

Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity

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Langerhans cells (LC) form a unique subset of dendritic cells (DC) in the epidermis but so far their in vivo functions in skin immunity and tolerance could not be determined, in particular in relation to dermal DC (dDC). Here, we exploit a novel diphtheria toxin (DT) receptor (DTR)/DT-based system to achieve inducible ablation of LC without affecting the skin environment. Within 24 h after intra-peritoneal injection of DT into Langerin-DTR mice LC are completely depleted from the epidermis and only begin to return 4 wk later. LC deletion occurs by

apoptosis in the absence of inflammation and, in particular, the dDC compartment is not affected. In LC-depleted mice contact hypersensitivity (CHS) responses are significantly decreased, although ear swelling still occurs indicating that dDC can mediate CHS when necessary. Our results establish Langerin-DTR mice as a unique tool to study LC function in the steady state and to explore their relative importance compared with dDC in orchestrating skin immunity and tolerance.

Introduction

Dendritic cells (DC) are professional antigen presenting cells specialized to induce and regulate T cell immunity and tolerance (Banchereau and Steinman, 1998; Steinman and Nussenzweig, 2002). Initiation of immune responses and maintenance of self-tolerance are believed to rely on the mobilization of sentinel DC to regional lymph nodes (LNs) where they interact with naive antigen-specific T cells. Langerhans cells (LC) are the resident DC population in skin epidermis where they form a contiguous network to detect any invading pathogen or antigen (Romani et al., 2003). Underlying the epidermis, the skin contains a second DC population, dermal DC (dDC), which are phenotypically, and may be functionally, distinct from LC. Due to their position in the epidermis, LC have classically been thought to play a pivotal role in the initiation and control of skin (auto-)immunity and allergy (Schuler and Steinman, 1985; Cumberbatch et al., 2003; Romani et al., 2003). However, this notion has recently been challenged by findings that LC do not initiate protective T cell responses to certain viral antigens

(Allan et al., 2003; Zhao et al., 2003) and *Leishmania major* parasites (Ritter et al., 2004). Instead, protection from these infections depends on LN antigen presentation and T cell activation by dDC. The functional role of LC in T cell priming thus remains unclear. Furthermore, the relative importance of LC compared with dDC in sensitization to chemical allergens and elicitation of cutaneous contact hypersensitivity (CHS) responses is still elusive.

Both LC and dDC constitutively migrate from the skin at a low rate (Hemmi et al., 2001) and skin-draining LN contain LC immigrants as well as interstitial DC derived from the dermis (Anjuere et al., 1999; Ruedl et al., 2000; Henri et al., 2001). This homeostatic turnover increases under inflammatory conditions and dDC appear in the LN as early as 24 h after skin painting, whereas LC require 3–4 d to migrate from the epidermis (Kamath et al., 2002). Recently, it has been shown that the first wave of antigen presentation to naive CD4 T cells occurs by the two LN-resident skin-derived DC subtypes, which acquire subcutaneously injected antigen from the lymph (Itano et al., 2003). Interestingly, this presentation only leads to transient T cell activation and the generation of a delayed type hypersensitivity response requires cell-associated trafficking of cutaneous antigen to the regional LN.

An effective approach to study the function of defined cell populations or lineages in the intact animal is by their

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Abbreviations used in this paper: CHS, contact hypersensitivity; DC, dendritic cells; dDC, dermal DC; DT, diphtheria toxin; DTR, DT receptor; ES, embryonic stem; LC, Langerhans cells; LN, lymph node; MHCII, major histocompatibility complex class II; TNCB, trinitrochlorobenzene; wt, wild-type.

The online version of this paper contains supplemental material.

inducible and specific ablation in vivo. One strategy exploits the insensitivity of mouse cells to killing by diphtheria toxin (DT; Pappenheimer et al., 1982). The cytotoxicity of DT is strictly dependent on receptor-mediated endocytosis (Naglich et al., 1992) and resistance of murine cells results from the low affinity of the toxin for the rodent DT receptor (DTR). Thus, transgenic expression of a primate DTR in mice confers DT sensitivity to murine cells (Saito et al., 2001), which are thought to die by apoptosis (Morimoto and Bonavida, 1992; Thorburn et al., 2003). This DTR/DT system has been successfully used to achieve conditional ablation of DC in CD11c-DTR mice (Jung et al., 2002). In these mice a single injection of DT lead to rapid depletion of CD11c⁺ cells for 2 d after which the DC compartment was gradually restored to wild-type (wt) numbers by day 6.

Langerin is a C-type lectin specifically expressed by LC (Takahara et al., 2002; Valladeau et al., 2002). To target DT sensitivity to LC, we generated mice that harbor a targeted insertion of a primate DTR in the Langerin locus. Here, we report the inducible ablation of LC in adult Langerin-DTR mice within 24 h after injection of DT. LC depletion is complete, specific and occurs by apoptosis without inducing inflammation of the skin. We show that reconstitution of the epidermal LC compartment in the steady state takes at least 4 wk and appears to originate in situ. Functional analysis of LC-depleted mice reveals that dDC are able to mediate a cutaneous CHS response, but supports an essential role for LC in the elicitation of optimal allergic reactions to topically applied hapten.

