Functional Redundancy of Langerhans Cells and Langerin$^+$ Dermal Dendritic Cells in Contact Hypersensitivity

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The relative roles of Langerhans cells (LC), dermal dendritic cells (DC), and, in particular, the recently discovered Langerin$^+$ dermal DC subset in the induction and control of contact hypersensitivity (CHS) responses remain controversial. Using an inducible mouse model, in which LC and other Langerin$^+$ DC can be depleted by injection of diphtheria toxin, we previously reported impaired transport of topically applied antigen to draining lymph nodes and reduced CHS in the absence of all Langerin$^+$ skin DC. In this study, we demonstrate that mice with a selective depletion of LC exhibit attenuated CHS only upon sensitization with a low hapten dose but not with a high hapten dose. In contrast, when painting a higher concentration of hapten onto the skin, which leads to increased antigen dissemination into the dermis, CHS is still diminished in mice lacking all Langerin$^+$ skin DC. Taken together, these data suggest that the magnitude of a CHS reaction depends on the number of skin DC, which have access to the hapten, rather than on the presence or absence of a particular skin DC population. LC and (Langerin$^+$) dermal DC thus seem to have a redundant function in regulating CHS.

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INTRODUCTION

Dendritic cells (DC) are a heterogenous family of antigen-presenting cells critical for the induction and control of T cell immunity and tolerance. Upon antigen encounter at epithelial borders to the environment, DC migrate to local lymph nodes (LN), where they activate naive T cells to become effector cells (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000; Steinman *et al.*, 2003). In mouse skin, at least three phenotypically distinct DC populations can be distinguished: Langerhans cells (LC) expressing the C-type lectin Langerin/CD207 form a contiguous network in the epidermis, whereas Langerin$^+$ and Langerin^{neg} DC subsets reside in the dermis (Bursch *et al.*, 2007; Ginhoux *et al.*, 2007; Poulin *et al.*, 2007). LC can be further separated from Langerin$^+$ dermal DC by their expression or lack of EpCam and CD103, respectively (Nagao *et al.*, 2009). Whether the different skin DC subsets exert specific functions in the regulation of cutaneous immune responses remains elusive (Romani *et al.*, 2006, 2010; Clausen and Kel, 2010).

Allergic contact dermatitis (ACD), one of the most common occupational diseases, leads to considerable health-related quality of life impairment in affected patients and its prevention therefore is of high socioeconomic impact. ACD, also referred to as contact hypersensitivity (CHS), is a T cell-mediated skin inflammation caused by repeated exposure to contact allergens, i.e., low-molecular-weight chemicals called haptens (Vocanson *et al.*, 2009). During the asymptomatic sensitization phase, haptens penetrating the skin bind to host proteins, which are then collected by resident DC that migrate to the LN to activate naive hapten-specific T cells. In sensitized individuals, re-exposure to the hapten initiates the effector phase and clinical expression of ACD, characterized by recruitment and activation of specific T cells at the site of hapten challenge. In mice, painting of a sensitizer onto the skin initiates production of proinflammatory cytokines that activate skin DC to prime hapten-specific T cells in draining LN (Grabbe and Schwarz, 1998). Re-exposure to the same hapten onto the ear induces a transient ear swelling reaction mediated by IFNγ-producing CD8$^+$ T cells and controlled by CD4$^+$ T cells secreting IL-4 and IL-10 (Gorbachev and Fairchild, 2004). LC have been critically linked to the initiation of contact sensitivity responses (Toews *et al.*, 1980), however, recent advances in the pathophysiology of CHS have revisited the dogma that LC are mandatory for T-cell priming.

The essential contribution of LC to hapten sensitization has recently been challenged in different LC ablation models (Bennett *et al.*, 2005; Kaplan *et al.*, 2005, 2008;

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Abbreviations: ACD, allergic contact dermatitis; CHS, contact hypersensitivity; DC, dendritic cell(s); DNFB, 2,4-dinitrofluorobenzene; DT, diphtheria toxin; DTR, diphtheria toxin receptor; LC, Langerhans cell(s); LN, lymph node(s); WT, wild type

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Kissenpfennig *et al.*, 2005; Bennett and Clausen, 2007). On one hand, knock-in mouse strains have been independently generated by two laboratories, in which expression of the high-affinity human diphtheria toxin (DT) receptor (DTR) is driven by the endogenous *langerin* promotor. In these Langerin-DTR mice, LC can be depleted for at least 2–4 weeks after a single injection of DT (Bennett *et al.*, 2005). Initial experiments demonstrated a similar and reduced CHS responses in the French (Kissenpfennig *et al.*, 2005) and Dutch (Bennett *et al.*, 2005) Langerin-DTR mice, respectively, as compared with wild type (WT). Later, a population of Langerin$^+$ dermal DC distinct of epidermal LC was discovered (Bursch *et al.*, 2007; Ginhoux *et al.*, 2007; Poulin *et al.*, 2007) and it was established in the French mice that these recover from the toxin treatment within 1–2 weeks after DT (Bursch *et al.*, 2007). Using the French Langerin-DTR mice, it was then shown that CHS responses were indeed decreased when all Langerin$^+$ skin DC were absent, but restored to WT levels once the Langerin$^+$ dermal DC subset had returned (Bursch *et al.*, 2007). In contrast, CHS was still impaired in the Dutch Langerin-DTR mice 4 weeks after DT (Bennett *et al.*, 2007).

