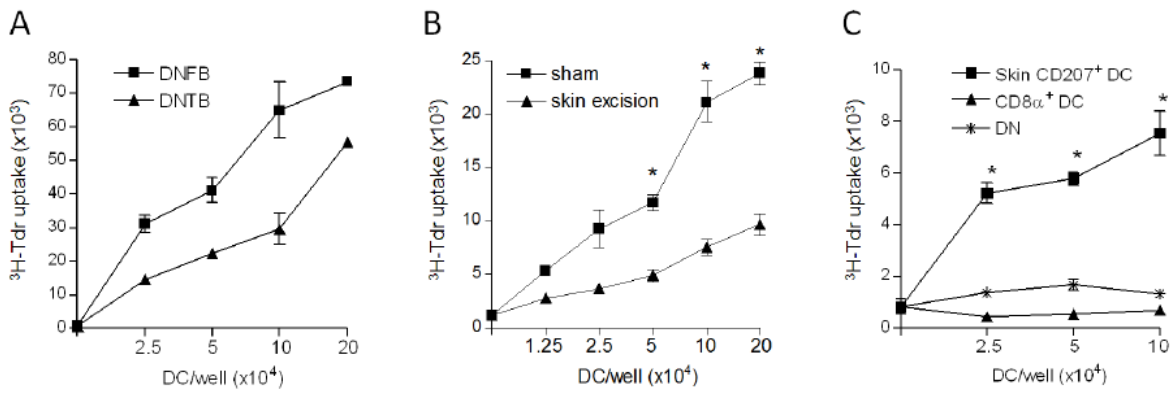
SUPPLEMENTAL FIGURES



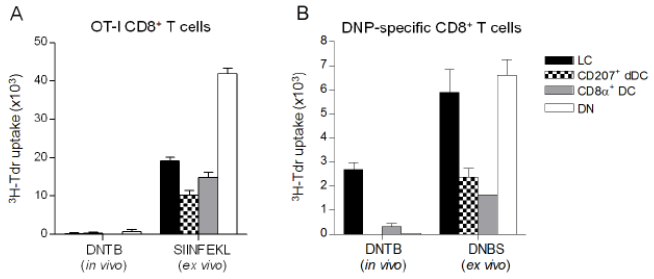
Supplemental Figure 1

Identity of EGFP$^+$ skin DC in BM chimeric mice. 8 weeks after generation of BM chimeras, several mice were sacrificed and epidermal and dermal cell suspensions were prepared by enzymatic digestion of ear skin and were analyzed by flow cytometry for the expression of pan-CD45, CD11c, EGFP, CD207, EpCAM and CD45.2. In B6 ly5a (CD45.1)→Lang-EGFP(CD45.2) chimeric mice, the vast majority of EGFP$^+$ cells present in the epidermis and dermis were host-derived (CD45.2$^+$) LC (CD207$^+$EpCAM$^+$) and CD207$^+$ dDC were EGFP$^-$. By contrast, the few EGFP$^+$ DC present in Lang-EGFP→ B6 ly5a mice were only detected in the dermis and corresponded to CD207$^+$ dDC (EpCAM$^-$), while all LC present in epidermis and dermis were EGFP$^-$.