Results and discussion

Generation of Langerin-DTR mice

A powerful means of investigating the functional role of defined cell populations is via their ablation in vivo. This can be achieved by cell type-specific expression of a primate DTR, which induces apoptotic cell death upon binding and internal-

ization of DT (Morimoto and Bonavida, 1992; Saito et al., 2001; Thorburn et al., 2003). Previously, we have identified the mouse homologue of the human Langerin gene and confirmed its LC-specific expression (Takahara et al., 2002). For highly efficient and inducible depletion of LC, we generated Langerin-DTR knockin mice (Fig. 1). Specifically, a simian DTR:EGFP cassette (Jung et al., 2002) was inserted into the second exon of the Langerin gene via homologous recombination in embryonic stem (ES) cells (Fig. 1 A). To ensure efficient translation of the DTR:EGFP fusion protein, all ATG codons upstream of the DTR cDNA were mutated. The linearized vector was electroporated into C57BL/6 ES cells (Bruce4; Köntgen et al., 1993). Correctly targeted ES cell clones were identified by genomic Southern blot using a 5' external probe outside the homology arm and subsequently confirmed to be single-copy integrants with the internal DTR:EGFP cDNA probe (Fig. 1, A and B; not depicted). Two independent targeted ES cell clones were injected into blastocysts for the generation of chimeras. After germline transmission of the mutation, the Langerin-DTR line was established by crossing one founder with an ACTFlpe mouse (Rodriguez et al., 2000) to excise the FRT-flanked neomycin (Neo^R) cassette. Deletion of the selection marker was confirmed by genomic Southern blot and sequence analysis (Fig. 1, A and C; not depicted). The Langerin-DTR mice are maintained as heterozygotes, are healthy, and breed normally. They are routinely genotyped by PCR (Fig. 1 D).

Efficient and specific long-term ablation of LC upon injection of DT

Both during initial seeding of the epidermis in fetal life (Tripp et al., 2004) and repopulation of the epidermis after mechanical disruption (Holzmann et al., 2004) major histocompatibility complex class II (MHCII) precedes expression of Langerin. Therefore, we monitored depletion and repopulation of LC by

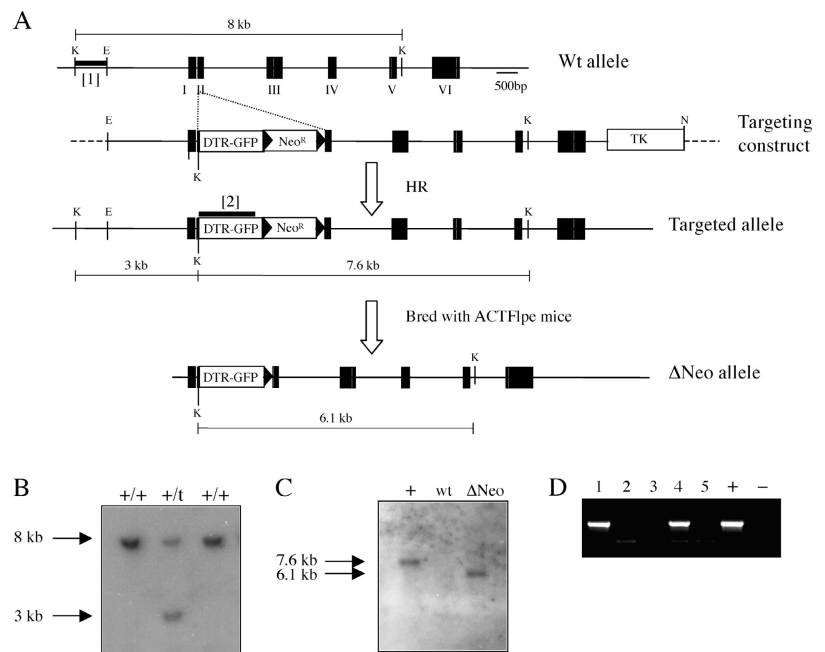


Figure 1. Generation of Langerin-DTR mice. (A) Schematic representation of the targeting strategy used for the generation of Langerin-DTR mice. HR, homologous recombination; K, KpnI; E, EcoRI; N, NotI. (B) Identification of targeted ES cell clones by Southern hybridization using probe [1] after digestion with KpnI, 8-kb wt allele and 3-kb targeted allele. +/+, wt; +/t, targeted heterozygote. (C) Deletion of the Neo^R cassette was verified by Southern blot analysis using the DTR:GFP cassette as probe [2]: +, Langerin-DTR founder; wt, wt control; ΔNeo, Langerin-DTRΔNeo. (D) Genomic PCR of Langerin-DTR mice, lanes 1–5, Langerin-DTR mutant and wt littermates; +, Langerin-DTR founder; –, water control.