On the other hand, transgenic mice expressing the toxic subunit of DT under control of the human *langerin* promotor constitutively lack epidermal LC from birth, whereas other Langerin$^+$ DC populations, in particular, Langerin$^+$ dermal DC are not affected (Kaplan *et al.*, 2005). These Langerin-DTA mice mount exaggerated CHS reactions suggesting that LC play a regulatory rather than a stimulatory role in this model. Therefore, to date the requirement of LC, Langerin$^+$ and Langerin^{neg} dermal DC during the sensitization of CHS remains controversial or unknown.

In this study, we take advantage of the differential repopulation kinetics between epidermal LC and Langerin$^+$ dermal DC and assess CHS responses in mice selectively lacking LC and mice lacking all Langerin$^+$ DC. In particular, we compare different haptens, hapten concentrations and, for the first time, the two inducible LC depletion mouse models side-by-side. Our data demonstrate that in both Langerin-DTR strains the magnitude of a CHS reaction depends on the antigen dose and the number of skin DC that have access to the hapten, rather than the presence or function of one of the three skin DC subsets.

RESULTS

Rapid steady state repopulation of dermal Langerin$^+$ DC

The skin contains at least three subsets of DC, namely epidermal LC and Langerin$^+$ and Langerin^{neg} dermal DC. In Langerin-DTR mice, all Langerin$^+$ cells are rapidly depleted upon injection with DT. We previously described that steady state LC reconstitution of the epidermis takes at least 2–4 weeks (Bennett *et al.*, 2005), whereas Langerin$^+$ LN DC recover faster from the toxin treatment (Bennett *et al.*, 2007). These data were obtained before the recent discovery of a distinct subset of Langerin$^+$ DC in the dermis, and in this study we further characterize the steady state repopulation of dermal Langerin$^+$ cells. Langerin-DTR mice were injected with DT and the repopulation kinetics of Langerin$^+$ cells in epidermis, dermis, and LN was assessed (Figure 1). As expected, 2 days after toxin treatment all Langerin$^+$ DC in epidermis, dermis, and LN were efficiently depleted. Already after 1 week, Langerin$^+$ cells were reappearing in the dermis and in skin-draining LN, and continued to increase in numbers over time, whereas the epidermis essentially remained devoid of LC. Nonetheless, we observe only a partial recovery of the Langerin$^+$ CD103$^+$ DC population in the dermis and skin-draining LN (Figure 1b). In fact, the rare Langerin$^+$ major histocompatibility complex II$^+$ (MHCII$^+$) DC present in the dermis 2 weeks after DT are difficult to make out in cross-sections of ear skin (Figure 1a, right panel, white arrowhead). Analysis of Langerin$^+$ cells among whole ear skin crawl-outs analyzed 15 days after DT confirms the profound LC deficiency and incomplete reconstitution of Langerin$^+$ CD103$^+$ dermal DC (Figure 1c). Notably, the French and Dutch Langerin-DTR mice show a similar Langerin$^+$ dermal DC recovery of 44.7 and 29.2%, respectively (calculated from absolute numbers of migratory cells, Figure 1c). Consequently, topical application of FITC 2 or 10 days after DT results in antigen transport to draining LN by Langerin^{neg} dermal DC or both Langerin$^+$ and Langerin^{neg} dermal DC subsets (Supplementary Figure S1 online). This difference in repopulation kinetics between LC and Langerin$^+$ dermal DC after toxin treatment enables us to study CHS responses in the absence of all Langerin$^+$ cells or in the absence of LC alone (see Table 1 for an overview).

Low-dose CHS is decreased in the absence of both all Langerin$^+$ DC and LC alone

We have previously shown that in the absence of all Langerin$^+$ cells CHS responses are diminished due to inefficient hapten transport to local LN for the priming of naive antigen-specific T cells (Bennett *et al.*, 2005, 2007). In this study, we investigated the differential roles (if any) of epidermal LC versus Langerin$^+$ and Langerin^{neg} dermal DC in mediating CHS. Taking advantage of the slow return of the epidermal LC in relation to dermal Langerin$^+$ DC, we injected mice with DT 2 days before inducing CHS to deplete all Langerin$^+$ cells, or 10 days before inducing CHS to selectively deplete epidermal LC only (Table 1). On the basis of our observation that a reduced dose of hapten preferentially targets LC in the epidermis (Bennett *et al.*, 2007), we sensitized mice with 0.5% oxazalone on the shaved abdomen and challenged with 0.25% of oxazalone on one ear 5 days later. Consistent with our previous findings, CHS responses are significantly reduced in Langerin-DTR mice, when they lack all Langerin$^+$ cells. Similarly, mice in which dermal Langerin$^+$ cells have recovered from the toxin, exhibit a diminished CHS response at this low oxazolone concentration as compared with WT (Figure 2a).

