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Langerhans Cells Are Required for Efficient Presentation of Topically Applied Hapten to T Cells

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Dendritic cells (DC) play a pivotal role in the control of T cell immunity due to their ability to stimulate naive T cells and direct effector function. Murine and human DC are composed of a number of phenotypically, and probably developmentally, distinct subsets, which may play unique roles in the initiation and regulation of T cell responses. The skin is populated by at least two subsets of DC: Langerhans cells (LC), which form a contiguous network throughout the epidermis, and dermal DC. LC have classically been thought vital to initiate T cell responses to cutaneous Ags. However, recent data have highlighted the importance of dermal DC in cutaneous immunity, and the requirement for LC has become unclear. To define the relative roles of LC and dermal DC, we and others generated mouse models in which LC were specifically depleted in vivo. Unexpectedly, these studies yielded conflicting data as to the role of LC in cutaneous contact hypersensitivity (CHS). Extending our initial finding, we demonstrate that topical Ag is inefficiently transported to draining lymph nodes in the absence of LC, resulting in suboptimal priming of T cells and reduced CHS. However, dermal DC may also prime cutaneous T cell responses, suggesting redundancy between the two different skin DC subsets in this model. The Journal of Immunology, 2007, 179: 6830–6835.

Dendritic cells (DC) are professional APCs that are essential for the initiation and control of T cell-mediated immunity (1). Not only are DC required for the activation of naive T cells, but it is becoming increasingly clear that they are also necessary for appropriate reactivation of the memory response. Furthermore, DC have important immunoregulatory functions both in maintaining steady state peripheral tolerance, and to control on-going immune responses, for example, via interaction with regulatory T cells (2). Murine and human DC can be divided into different phenotypically distinct subsets (3, 4) and, at present, the developmental and functional relationships between these populations are not clear.

The skin contains at least two phenotypically distinct populations of DC: epidermally resident Langerhans cells (LC) and dermal DC. The skin therefore provides a tractable system with which to dissect the functional relevance of the presence of different DC subsets. Early work on skin DC focused largely on LC, which were described as prototypic immature DC (5). It was assumed that pathogens or allergens entering the body via the skin would encounter, be taken up, processed, and subsequently presented in draining lymph nodes (LN) by epidermal LC, which were therefore responsible for initiating T cell immune responses to cutaneous Ags (6–8). More recently, however, dermal DC have been shown to be required for initiation of T cell responses to protein and viral Ags (9, 10), while alternative DC populations prime other cutaneous immune responses (11, 12), obviating a need for LC in these models. In the light of these data, it has been proposed that the primary function of LC may be the transport of skin Ags to draining LN for transfer to, and presentation by, LN-resident DC populations (13). Alternatively, a regulatory role could be considered for LC, either within draining LN or in situ in the skin, although it was recently shown that LC may be the only nontolerogenic DC population in the steady state (14).

To investigate whether LC are specifically required for T cell responses to topical Ags, or whether dermal DC can also fulfill this role, we generated an inducible mouse model in which LC could be specifically depleted in vivo upon injection of diphtheria toxin (DT). LC are completely ablated from the epidermis of Langerin-DTR mice within 48 h of injection of DT and the epidermis remains largely devoid of LC for at least 4 wk post-DT (15). Depletion of LC in these mice therefore provides us with a window in which to specifically address the role of LC in immune responses to cutaneous Ags. In this study, we further investigated the induction of cutaneous contact hypersensitivity (CHS) following topical application of hapten Ag, as a model cutaneous immune response.

CHS is a delayed-type hypersensitivity response to a topically applied hapten. Painting of a “sensitizer” onto the skin initiates local production of proinflammatory cytokines and chemokines that activate skin DC to prime hapten-specific T cells in draining LN (16). CHS is a transient response mediated by IFN-γ-producing CD8+ T cells and controlled in the skin by CD4+ T cells producing IL-4 and IL-10 (17). Early studies suggested a correlation between low density of LC and a reduction in CHS (18, 19). Furthermore, LC activation has been linked with CHS in a number of murine models in which cytokine or chemokine levels are altered in the skin (20–23). More recently, however, models in...
which LC are inducibly depleted in vivo (15, 24), or are constitutively absent (25), have yielded conflicting results rather than clarifying the issue. Thus, whether LC are required for presentation of topically applied hapten is remains unclear.

Therefore, we set out to thoroughly investigate and characterize the relative functions of LC and dermal DC for the induction and control of CHS using the Langerin-DTR mouse. We demonstrate that topical Ag is inefficiently trafficked to draining LN in the absence of LC, resulting in suboptimal priming of CHS-effector T cells. However, CHS is still initiated in the absence of LC, indicating that, within the skin, redundancy between different DC subsets may exist.