MHCII. As investigated by fluorescence microscopy of epidermal ear sheets, LC develop normally in Langerin-DTR mice (Fig. 2 A and Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200501071/DC1>). However, highlighting their toxin sensitivity LC are rapidly and completely ablated upon

i.p. injection of DT (Fig. 2 A). The vast majority of LC appear to be depleted within 24 h of injection and at 48 h the ear epidermis is devoid of LC. Interestingly, LC do not repopulate the epidermis during 4 wk after injection of DT (Fig. 2 B) and at this time point individual foci (“patches”) of MHCII⁺ LC be-

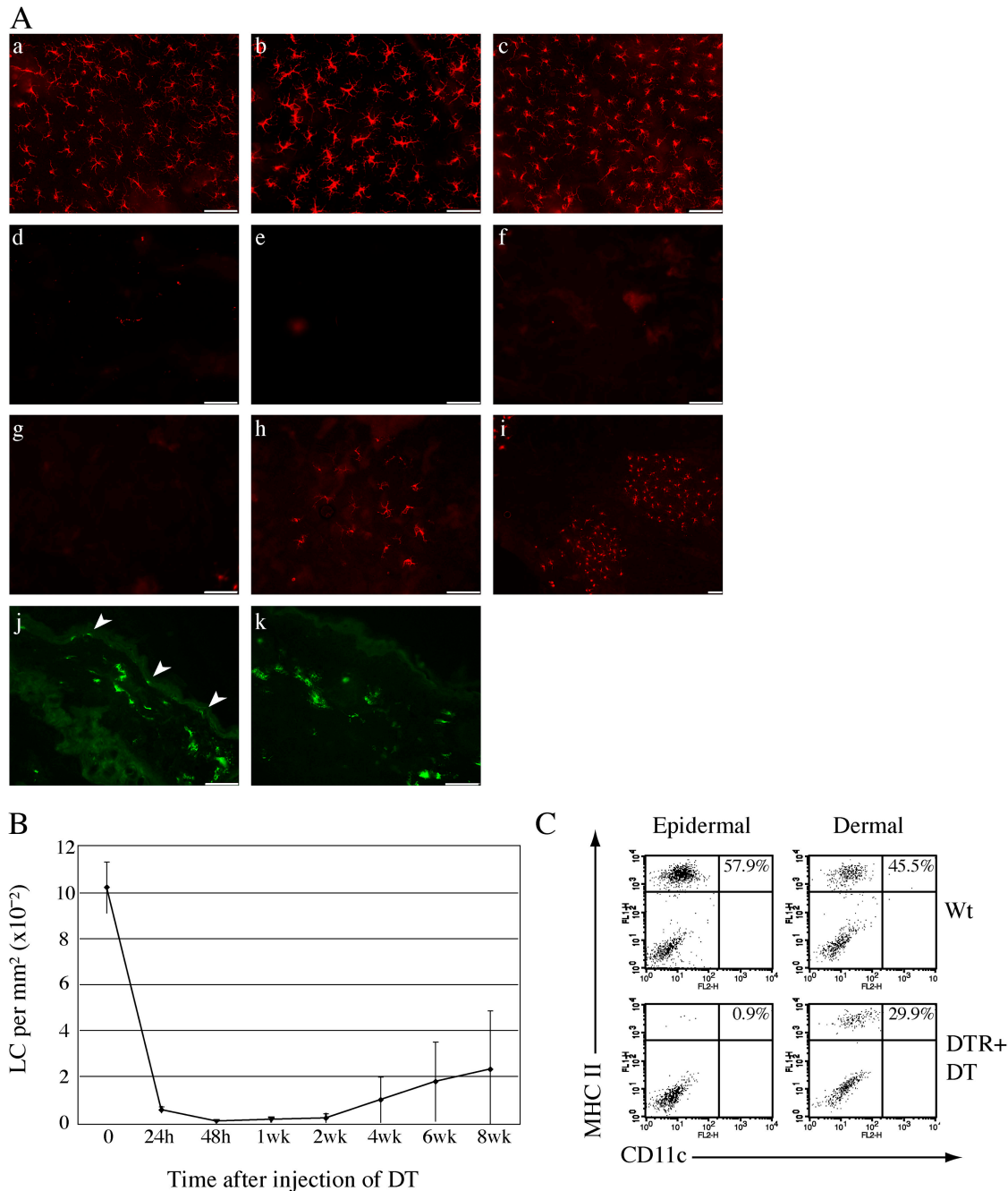
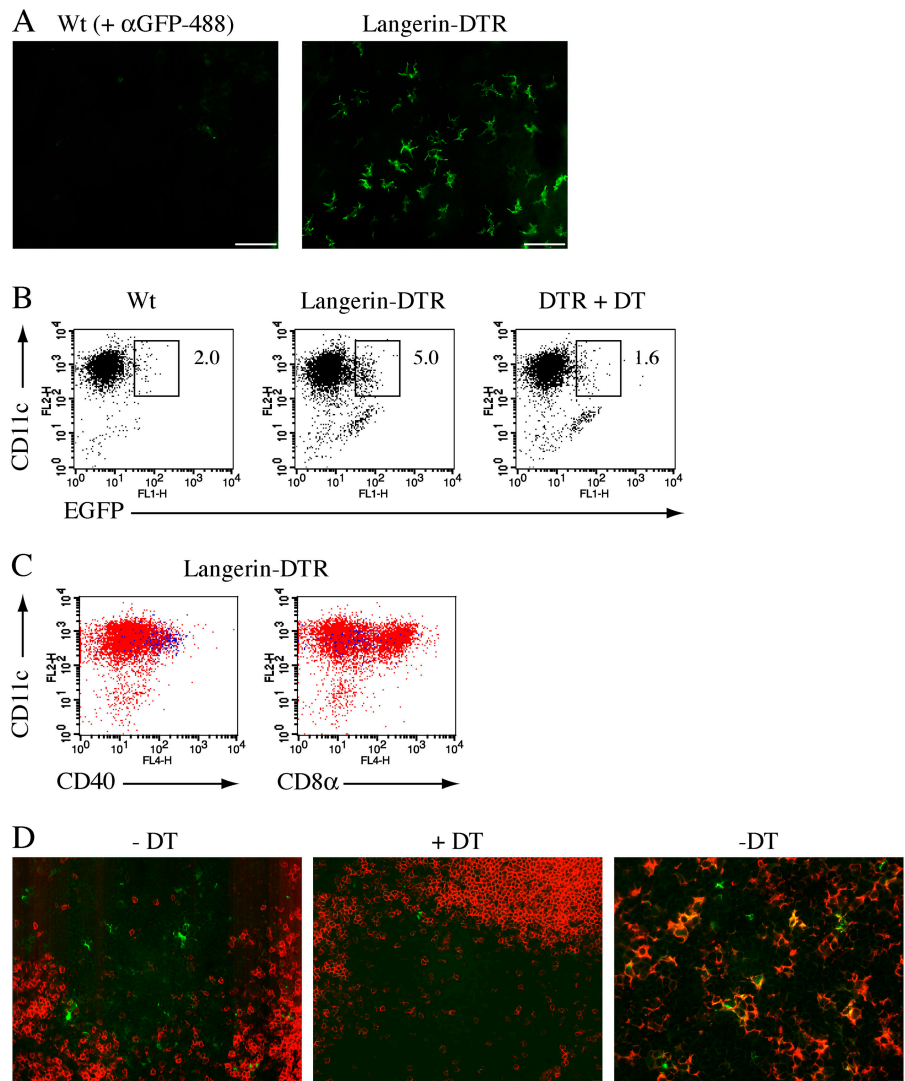