To exclude any bias of a particular hapten (oxazolone) on the results of this study, we also induced low-dose CHS with 2,4-dinitrofluorobenzene (DNFB). As depicted in Figure 2b, upon sensitization with 0.5% followed by challenge with 0.25% DNFB, CHS responses are significantly impaired 2 days as well as 10 days after injection of DT. These data indicate that at low-hapten concentrations, CHS is reduced

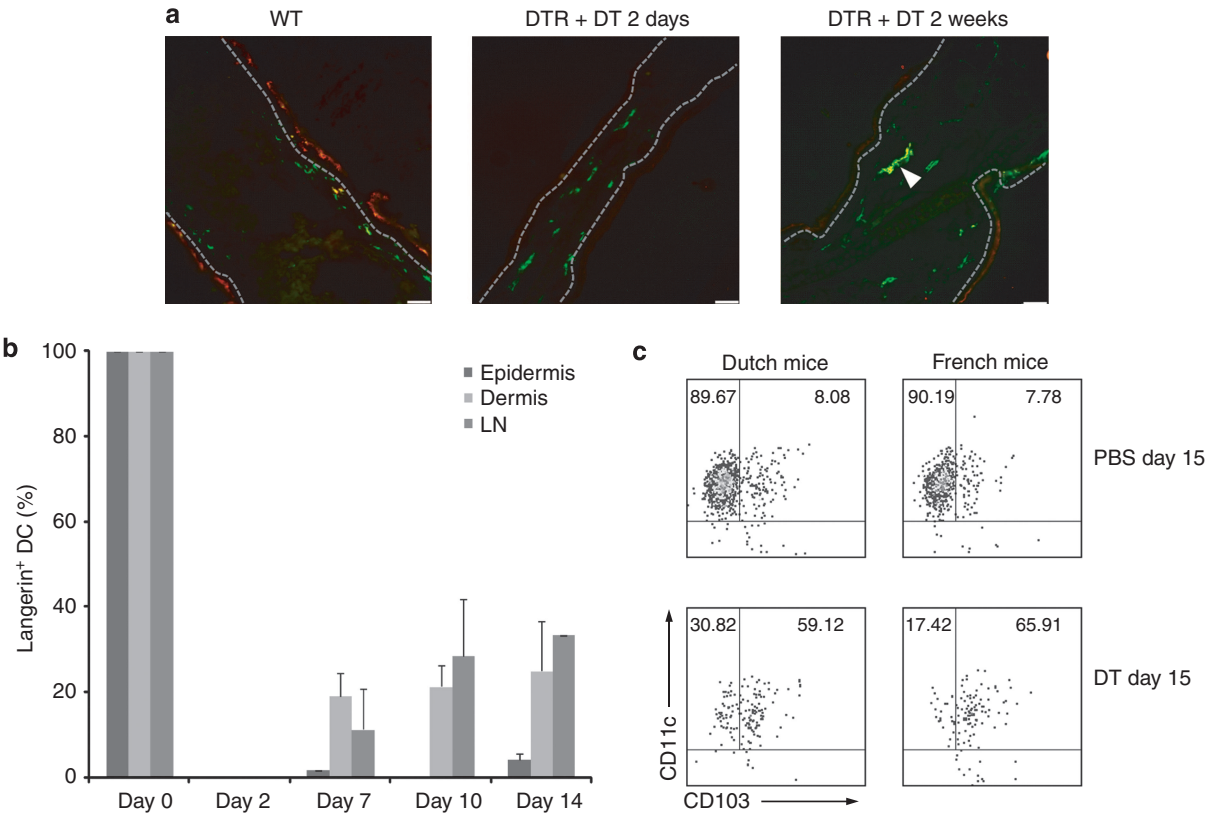

Figure 1. Steady state repopulation of Langerin$^+$ dendritic cell (DC) populations. (**a**) Anti-Langerin (red fluorescence) and anti-major histocompatibility complex II (MHCII) (green fluorescence) staining of ear cross-sections of wild type (WT) (left panel) and Langerin-diphtheria toxin receptor (DTR) mice (''Dutch mice'') injected with diphtheria toxin (DT) at 48 hours (middle panel) or 14 days (right panel) earlier. The arrowhead identifies one of the rare Langerin$^+$ dermal DC (yellow fluorescence) that had recovered from the toxin at 2 weeks. Bars = 50 μm. (**b**) Recovery of Langerin$^+$ cells in epidermis, dermis, and cutaneous lymph node (LN). Mice were injected with DT at day 0. Single-cell suspensions were prepared at different time points and analyzed for the presence of Langerin$^+$ cells. Values of untreated mice (day 0) were set equal to 100%. The graph shows the percentages of MHCII$^+$ Langerin$^+$ cells (mean ± SD). In dermal suspensions, epidermal Langerhans cells (LC) in transit and Langerin$^+$ dermal DC were distinguished by differential expression of EpCam and CD103 (not shown). Only numbers of CD103$^+$EpCam^{neg} dermal Langerin$^+$ cells are considered in the columns for dermis. Four mice per time point were analyzed. (**c**) At 15 days after injection of phosphate-buffered saline (PBS) or DT, whole-skin explants were prepared from the ears of French or Dutch Langerin-DTR mice, and further cultured for 4 days. Migratory cells from 4–6 skin explants were pooled and stained for CD11c, Langerin and CD103. At least 3,000 live migratory cells were acquired by fluorescence activated cell sorting. Dot plots display migratory cells gated for Langerin expression. Percentages represent the proportions of CD11c$^+$ CD103^{neg} LC and CD11c$^+$ CD103$^+$ Langerin$^+$ dermal DC. Results are representative of two independent experiments.

both in the absence of all Langerin$^+$ DC and of LC alone, irrespective of the type of hapten.