Materials and Methods

**Mice**

C57BL/6 (B6) mice were obtained from Harlan Sprague Dawley. Langerin-DTR mice (on a B6 background) express the high-affinity human DTR in the Langerin locus and have been described elsewhere (15). LC were depleted, as stated previously, upon i.p. injection of 400 ng DT in PBS (15). Mice were housed in the animal facility of the Academic Medical Center (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 (Dier Experimenter Commissie, AMC).

**Induction of CHS and irritant responses**

To elicit CHS, mice were sensitized with 50 μl of either 0.5 or 2% Oxazolone (Sigma-Aldrich) in acetone/oil (AOO) 4/1 on the shaved abdomen and challenged 5 days later with 20 μl of, respectively, 0.25 or 0.5% Oxazolone 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 DT i.p. either 72 h before sensitization or 72 h before challenge. To test irritant responses, 1% Croton oil (in olive oil; Sigma-Aldrich) was painted onto the back of the right ear and ear swelling was measured 24 and 48 h later.

**Preparation and transfer of T cells**

For adoptive transfer of Oxazolone-induced CHS, T cells (from draining LN) were taken from wt or Langerin-DTR mice injected with DT that had been sensitized on the abdomen with 2% Oxazolone (Sigma-Aldrich) in acetone/oil (AOO) 4/1 on the shaved abdomen and challenged 5 days later with 20 μl of, respectively, 0.25 or 0.5% Oxazolone 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 DT i.p. after either 72 h before sensitization or 72 h before challenge. To test irritant responses, 1% Croton oil (in olive oil; Sigma-Aldrich) was painted onto the back of the right ear and ear swelling was measured 24 and 48 h later.

**FACS analysis of FITC**

FITC (Sigma-Aldrich) was dissolved in DMSO and diluted to 0.5% in 1/1 acetone/dibutylphthalate (Sigma-Aldrich). Twenty-five microtiter plates were coated onto the dorsal sides of both ears. Forty-eight or 96 h later, draining LN were taken and incubated with collagenase D (Worthington Biochemical) and EDTA to facilitate release of DC. For flow cytometric analysis, cells were incubated with FcBlock (2.4G2; BD Pharmingen) and stained with the following mAbs: anti-MHCII-PE (M5/114), anti-CD8α-PE (53-6.7), anti-CD40-biotin (3/23) (all BD Pharmingen), anti-CD11c-alexa647 (N418, eBioscience), anti-Langerin-biotin (929-F3, www.dendritics.net), anti-DEC205 purified from the NLDC-145 hybridoma (provided by J. Sansom, VU Medical Center, Amsterdam, The Netherlands) and conjugated to biotin in our laboratory. PE-Cy7-conjugated streptavidin was used to visualize biotin-conjugated Abs (BD Pharmingen). Experiments were done on a FACSCanto and analyzed using FlowJo software (BD Biosciences).

**Staining for intracellular cytokines**

LN cells from sensitized mice were restimulated in vitro with 2.5 μg/ml plate-bound αCD3 (BD Pharmingen) for 18 h. Four hours before harvesting brefeldin-A (Sigma-Aldrich) was added to the cells at a final concentration of 10 μg/ml. Cells were stained with anti-CD8 (BD Pharmingen), fixed, permeabilized with saponin, and incubated with anti-IFN-γ-APC (clone XM1G1.2; BD Pharmingen).

**Immunofluorescence on LN cryosections**

LN sections were fixed in acetone and stained according to standard procedures with anti-Langerin (929.F3, www.dendritics.net), followed by anti-rat-Cy3 (Jackson ImmunoResearch Laboratories) and anti-MHCII-FITC (M5/114, BD Pharmingen). Images were viewed at room temperature using a Leica TCS-SP2 confocal microscope.

**Results**

**LC are required for the optimal initiation, but not the elicitation, of CHS**

We have previously reported that CHS responses following painting of 1% trinitrochlorobenzene are diminished in the absence of LC, but that dermal DC can also mediate the response (15). We next wanted to extend this observation to other haptons and, in addition, reasoned that using a reduced dose of hapten may target LC in the epidermis more selectively. After a careful dose titration of Oxazolone (data not shown), we repeated the experiments using 0.5% Oxazolone to sensitize the mice. Fig. 1A demonstrates that, in the absence of LC, CHS responses to Oxazolone are significantly reduced, and in most mice no ear swelling is detected. However, some animals clearly do respond to Oxazolone in the absence of LC, indicating that dermal DC can also present haptenized proteins to LN T cells after painting of a low hapten dose. This finding is supported by earlier work suggesting that LC are more efficient at priming CHS responses than dermal DC at low hapten doses (26). Langerin-DTR mice depleted of LC do not show diminished irritant responses, demonstrating that the reduction in CHS is not due to a general nonspecific decrease in ear swelling responses in these mice (Fig. 1B). No qualitative differences could be detected in CHS responses initiated in the presence or absence of LC; in particular, there was no significant decrease in IFN-γ production by CD8+ cells from mice primed with Oxazolone following depletion of LC, whereas IL-4 and IL-10 could not be detected (Fig. 1C).