Figure 2. Efficient and specific ablation of LC from Langerin-DTR mice. (A) Epidermal sheets were separated from mouse ears and stained for MHCII to visualize LC: (a) wt; (b) wt + DT (72 h); (c) Langerin-DTR. Mutant mice were subsequently injected i.p. with DT and LC visualized to determine the kinetics of depletion and repopulation; (d) 24 h; (e) 48 h; (f) 2 wk; (g–i) 4 wk. Pictures are representative of four different mice for each time point, γ adjustments were made to reduce background on epidermal sheets. Cross sections through ear skin were stained with MHCII to demonstrate selective depletion of LC and an intact dDC compartment after DT. (j) Langerin-DTR; (k) DTR + DT (24 h). Arrowheads identify MHCII⁺ LC in the epidermis. Bars, 50 μ m. (B) Quantification of depletion and repopulation of LC after injection of DT. Numbers of MHCII⁺ LC were counted using a gridded eyepiece with the 40 \times objective. 20 fields of evenly spaced cells were counted from four different mice for each time point. At 6 wk after injection of DT, LC numbers are still significantly decreased compared with wt mice (Mann-Whitney test wt vs. Langerin-DTR mice at 6 wk: $P = 0.0286$). Error bars represent SD. (C) LC but not dDC are ablated in Langerin-DTR mice. Emigrant cells from the epidermis or dermis of wt and Langerin-DTR mice injected with DT 48 h earlier were analyzed by flow cytometry. Numbers show the percentage of CD11c/MHCII double-positive cells, FACS plots are representative of three separate experiments.