High-dose CHS is only diminished in mice lacking all Langerin$^+$ DC

To address the contribution of Langerin$^+$ dermal DC to CHS reactions, we painted a higher concentration of hapten onto the skin leading to increased antigen dissemination into the dermis. We sensitized and challenged the mice with 2 and 0.5% oxazolone or 1 and 0.5% DNFB, respectively. As expected, mice lacking all Langerin$^+$ cells still develop significantly diminished CHS to both haptens (Figure 3a and b, left panels). In contrast, when only epidermal LC are absent, CHS responses are comparable with WT mice, and when they are reduced, this difference often is not significant (Figure 3, right panels). These findings suggest that once a hapten efficiently reaches the dermis, LC are dispensable and (Langerin$^+$) dermal DC are sufficient for the induction of an effective CHS response.

Table 1. Low- and high-dose CHS in the presence or absence of distinct skin DC subsets

Composition of skin DC compartment	Low-dose CHS	High-dose CHS
WT	++	+++
Lack of epidermal LC only (DT day −10 or −15)	↓	Similar (++)
Lack of all Langerin$^+$ cells (DT day −1 or −2)	↓	↓

Abbreviations: CHS, contact hypersensitivity; DC, dendritic cell; DT, diphtheria toxin; LC, Langerhans cells; WT, wild type.
+ Degree of ear swelling; ↓ reduction in ear swelling as compared with WT mice.

Similar reduction of low- and high-dose CHS in French and Dutch Langerin-DTR mice

Performing similarly timed DT treatments, using the French Langerin-DTR mice, Bursch *et al.* (2007) reported diminished

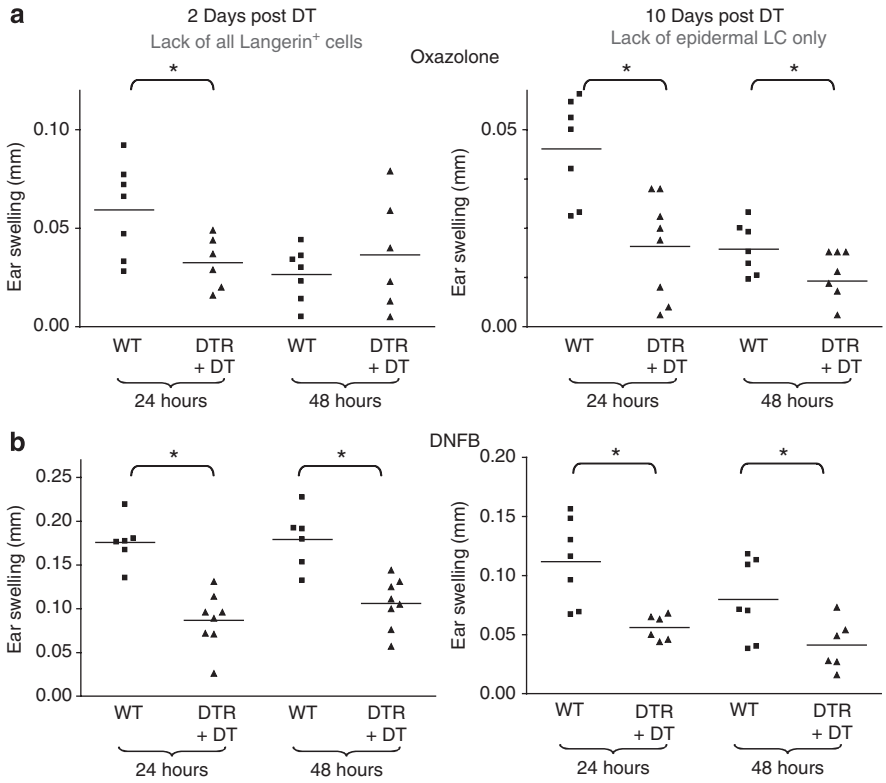

Figure 2. Contact hypersensitivity reactions to low-dose hapten. Wild type (WT) or Langerin-diphtheria toxin receptor (DTR) mice (''Dutch mice'') injected with diphtheria toxin (DT) at day −2 or −10 ($n = 6$–8) were (**a**) sensitized on the abdomen with 0.5% oxazolone and challenged 5 days later with 0.25% oxazolone on one ear, or (**b**) sensitized with 0.5% 2,4-dinitrofluorobenzene (DNFB) and challenged with 0.25% DNFB. Ear swelling was measured as the difference between before and after challenge. One representative experiment out of three is shown; symbols represent individual mice. Ear swelling responses were compared using a Student's *t*-test; WT versus DTR + DT: $*P \leqslant 0.05$. LC, Langerhans cells.

CHS in the absence of all Langerin$^+$ DC, but no reduction in CHS when only LC are absent from the skin. Therefore, it was important to compare low- and high-dose CHS responses in the French and Dutch Langerin-DTR mice immediately or 10 days after DT. As shown in Figure 4, following a low-dose protocol of inducing CHS to oxazolone both Langerin-DTR mice mount attenuated ear swelling reactions in the absence of all Langerin$^+$ DC (Figure 4a) and LC alone (Figure 4b). These results demonstrate that when tested in the same experiment, under conditions that limit hapten delivery preferentially to the epidermis, the French and Dutch Langerin-DTR mice develop equally reduced CHS after ablation of all Langerin$^+$ DC, but also in the selective absence of LC. In addition, when analyzed side-by-side both Langerin-DTR mutants respond similarly in high-dose CHS (Figure 5).

DISCUSSION

CHS, in the form of occupational ACD to low-molecular-weight chemicals (haptens), leads to a considerable impairment of the quality of life in affected patients (Vocanson *et al.*, 2009). Initiation of disease is critically linked to the migration of skin DC to local LN and activation of naive hapten-specific T cells during the asymptomatic sensitization phase. Re-exposure to the relevant hapten induces the effector phase and clinical expression of ACD. DC are a heterogenous family of antigen-presenting cells and as such key regulators of T cell immunity and tolerance (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000; Steinman *et al.*, 2003; Steinman and Banchereau, 2007). Therefore, a better understanding of DC function in CHS may open improved immunotherapeutic approaches to treat patients with ACD.