Supplemental Figure 2

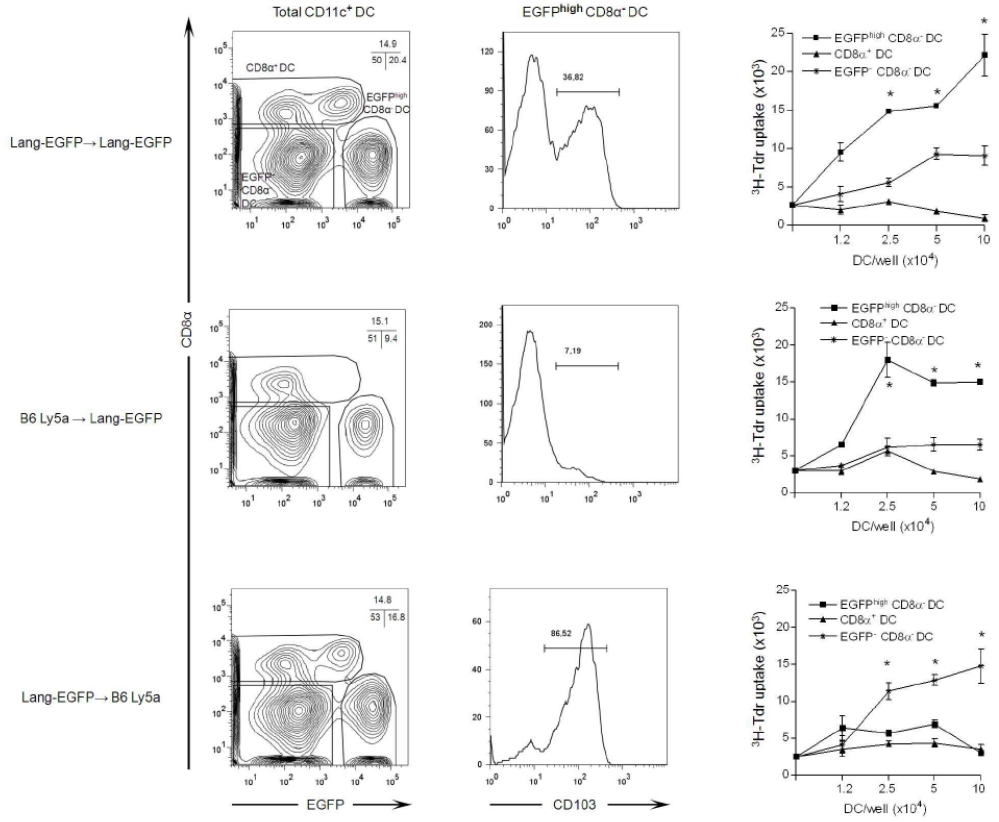
Presentation of haptenated-Ag to CD8$^+$ T cells after skin exposure to DNTB is mostly restricted to skin-derived CD207^{high} DC. B6 (**A, B**) or Lang-EGFP (**C**) mice were skin-painted with either DNFB (**A**) or DNTB (**A-C**) and, 72h later, DC were enriched from cutaneous LN and further sorted by flow cytometry into skin derived CD207$^+$ DC (CD8α$^-$EGFP^{high}), LN resident CD8α DC (CD8α$^+$EGFP$^{-/low}$) and DN DC ((CD8α$^-$EGFP$^-$), as shown in **Figure 3A**. For some experimental groups, the DNTB-painted ear skin was surgically excised 5 hours after hapten delivery (**B**). Various numbers of total DC (**A, B**) or isolated DC subsets (**C**) were cultured with DNP-specific CD8$^+$ effector T cells isolated from B6 mice at d+5 after DNFB immunization. Proliferation of CD8$^+$ T cells was determined after 3 days by Thymidine uptake and is expressed as mean cpm±SD of triplicate wells. Data are representative of 1 out of 2 (**B-C**) to 4 (**A**) independent experiments. *p< 0.05



GOMEZ DE AGUERO ET AL. (FIG. S3)