Figure 3. EGFP expression in Langerin-DTR mice. (A) Expression of EGFP in Langerin-DTR epidermal sheets. Visualization of EGFP expression was enhanced by incubation of an anti-GFP-488 antibody, which specifically labeled cells from Langerin-DTR mice. Bars, 50 μ m. (B) EGFP expression in skin draining LN. CD11c⁺ LN cells from wt, Langerin-DTR, and Langerin-DTR mice injected with DT (48 h) were enriched and analyzed for EGFP expression, 3.34% \pm 0.375 (mean \pm SEM, $n = 4$) of LN cells were gated EGFP-positive compared with 1.47% \pm 0.121 ($n = 4$) background fluorescence in wt mice. Mann-Whitney test wt vs. Langerin-DTR, $P = 0.0159$. FACS plots are representative of four different experiments. (C) Cells from Langerin-DTR mice were analyzed for expression of surface markers and CD11c. Blue dots are gated EGFP⁺ cells from B. Plots are representative of at least five different experiments. (D) LNs from Langerin-DTR mice or Langerin-DTR mice injected with DT for 48 h were sectioned and analyzed for the presence of EGFP⁺ LC. All sections were stained with anti-GFP-488 in addition to anti-B220 (a and b) or anti-MHCII (c) to demarcate B and T cell areas, respectively. Bars, 10 μ m.



come visible in the epidermal sheets (Fig. 2 A). Even at 8 wk LC reconstitution is incomplete and, in addition, the cells that return from 4 wk onwards appear to have more extended dendrites “reaching out” into the intercellular space. Therefore, fewer LC cover the same surface area compared with before DT deletion (unpublished data). As expected, there is no adverse effect of DT on LC of wt mice and, importantly, dDC are still present after injection of DT into Langerin-DTR mice (Fig. 2 A).

These histological observations were confirmed by flow cytometry of cells that emigrated in vitro from epidermal and dermal sheets, respectively (Fig. 2 C). At 48 h after DT injection, CD11c⁺MHCII⁺ LC are reduced by 95.4% \pm 1.18 (mean \pm SEM, $n = 6$). The reduction of dermal CD11c⁺MHCII⁺ cells (19.4% \pm 4.9, $n = 6$) can be attributed to loss of LC in transit on their homeostatic migration to the local LN. In addition, upon separation small areas of epidermis inevitably remain attached to the dermal sheet (Stoitzner et al., 2003).

Recently, the DTR/DT system was used to efficiently delete DC within 24 h of systemic injection of DT (4 ng/g body weight) into CD11c-DTR transgenic mice (Jung et al., 2002).

To ensure that sufficient concentrations of DT were reached at the epithelial border to the outside world, Langerin-DTR mice were treated with 16 ng DT/g body weight throughout our experiments. However, doses as low as 2 ng/g body weight yield near complete depletion of the LC after 48 h (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200501071/DC1>).

In contrast to CD11c-DTR mice, in which DC numbers recover to pre-DT treatment levels within 5–6 d (Jung et al., 2002), the first signs of LC repopulation of the epidermis in DT-injected Langerin-DTR mice become evident only after 4 wk (Fig. 2 A), in agreement with the low steady-state turnover of LC (Merad et al., 2002). There are multiple hypotheses to explain the return of LC in LC-depleted mice: On one hand, LC may recover due to in situ proliferation of a dedicated Langerin^{negative} LC precursor, or rare LC which escaped DT-mediated deletion; alternatively, LC reconstitution may be the result of de novo immigration, differentiation, and proliferation of LC precursors and/or monocytes into the epidermis (Merad et al., 2002; Holzmann et al., 2004). In the absence of any inflammatory mediators attracting LC precursors to move into the epidermis and considering the focal nature of initial repopulation

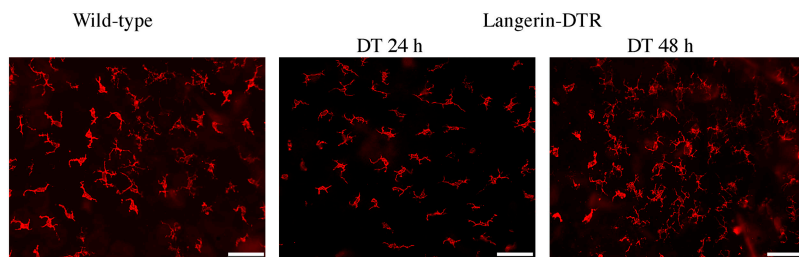


Figure 4. **Depletion of LC in the absence of inflammation.** Epidermal sheets from wt and Langerin-DTR mice injected with DT were stained with an antibody against the $\gamma\delta$ TCR to visualize dendritic epidermal $\gamma\delta$ T cells. Pictures are representative of four different mice for each time point. γ adjustments were made to reduce background on epidermal sheets. Bars, 50 μ m.

of the epidermal LC niche, we currently favor local differentiation and proliferation of in situ LC or LC precursors as the most likely mechanism of LC reappearance, and are investigating this hypothesis further.

Langerin-driven EGFP expression in Langerin-DTR mice

In Langerin-DTR mice, the Langerin promoter drives expression of a DTR:EGFP fusion protein (Fig. 1 A). By fluorescence microscopy of epidermal sheets, we can detect EGFP⁺ LC, which vanish upon injection of DT (Fig. 3 A and not depicted). However, even using an anti-GFP antibody for detection, the staining appears to be less intense than that observed with anti-MHCII and we are unable to visualize all LC which, based on their susceptibility to DT-mediated killing, we would expect to be EGFP⁺ (compare Fig. 2 A with Fig. 3 A).