The skin contains at least three phenotypically distinct DC populations (Merad *et al.*, 2008). A network of Langerin$^+$ EpCam$^+$ CD103^{neg} LC is located in the epidermis and Langerin$^+$ EpCam^{neg} CD103$^+$ and Langerin^{neg} prototypic tissue DC reside in the dermis (Nagao *et al.*, 2009). All three skin DC subsets can migrate to draining LN in response to local inflammation, but whether they have specific roles in inducing or regulating an immune response is still largely unknown. LC have long been considered mandatory for CD8$^+$ T-cell priming in CHS, however, this paradigm has recently been challenged in experiments using different LC-deficient mouse models (Bennett and Clausen, 2007; Kaplan *et al.*, 2008).

To date, two types of LC ablation models have been generated and analyzed in CHS. On one hand, LC-deficient Langerin-DTA transgenic mice, which constitutively lack LC from birth, whereas other Langerin-expressing DC populations, in particular, Langerin$^+$ dermal DC are not affected (Kaplan *et al.*, 2005). On the other hand, two Langerin-DTR knock-in strains, which enable the inducible depletion of LC and other Langerin$^+$ DC by the injection of DT

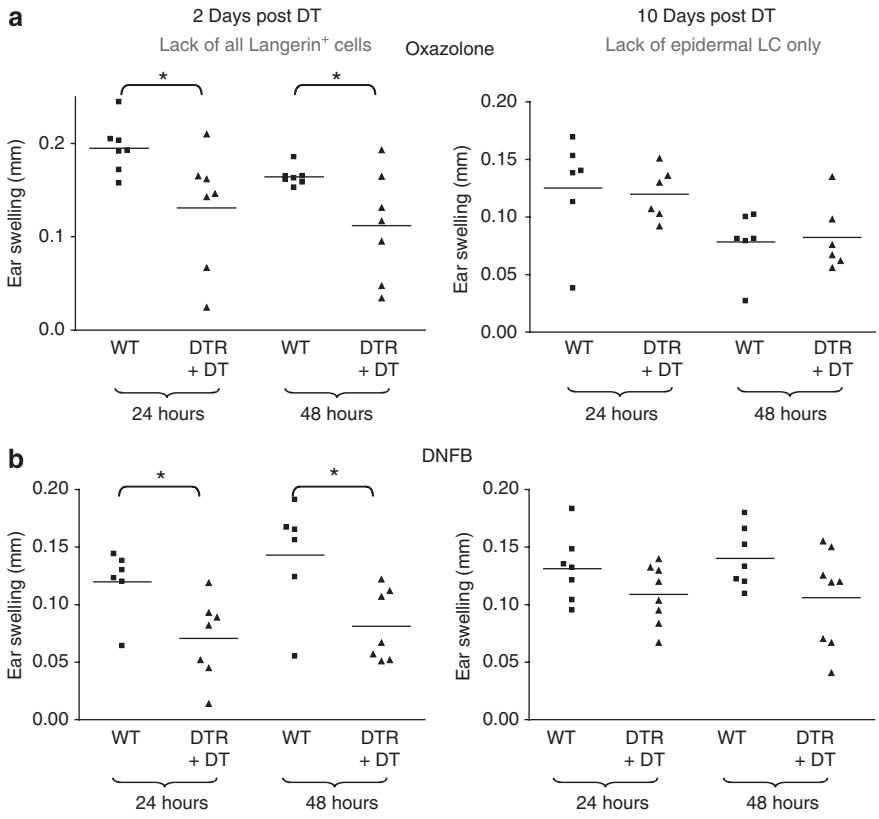

Figure 3. Contact hypersensitivity responses elicited by high-dose hapten. Wild type (WT) or Langerin-diphtheria toxin receptor (DTR) mice (''Dutch mice'') injected with diphtheria toxin (DT) at day −2 or −10 ($n = 6$–8) were (**a**) sensitized on the abdomen with 2% oxazolone and challenged 5 days later with 0.5% oxazolone on one ear, or (**b**) sensitized with 1% 2,4-dinitrofluorobenzene (DNFB) and challenged with 0.5% DNFB on one ear. Ear swelling was measured as the difference between before and after challenge. One representative experiment out of three is shown; symbols represent individual mice. Ear swelling responses were compared using a Student's *t*-test; WT versus DTR + DT: $*P \leqslant 0.05$. LC, Langerhans cells.

(Bennett *et al.*, 2005; Kissenpfennig *et al.*, 2005). Against common expectation from the 'LC paradigm' (Wilson and Villadangos, 2004), CHS reactions were not abolished in any of these LC ablation models indicating that dermal DC can contribute to the priming of hapten-specific $CD8^+$ T cells in CHS (Bennett and Clausen, 2007; Kaplan *et al.*, 2008). The reasons why Langerin-DTA mice show enhanced CHS are not clear. It has been interpreted to suggest that LC may have a regulatory function *in vivo* (Kaplan *et al.*, 2005). Alternatively, one may speculate that exaggerated T cell immunity in Langerin-DTA mice results from the constitutive LC deficiency and failing peripheral tolerance mechanisms during development of the immune system. Therefore, whether or not LC are, e.g., required for efficient skin graft rejection remains controversial (Merad *et al.*, 2004; Obhrai *et al.*, 2008).