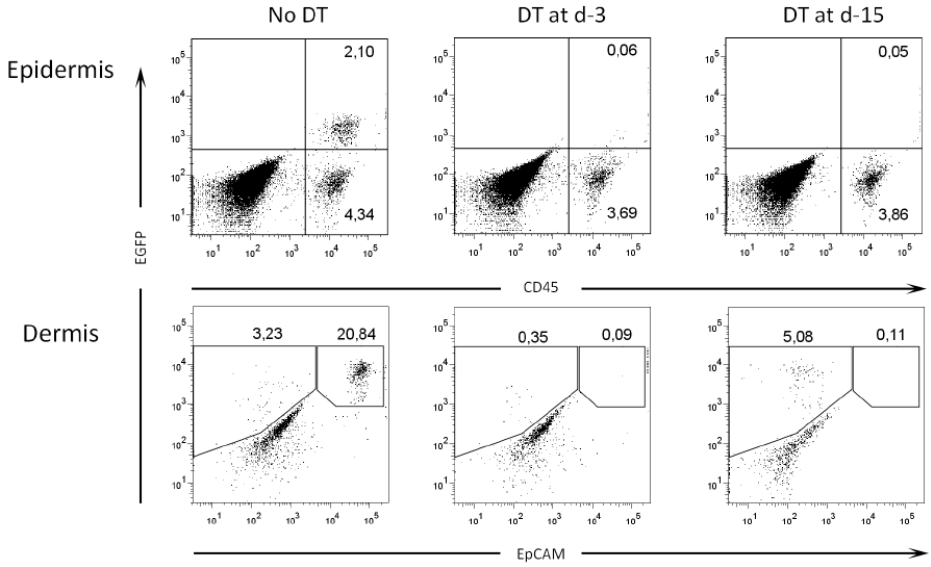
Supplemental Figure 3

All LN DC induce proliferation of specific CD8$^+$ T cells when pulsed *ex vivo* with Ag. Various DC subsets (**Figure 4A**) were purified from axillary and inguinal LN of Lang-EGFP mice, either naïve or 72h hours after abdominal skin painting with DNTB. They were then pulsed either with the SIINFEKL OVA peptide (**A**) or with DNBS (**B**), and 2.5x10^4 of each DC subsets were cultured with 1x10^5 OVA-specific CD8$^+$ T cells from OT-I transgenic mice (**A**) or DNP-specific CD8$^+$ effector T cells (**B**), respectively. Proliferation of CD8$^+$ T cells was determined after 3 days of culture by Thymidine uptake and is expressed as mean cpm±SD of triplicate wells. Data are representative of 1 out of 2 independent experiments.



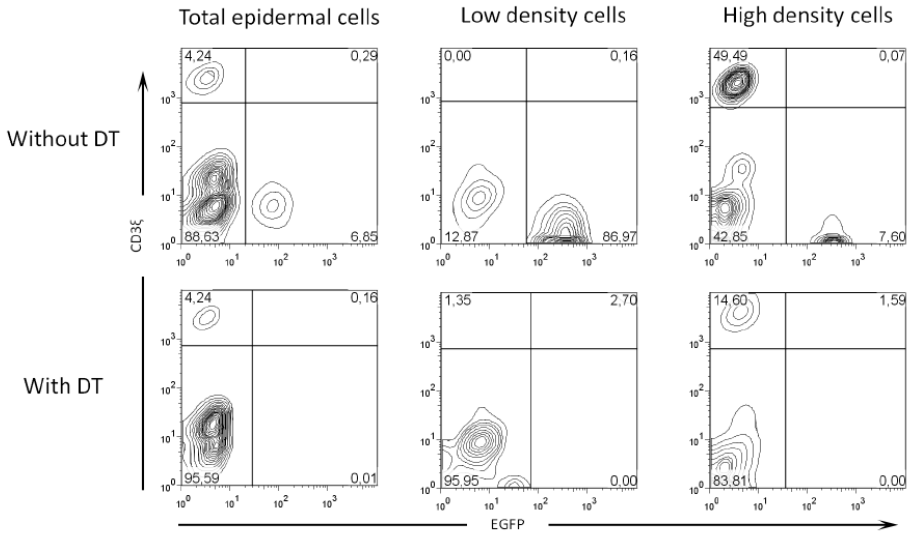
Supplemental Figure 4

In BM chimeric mice, presentation of DNTB in LN is mediated by host-derived CD207$^+$ LC but not donor-derived CD207$^+$ dDC. 3 types of Lang-EGFP BM chimeras were painted on the abdominal skin with DNTB and, 72 hours later, CD11c$^+$ cells were enriched from pooled inguinal and axillary LN and further separated into CD8α^++EGFP$^{-/low}$ DC, CD8α^-EGFP$^-$ DN DC and EGFP^{high} DC (left panels). Numbers indicate the percentage of each DC subset in gated CD11c$^+$ cells. LN EGFP^{high} DC from B6 ly5a (CD45.1)→Lang-EGFP(CD45.2) BM chimeras correspond to host-derived radioresistant LC and mostly contained CD103$^-$ cells, while those from Lang-EGFP→ly5a B6(CD45.1) BM chimeras represent donor-derived CD207$^+$ dDC and were mostly CD103$^+$ (middle panels). Serial dilutions of the DC subsets were cultured with DNP-specific CD8$^+$ T cells. The proliferative response was quantified by ^{3}H-TdR uptake after 3 days and is expressed as mean cpm±SD of triplicate wells. Data are representative of one out of two individual experiments. *p* values: *< 0.05