Flow cytometry of skin-draining LN of Langerin-DTR mice reveals a distinct CD11c⁺EGFP⁺ DC subpopulation, which corresponds to $3.34\% \pm 0.375$ (mean \pm SEM) of all CD11c⁺ LN cells (Fig. 3 B). As demonstrated in Fig. 3 C, these EGFP⁺ LN DC are CD40^{high} and CD8 α ^{low} and, thus, most likely represent recent epidermal skin immigrants (Henri et al., 2001; Stoitzner et al., 2003). By immunohistochemistry, the Langerin-EGFP⁺ DC are confined to the T cell area where LC-derived DC interact with naive antigen-specific T cells (Fig. 3 D). Upon injection of DT this skin-derived EGFP⁺ LN DC subset is readily depleted (Fig. 3, B and D). Careful examination of EGFP expression by colocalization with MHCII reveals that there are very few EGFP⁺ cells in thymus and spleen; although they are clearly present in the thymus, it is very difficult to detect any EGFP⁺ cells in the spleen (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200501071/DC1>). These observations are in contrast to published data using anti-Langerin antibodies (Valladeau et al., 2002; Stoitzner et al., 2003). As is evident from Fig. 3 A, Langerin-driven EGFP expression may not allow detection of all cells that should be EGFP⁺. Therefore, we do not know if this discrepancy is due to an underestimation in the Langerin-DTR mice or reflects a genuine functional difference.

Depletion of LC in the absence of inflammation

One of the important advantages of DTR/DT-mediated LC depletion compared with conventional techniques, namely steroids (Grabbe et al., 1995), UV-B irradiation (Merad et al., 2002) or repeated tape stripping (Holzmann et al., 2004), is efficient ablation of LC without any apparent adverse side ef-

fects. In particular, there is no induction of skin inflammation as DT induces apoptosis by inhibition of protein synthesis and activating components of the death receptor pathway (Morimoto and Bonavida, 1992; Thorburn et al., 2003). Tissue integrity was demonstrated by visualizing a wt dendritic epidermal $\gamma\delta$ T cell network in DT-treated Langerin-DTR mice (Fig. 4). In addition, we failed to detect any cell infiltration or inflammatory ear swelling in response to DT-induced LC death (unpublished data). Upon injection of DT, LC disappear from the epidermis so rapidly that it would be difficult to demonstrate their death by apoptosis in situ. Therefore, epidermal emigrants from ears of Langerin-DTR and wt mice were incubated with DT in vitro. The specific appearance of AnnexinV⁺ cells in cultures from Langerin-DTR, but not wt, mice supports our hypothesis of apoptotic LC death (unpublished data). Thus, LC-depleted Langerin-DTR mice represent immunologically naive, resting animals with no other obvious phenotype except their lack of LC. Therefore, they provide a unique tool to study the role of LC and their LN descendants in the steady state, for instance, in the induction of peripheral tolerance to cutaneous antigens.

Reduced CHS in the absence of LC

Murine CHS is a prototypic T cell response to haptens painted onto the skin. Priming of naive hapten-specific T cells, during the asymptomatic sensitization phase, is thought to be mediated by LC. Re-exposure to the relevant hapten then initiates the efferent phase and development of disease (Grabbe and Schwarz, 1998). Although LC have been strongly linked to the initiation of CHS in vivo (Enk and Katz, 1992; Enk et al., 1993), their function relative to dDC has not been determined. LC may also play an, as yet undefined, role regulating the effector phase of the allergic response.

To test the requirement for LC we induced a CHS response in LC-depleted mice. 3 d after i.p. injection of DT, when the epidermis is devoid of LC (Fig. 2, A and B), Langerin-DTR mice were sensitized to hapten by painting 1% trinitrochlorobenzene (TNCB) on the abdomen. 5 d later, the animals were challenged by topical application of 0.5% TNCB onto one ear and ear swelling was measured 24 and 48 h later. As shown in Fig. 5, an allergic response is induced both in the presence and absence of LC, which subsides with time. It is important to point out that hapten sensitization (abdomen) is spatially separated from the site of elicitation of disease (ear) and LC precursors do not repopulate the ear epidermis after sensitization (unpublished data). Therefore, LC are absent throughout the entire CHS response and our data demonstrate that dDC are sufficient for the initiation and elicitation of CHS.