By injecting DT after sensitization, but before hapten challenge into the two inducible LC depletion models, it was consistently found that LC (and $Langerin^+$ dermal DC) have no role in regulating the effector T cell response in CHS. In contrast, when DT was given during or shortly before sensitization CHS reactions were reported to be similar (Kissenpfennig *et al.*, 2005) or diminished (Bennett *et al.*, 2005) as compared with WT. In 2007, it was discovered that not all $Langerin^+$ cells in the dermis were transmigrating LC, but that a second phenotypically distinct $Langerin^+$ DC subset was located in the dermis (Bursch *et al.*, 2007; Ginhoux *et al.*, 2007; Poulin *et al.*, 2007). Thus, the initial CHS experiments in Langerin-DTR mice were in fact performed in the absence of both $Langerin^+$ skin DC subsets. After it was described in the French mice that the dermal $Langerin^+$ DC recover faster from the toxin treatment than the LC, these discordant CHS results were reconciled by documenting reduced CHS in the absence of all $Langerin^+$ skin DC, whereas CHS was indistinguishable from WT when only LC were absent (Bursch *et al.*, 2007). From this it was proposed that dermal $Langerin^+$ DC, but not LC, are critical to mediate CHS.

In this study, we establish that also in the Dutch Langerin-DTR mice a fraction of dermal $Langerin^+$ DC has returned already 2 weeks after DT, when the epidermis is still devoid of LC. This confirms earlier observations (Nagao *et al.*, 2009) and is in line with the repopulation kinetics of DC depleted from CD11c-DTR mice, which fully recover to pre-DT levels within 1 week (Jung *et al.*, 2002). Although it is not clear why in the skin there is only partial (<50%) reconstitution of LC and $Langerin^+$ dermal DC in the steady state (Figure 1b and (Bennett *et al.*, 2005; Bursch *et al.*, 2007)), the two inducible

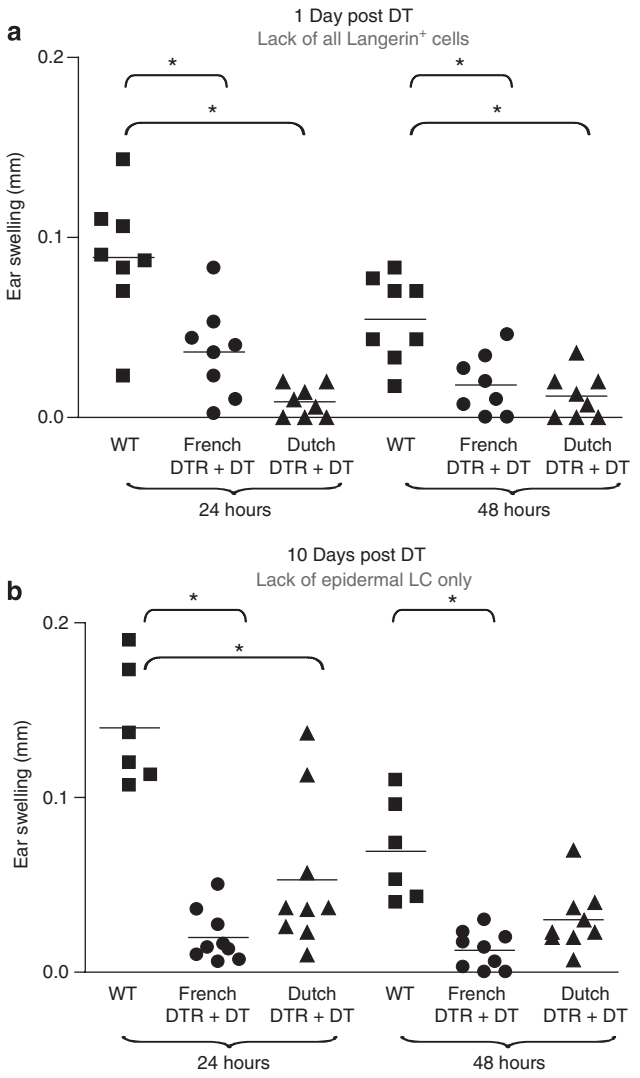

Figure 4. Comparison of low-dose contact hypersensitivity in French and Dutch Langerin-diphtheria toxin receptor (DTR) mice. Wild type (WT), French or Dutch Langerin-DTR mice injected with diphtheria toxin (DT) at (**a**) day −1 or (**b**) day −10 ($n = 6$–9) were sensitized on the abdomen with 0.5% oxazolone and challenged 5 days later with 0.25% oxazolone on one ear. Swelling was measured as the difference between challenged (right) and unchallenged (left) ear. The summary of 2–3 experiments is shown; symbols represent individual mice. Ear swelling responses were compared using a Student's *t*-test; WT versus DTR + DT: $*P \leqslant 0.05$. LC, Langerhans cells.