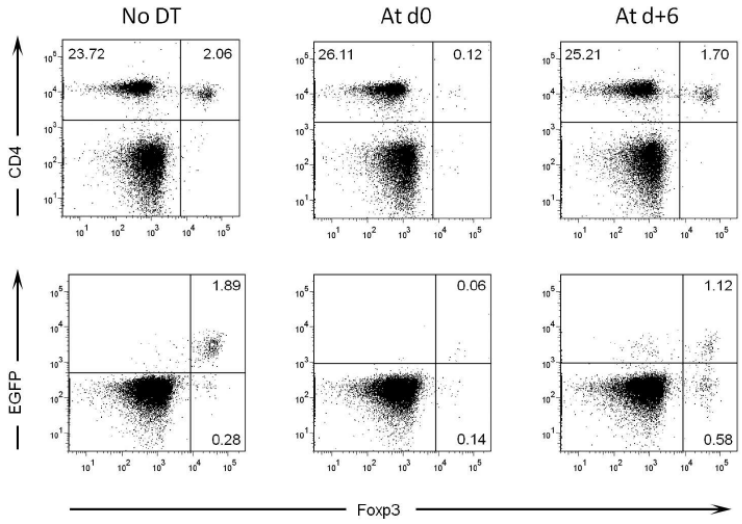
Supplemental Figure 5

Frequency of epidermal LC and CD207$^+$ dDC after DT injection at day-3 versus day-15. Epidermal and dermal cell suspensions were prepared from ears of Lang-DTR mice 3 or 15 days after DT injection and were analyzed by flow cytometry using anti-CD45, -CD11c and – EpCAM Abs. Total epidermal EGFP$^+$ cells correspond to LC, while EGFP$^+$ cells present in the dermis contain both CD207$^+$ dDC (EGFP$^+$EpCAM$^-$) and LC on their way to LN (EGFP$^+$ EpCAM $^+$). CD45 versus EGFP expression on total cells is shown for the epidermis (upper panels), while EpCAM versus EGFP expression on CD45$^+$CD11c$^+$ cells is shown for the dermis (lower panels). Whereas day-3 DT injected mice are devoid of the majority of LC and CD207$^+$ dDC, day-15 DT injected mice are deficient in LC in both epidermis and dermis, but harbor a normal proportion of CD207$^+$ dDC.