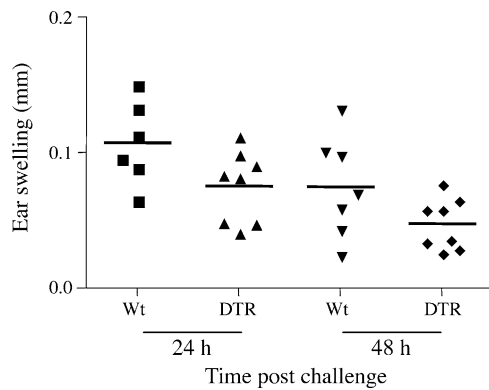


Figure 5. **Depletion of LC results in sub-optimal CHS responses.** Wt or Langerin-DTR mice ($n = 6-8$) injected with DT at day -3 , were sensitized on the abdomen with 1% TNCB and challenged 5 d later with 0.5% TNCB on one ear. Swelling was measured as the difference between the challenged and nonchallenged ear. Ear swelling responses were compared using a repeated measures ANOVA; wt vs. DTR + DT: $P = 0.039$. One representative experiment out of three is shown. The two additional experiments were performed with four mice per group.

It is also evident from Fig. 5 that although dDC alone are able to mediate CHS, the magnitude of the allergic response is significantly reduced in the absence of LC. This finding contradicts published data (Grabbe et al., 1995), which were, however, obtained using experimental conditions (steroids) known to disturb the local microenvironment. It has recently been discovered that distinct skin-derived DC populations sequentially present antigen to $CD4^+$ T cells with dDC, not LC, being the first to carry cell-associated antigens to the LN for stimulation of a delayed type hypersensitivity response (Itano et al., 2003). Ongoing experiments extend these studies and aim to dissect whether LC are required during the afferent and/or efferent phase of CHS, to mediate an optimal allergic response.

To date it has not been possible to definitively assess the relative antigen presentation contributions of LC and dDC in skin immunity and tolerance. By inducible LC ablation in mice, we show here that dDC are able to induce CHS to chemical allergens, but LC are mandatory to provoke aggravated allergic disease. Although the former may not be entirely unexpected for hapten antigens likely to penetrate into the dermis, the latter may allow the LC to redeem some of the credit they recently seem to have lost (Serbina and Pamer, 2003). Our Langerin-DTR mice represent a unique tool with which to unravel the long-standing and more recent mysteries of LC function and development, in particular, in relation to their dDC neighbors.

Materials and methods

Mice

Mice were housed in the animal facility of the AMC (Animal Research Institute Amsterdam [ARIA], University of Amsterdam). All animal experimentation was in compliance with EU as well as national laws and approved by the local ethical committee (Dierexperimentencommissie, AMC).

Generation of Langerin-DTR mice

A 5-kb EcoRI fragment containing the putative promoter region and first three exons of the Langerin gene was identified from a murine BAC clone (Research Genetics) and subcloned into pBS as a base for the Langerin-DTR targeting construct. All ATG codons before the intended DTR insertion

point in the second exon were subsequently mutated using two successive sets of mutagenic primers, which included unique restriction sites for later cloning steps. HSV-TK was released from pIC19R-MC1tk (Mansour et al., 1988) and FRT2Neo from pFRT2neoA (Jung et al., 1993). Both selection markers were cloned downstream of the Langerin homology arm, leaving a unique Sall restriction site between the two cassettes. This restriction site enabled introduction of the 3' homology arm of the targeting vector after PCR amplification from C57Bl/6 genomic DNA. The targeting construct was completed by inserting DTR:EGFPbpA (modified from Jung et al. (2002) by addition of a bpA tail) into a unique PmeI site created in exon 2. The final construct was linearized using NotI, precipitated, and resuspended in 100 μ l of sterile PBS (25 μ g DNA per 10^7 ES cells). Electroporation of ES cells (Bruce4; Köntgen et al., 1993; gift of A. Waismann, University of Mainz, Mainz, Germany) and the expansion of Neo^R clones was performed at the AMC targeting facility.

Southern blot analysis of ES cell clones

Genomic DNA from ES cell clones was digested with KpnI and blotted onto a nylon membrane (Genescreen plus; NEN Life Science Products). The DNA was hybridized to an ~ 800 -bp 5' external probe, generated by PCR (Lang-1, 5'-GGAATGGTACCATCAGCAITTCAGGG-3'; Lang-2, 5'-AGTGTTCAGGGGAATTCCTGGTTG-3'), which yielded an 8-kb wt and 3-kb targeted band. Hybridization was performed using RapidHyb buffer (Amersham Biosciences) at 65°C overnight. Membranes were washed with $1 \times$ SSC/0.1% SDS and exposed to Kodak XOMAT-AR film. Stripped membranes were reprobed with a 1.4-kb EcoRI DTR:EGFP fragment to exclude multiple integrants. Southern analysis revealed a single 7.6-kb and 600-bp bands. Positive clones were confirmed by digest with a different enzyme (NcoI) and hybridization to a 425-bp DTR probe, giving a correctly targeted band size of 3.5 kb.