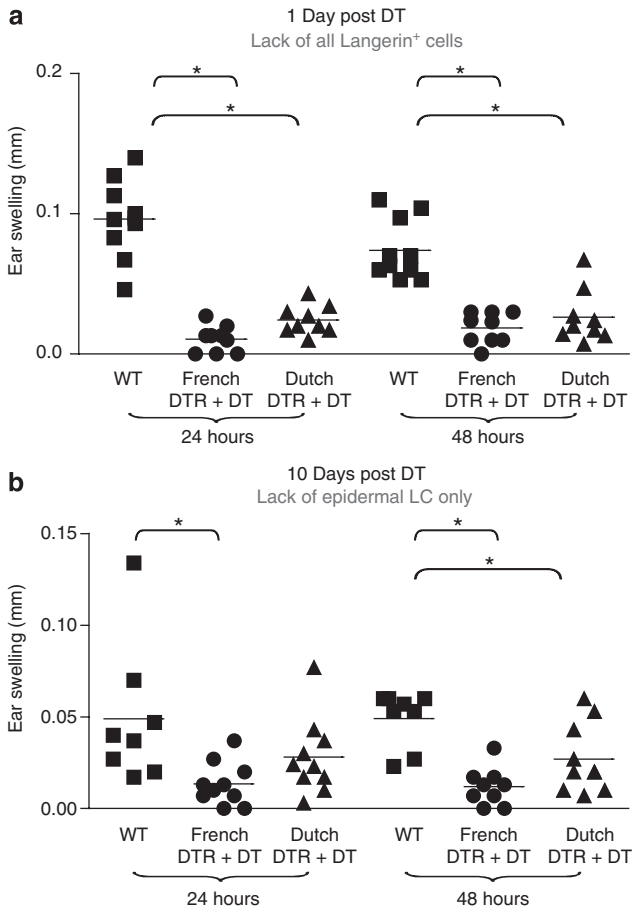

Figure 5. High-dose contact hypersensitivity in French and Dutch Langerin-diphtheria toxin receptor (DTR) mice. Wild type (WT), French or Dutch Langerin-DTR mice injected with diphtheria toxin (DT) at (**a**) day −1 or (**b**) day −10 ($n = 8$–10) were sensitized on the abdomen with 2% oxazolone and challenged 5 days later with 0.5% oxazolone on one ear. Ear swelling was measured as the difference between challenged (right) and unchallenged (left) ear. The summary of two experiments is shown; symbols represent individual mice. Ear swelling responses were compared using a Student's *t*-test; WT versus DTR + DT: $*P \leqslant 0.05$. LC, Langerhans cells.

Langerin-DTR models show similar depletion and repopulation dynamics of Langerin$^+$ (skin) DC populations (Figure 1c).

During our earlier experiments, we observed that differences in ear swelling between DT-treated and -untreated mice were less significant at standard concentrations as compared with low concentrations of hapten, e.g., trinitrochlorobenzene (Bennett *et al.*, 2005) and oxazolone (Bennett *et al.*, 2007) and proposed that LC are mainly required for efficient induction of CHS when antigen dissemination into the dermis is limited. This observation was anticipated by Bacci *et al.* (1997), who also suggested that the requirement for LC in CHS was only given at low doses of hapten. In elegant syngeneic grafting experiments, the authors demonstrate that epidermal grafts obtained from ear skin treated with DNFB at a low dose sensitized the recipient mice. In contrast, dermal grafts failed to induce CHS when the donor skin received an epicutaneous application of a low dose of DNFB, whereas vigorous CHS was detected by dermal grafts prepared from skin treated with a high dose of DNFB. This hypothesis of antigen diffusion/access is in agreement with the requirement of LC for effective tumor immunotherapy after epicutaneous immunization with low dose of antigen (Stoitzner *et al.*, 2008), however, our previous studies were carried out before the Langerin$^+$ dermal DC were described. To determine whether the magnitude of a CHS reaction is a function of antigen dose or the presence of Langerin$^+$ dermal DC, we assessed both low- and high-hapten dose CHS in mice lacking all Langerin$^+$ DC or selectively depleted of epidermal LC (Figures 2 and 3). Consistent with our previous data and with Bursch *et al.* (2007), CHS responses are attenuated in the absence of all Langerin$^+$ cells applying

both a low and a higher dose of oxazolone or DNFB onto the skin. In contrast, when only epidermal LC are absent CHS is still diminished at a low-hapten dose, but restored to WT at a higher concentration of both oxazolone and DNFB (summarized in Table 1). Notably, low-dose CHS is similarly reduced in DT-treated French Langerin-DTR mice irrespective of the presence or absence of Langerin$^+$ dermal DC (Figure 4). Furthermore, both Langerin-DTR models equally respond in high-dose CHS (Figure 5), which excludes any ambiguity concerning the different Langerin-DTR strains.

While this paper was under revision, a study by Honda *et al*. (2010) using the French Langerin-DTR model reported reduced CHS to DNFB in the absence of all Langerin$^+$ skin DC, whereas CHS was similar to WT in mice lacking either LC or Langerin$^+$ dermal DC. In agreement with the present study, this suggests that both Langerin$^+$ skin DC subsets play compensatory roles in the sensitization phase of CHS. However, it remains elusive why the authors fail to detect the essential role of LC for efficient sensitization at a low antigen dose as described in this study and observed by others (Bacci *et al*., 1997 and Stoitzner *et al*., 2008). Since we have assessed the same antigen (DNFB), depletion timing (DT day −1 and day −10) and LC ablation model (French Langerin-DTR mice) as used by Honda *et al*., a difference in antigen concentration seems to be the only remaining explanation for this discordant observation.