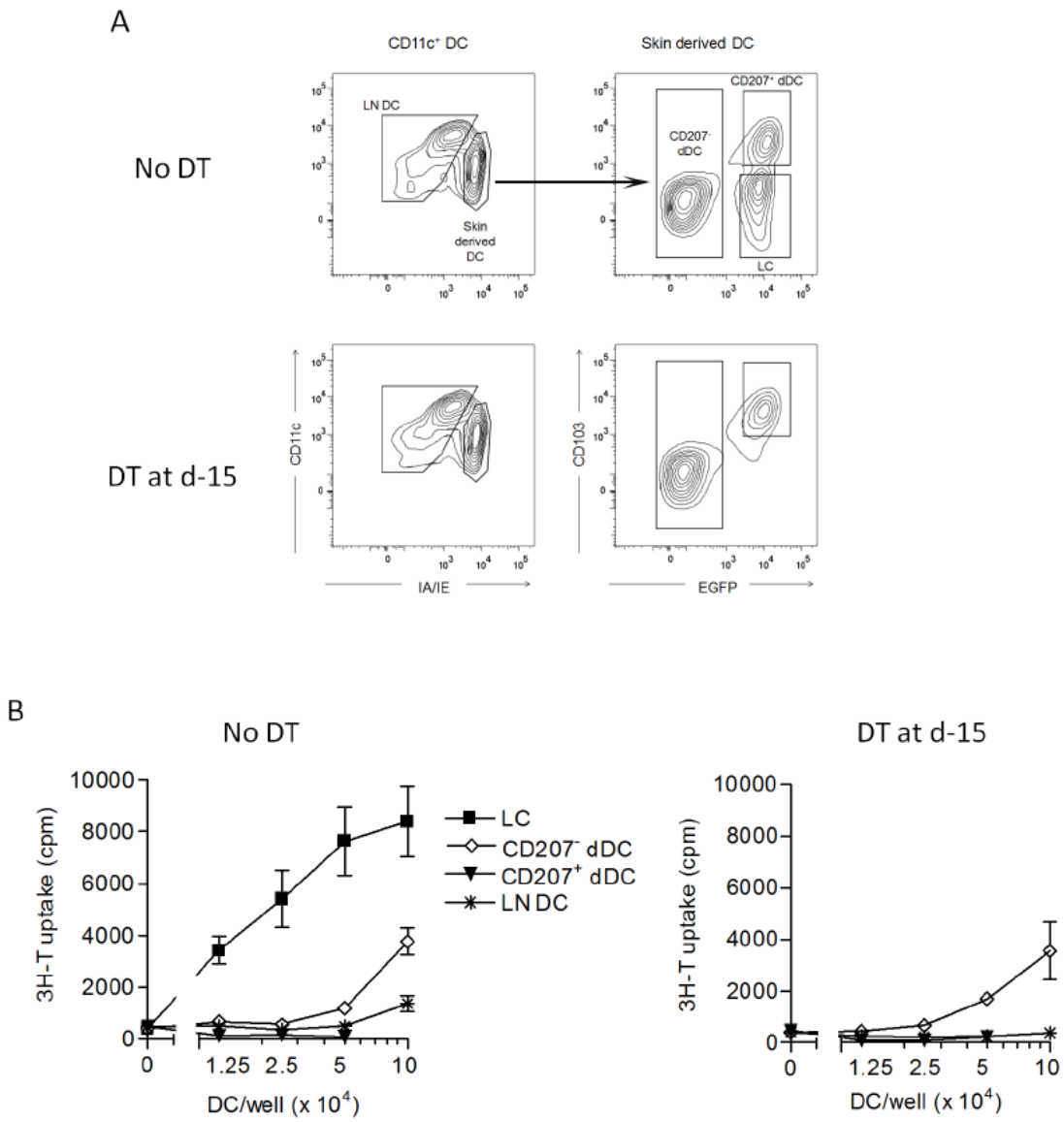
Supplemental Figure 6

Enrichment of epidermal LC and DETC by density gradient centrifugation. Lang-DTR mice were treated (lower panels) or not (top panels) with DT 3 days before DNTB ear painting. 4 hours later, ear epidermal cells were prepared by enzymatic digestion and centrifuged over an Optiprep® gradient. Flow cytometry analysis of EGFP (CD207) versus CD3ε expression revealed that the low density fraction contained 80-90% LC ($EGFP^{+}CD3\varepsilon^{-}$) and some keratinocytes (DN cells), while high density cells contained mostly DETC ($EGFP^{-}CD3\varepsilon^{+}$) and keratinocytes, but few LC. Low density cells prepared from DT-injected donor mice comprised almost exclusively keratinocytes and no LC. Similar purities were obtained when donor mice were painted with AOO as control.



Supplemental Figure 7

$CD4^+Foxp3^+$ Tregs recovered in DEREG mice within a week after DT injection. Foxp3-DTR/EGFP mice (DEREG) were injected with DT twice daily at days -2 and -1 and the frequency of $CD4^+Foxp3^+$ Tregs was determined in skin LN at day 0 (middle panel) and day 6 (right panel). Most $CD4^+Foxp3^+$ Tregs in non-injected DEREG mice were $EGFP^+$ and were efficiently depleted in LN at day 0. At day 6, $Foxp3^+$ Tregs have recovered to >80% of their normal frequency in LN and a significant proportion of them do not express EGFP.



Supplemental Figure 8

Dermal CD207$^-$ DC are the sole DC responsible for presentation of DNTB-modified peptides in LN of LC-depleted mice. At day-15 before abdominal skin painting with DNTB, Lang-DTR mice were injected with DT to selectively deplete LC. At day+3, LN resident DC (CD11c$^+$MHC-II^{int}), CD207$^-$ dDC (CD11c$^+$MHC-II^{high}EGFP$^-$CD103$^-$), CD207$^+$ dDC (CD11c$^+$MHC-II^{high}EGFP^{high}CD103$^+$) and LC (CD11c$^+$MHC-II^{high}EGFP^{high}CD103$^-$) were FACS sorted from pooled inguinal, axillary and brachial LN (**A**) and were cultured at serial dilutions with 10^5 DNP-specific CD8$^+$ effector T cells (**B**). Proliferation of CD8$^+$ T cells was determined after 3 days by Thymidine uptake and is expressed as mean cpm±SD of triplicate wells. Data are representative of 1 out of 2 independent experiments.