Genomic analysis of Langerin-DTR mice

Two correctly targeted ES cell clones were selected for the production of chimeras. Germline transmission was verified by PCR and Southern blot analysis as above. Mice were genotyped as described by Jung et al. (2002) (DTR1, 5'-GCCACCATGAAGCTGCTGCCG-3'; DTR2, 5'-TCAGTGGGAATTAGTCATGCC-3'). To delete the Neo^R cassette founder mice were bred with ACTFlpe mice (Rodriguez et al., 2000) purchased from The Jackson Laboratory. F1 offspring were analyzed by Southern: KpnI-digested DNA was hybridized with the DTR:EGFP probe to give a 7.6-kb fragment in the original targeted allele, and a 6.1-kb fragment in the Δ Neo allele. This was confirmed by sequence analysis of the region surrounding the resolved FRT site.

Injection of DT

DT was provided by B. Metz (The Netherlands Vaccine Institute, Bilthoven, NL) as a concentrated *Diphtheria* culture supernatant, diluted in sterile PBS, and 400 ng were injected i.p. per mouse corresponding to 16 ng/g body weight.

Preparation of epidermal sheets and fluorescent microscopy

The dorsal and ventral halves of mouse ears were split mechanically and floated on 20 mM EDTA/PBS at 37°C for 90 min. Epidermal sheets were then removed, fixed with ethanol, rehydrated in PBS, blocked with PBS/0.5% BSA, and stained with anti-MHCII-PE, anti- $\gamma\delta$ TCR-PE, or anti-GFP-488, as appropriate, for 30 min at RT. After washing with PBS the epidermal sheets were mounted on slides with Vector shield mounting medium (Vector Laboratories). Cryosections (5–6 μ m thick) were fixed in 4% PFA and stained according to standard procedures with anti-MHCII-FITC (skin) or anti-MHCII and anti-B220 followed by an anti-rat-Cy3 secondary antibody (lymphoid organs). Images were viewed at RT using a DMRA fluorescent microscope (objectives of 10 \times , 25 \times , or 40 \times ; Leica). Images were captured using a Kx14 camera (Apogee Instruments, Inc.) and Image-Pro Plus software. All antibodies were from BD Biosciences except anti-GFP-488 (Molecular Probes, Inc.) and anti-rat Cy3 (Jackson ImmunoResearch Europe Ltd.).

Monitoring EGFP fluorescence

All FACS data represent genuine Langerin-driven EGFP expression. Throughout our immunohistochemical experiments, using an anti-GFP antibody did not alter the staining pattern compared with direct EGFP fluorescence. However, although EGFP⁺ LC were visible by themselves, the staining intensity was increased with the anti-GFP-488 antibody (Fig. 3 A). To our surprise, F1 mice retaining the Neo^R cassette (Fig. 1 A) exhibited brighter anti-GFP staining than Neo-deleted animals. This observation was critical to enable visualization of EGFP⁺ cells in lymphoid organs by immu-

nohistochemistry. Having extensively checked both mice we are certain that there is no difference in the expression pattern of Langerin-driven EGFP in the presence of the *neo* gene.

Isolation of LC and dDC

LC and dDC were isolated after washing mouse ears in 70% ethanol and digestion of split ears in 1.2 U/ml dispase (Boehringer) for 30 min at 37°C. Epidermal and dermal layers were separated and cultured in 24-well plates (one ear per well) in RPMI (GIBCO BRL)/10% FCS (PAA) with β -mercaptoethanol and gentamycin (Sigma-Aldrich). 48 h later the cells were passed through a 70- μ m cell strainer (BD Biosciences) to remove any debris, and resuspended in PBS/1% FCS for flow cytometric analysis.

Enrichment of CD11c⁺ cells from lymphoid organs and flow cytometry

Lymphoid organs were incubated with Collagenase D (Worthington) and EDTA, and stained with anti-CD11c-PE and anti-PE-microbeads (Miltenyi Biotec). CD11c⁺ cells were enriched via application of a magnetic field. For flow cytometric analysis cells were blocked with FcBlock (2.4G2; BD Biosciences) and stained with the relevant antibodies (all obtained from BD Biosciences). Experiments were done on a FACSCalibur and analyzed using CellQuest software (Becton Dickinson).

CHS responses

To elicit CHS mice were sensitized with 50 μ l 1% TNCB (a gift from K. Mahnke, University of Heidelberg, Heidelberg, Germany) in acetone/olive oil (AOO) 4:1 on the shaved abdomen and challenged 5 d later with 20 μ l 0.5% TNCB in AOO on the back of the right ear. The left ear was untreated and swelling responses were measured as the difference between left and right ears at 24 and 48 h after challenge. To investigate ear swelling responses in the absence of LC, Langerin-DTR mice were injected with 400 ng DT i.p. 72 h before sensitization. In initial experiments we have excluded the possibility that untreated Langerin-DTR mice have an intrinsically lower capacity to mount a CHS response.

Online supplemental material

Fig. S1 demonstrates wt numbers of LC in Langerin-DTR mice, Fig. S2 provides a dose titration of DT to deplete LC, and Fig. S3 shows the presence or absence of EGFP⁺ cells in thymus and spleen. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200501071/DC1>.

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