Taken together, we demonstrated that the population dynamics of, in particular, Langerin$^+$ dermal DC is similar in the Dutch and French Langerin-DTR mice and that both strains mount comparable CHS responses when tested side-by-side. These data strongly suggest that the magnitude of a CHS reaction depends on the concentration of a given hapten and the number of DC present in the skin, which activate naive hapten-specific T cells in the LN. This confirms and extends classical observations on the role of LC in CHS (Toews *et al*., 1980; Bacci *et al*., 1997). In particular, the degree of CHS is not dependent on a specific skin DC subset, i.e., Langerin$^+$ dermal DC. Therefore, LC and (Langerin$^+$) dermal DC exert redundant functions in mediating CHS.

MATERIALS AND METHODS

Mice

WT C57Bl/6 mice were obtained from Harlan. Generation of the Dutch Langerin-DTR knock-in mice expressing the high-affinity human DTR has been described previously (Bennett *et al*., 2005). Mice were housed in the animal facilities of the Academic Medical Center (Animal Research Institute Amsterdam, University of Amsterdam) or Innsbruck Medical University, except for the French Langerin-DTR mice (Kissenpfennig *et al*., 2005), which were only kept in Innsbruck. All animal experimentation was in compliance with EU as well as national laws and approved by the local ethical committees.

Toxin treatment

As reported previously, mice were intraperitoneally injected with 400 ng DT (Sigma-Aldrich, Zwijndrecht, the Netherlands) in phosphate-buffered saline (Bennett *et al*., 2005). For the inducible depletion of all Langerin$^+$ DC, mice were treated with DT 2 days prior to induction of CHS. To specifically deplete epidermal LC, mice were injected 14 days before hapten sensitization.

FITC painting

FITC (Sigma-Aldrich) was dissolved in DMSO and diluted to 1% in 1/1 acetone/dibutylphthalate (Sigma-Aldrich). A volume of 25 µl was painted on the dorsal side of both ears. After 48 hours, draining LN were collected and DC were isolated and analyzed by flow cytometry.

Induction of CHS

To elicit CHS, WT and DT-treated Langerin-DTR mice were sensitized with 50 µl of either 0.5 or 2% oxazolone (Sigma) in acetone:olive oil (4:1) on the shaved abdominal skin. Five days later, mice were challenged with 20 µl of 0.25 or 0.5% oxazolone on the right ear. Ear swelling was measured at 24 and 48 hours post challenge. CHS responses were calculated as the difference between ear thickness before and after challenge. Alternatively, mice were sensitized with 50 µl of either 0.5 or 1% DNFB (Sigma-Aldrich) on day 0 and challenged with 20 µl of 0.25 or 0.5% DNFB on the right ear 5 days later. In the experiments depicted in Figures 4 and 5, the mice were sensitized with 50 µl of 0.5% or 2% oxazolone and challenged with 20 µl of 0.25% or 0.5% oxazolone on both sides of one ear. Swelling was measured as the difference of challenged (right) and unchallenged (left) ear.

Isolation of DC

Epidermal and dermal cells were prepared from the ears of C57BL/6 and DT-treated Langerin-DTR mice. Dorsal and ventral sides of ears were floated on a solution of 1.2 U ml^{-1} dispase (Roche Applied Bioscience, Almere, the Netherlands) in RPMI for 90 minutes at 37 °C, in order to separate dermis and epidermis. Next, the epidermis was incubated in 0.5% trypsin in RPMI and the dermis in 500 U ml^{-1} collagenase D (Worthington Biochemicals, Lakewood, NJ) in RPMI for 45 minutes at 37 °C. Epidermis and dermis were then mechanically scraped and passed through a 70 µm filter before the cells were washed and resuspended in fluorescence activated cell sorting buffer. Skin-draining LN were harvested and incubated with collagenase D (Worthington Biochemicals) and EDTA to facilitate release of DC.

Whole-skin explant culture

Briefly, ears of mice previously treated with phosphate-buffered saline or DT were cut off at the base, and ear skin was split into dorsal and ventral halves (Stoitzner *et al*., 2003). The dorsal halves were cultured in 24-well plates (one per well) in complete culture medium, without additional chemokines. After 4 days, emigrant DC were pooled from four to six individual ear skin explants, and investigated by flow cytometry.

Flow cytometry

For flow cytometric analysis, cells were incubated with Fc Block (2.4G2, BD Pharmingen, Rotterdam, the Netherlands) and stained with anti-CD11c-PE, (N418, eBioscience, Frankfurt, Germany), anti-MHCII-PE (M5/114, BD Pharmingen), anti-EpCam-PerCp-Cy5.5 (G8.8, Biolegend, Uithoorn, the Netherlands) and anti-CD103-Alexa647 (2E7, Biolegend). For intracellular staining with anti-Langerin-Alexa488 or anti-Langerin-biotin (929.F3, Dendritics,

Lyon, France), cells were fixed in 2% paraformaldehyde and stained in 0.5% saponin buffer. PE-Cy7-conjugated streptavidin (BD Pharmingen) was used to visualize biotin-conjugated antibody. Experiments were performed on a FACSCanto and analyzed using FlowJo software (Becton Dickinson, Breda, the Netherlands).

Immunofluorescence on ear cryosections

Cross-sections from ears were fixed in acetone and stained according to standard procedures with anti-Langerin (929.F3, Dendritics) followed by anti-rat-Cy3 (Jackson Immunoresearch Laboratories, Suffolk, UK) and anti-MHCII-Alexa488 (M5/114, BD Pharmingen). Images were viewed using a DMRA fluorescence microscope (Leica, Rijswijk, the Netherlands). Pictures were taken with a Kx14 camera (Apogee Instruments, Roseville, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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