Because LC would be absent for the entire time course of these experiments, we wanted to determine whether they were required during sensitization, elicitation, or both. To address this issue, mice were sensitized with a high dose of Oxazolone to ensure efficient priming of the T cell response. Three days later, they were injected with DT to deplete LC, and 3 days post depletion challenged with 0.5% Oxazolone to elicit ear swelling. Fig. 1D shows that when LC are absent during elicitation on the ear, there is no difference in the CHS response between depleted and wild-type (wt) mice. These data demonstrate that LC are required for priming but not elicitation of the T cell response following topical application of Oxazolone.

To verify that the requirement for LC was specific for the sensitization phase of CHS, mice were painted with Oxazolone in the presence or absence of LC and primed T cells adoptively transferred into wt (LC-competent) mice for the elicitation of the response. As shown in Fig. 2, T cells primed in the absence of LC cannot be activated to induce wt levels of CHS following transfer. These data confirm the specific role of LC during the priming of T cell responses to topically applied Ag.

**Ag is inefficiently transported to the draining LN in the absence of LC**

It has previously been shown that diminished CHS occurs in mice in which DC do not efficiently migrate to skin-draining LN (27). Therefore, to investigate whether the lack of T cell priming in our model was due to a reduction in presentation of Ag by skin-derived DC in the draining LN, we painted 0.5% FITC onto the ears of Langerin-DTR mice and followed the appearance of FITC+ MHCIhigh LN DC. In the absence of LC, there was a reduction of 67% (±7.4 SEM; n = 4) in the number of...
FITC^+^ MHCI^high^ cells in draining LN 48 h after application of FITC (Fig. 3A). Interestingly, this is the case when LN cells are harvested 48 and 96 h after FITC painting (65% (±6.5) reduction in FITC^+^ MHCI^high^ LN cells in LC-depleted mice 96 h after painting; n = 2, data not shown). This was unexpected in the light of previous data that suggested that LC require 96 h to reach the skin-draining LN (24, 28). FITC is clearly present 48 h after painting on CD11c^+^ cells with a defined LC phenotype: CD8α^−^ low, CD40^high^, (CD86^high^ data not shown), and DEC205^+^ (Fig. 3B). These cells are reduced upon depletion of LC, although not absent because dermal DC share a similar phenotype in draining LN. FITC^+^ Langerin^+^ cells are, however, completely depleted upon injection of DT (Fig. 3C).

**FIGURE 1.** LC are required for optimal initiation, but not elicitation, of CHS responses to Oxazolone. A, Wt or Langerin-DTR mice injected with DT at day –3 (n = 5–6), 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 the challenged and nonchallenged ear. One representative experiment of four is shown; points represent individual mice. Ear swelling responses were compared using a repeated measures ANOVA; wt vs DTR + DT, p < 0.05 in all four experiments. B, One percent Croton oil was painted on the right ears of wt or Langerin-DTR mice injected with DT at day –3 (n = 4). Swelling was measured as the difference between the painted and nonpainted ear. One representative experiment of two is shown; Mann-Whitney U test wt vs DTR + DT, p > 0.2 in both experiments. C, IFN-γ production by hapten-primed T cells is undiminished in Langerin-DTR mice sensitized in the absence of LC. Wt (n = 11) or Langerin-DTR mice injected with DT on day –3 (n = 12), were sensitized on the abdomen with 1% Oxazolone on day 0. On day +5, draining LN cells were purified from sensitized mice and polyclonally restimulated in vitro with plate-bound anti-CD3. Eighteen hours later, LN cells were stained for intracellular IFN-γ. The graph represents cumulative data from three independent experiments analyzed by an unpaired Student’s t test; wt vs DTR + DT, p = 0.3882. D, wt or Langerin-DTR mice (n = 6) were sensitized with 2% Oxazolone on day 0, and 3 days later, Langerin-DTR mice were injected with DT to deplete LC. On day +6, mice were challenged with 0.5% Oxazolone on the right ear. One representative experiment of two is shown; repeated measures ANOVA wt vs DTR + DT, p > 0.15 in both experiments.

**FIGURE 2.** Oxazolone-specific T cells are inefficiently primed in the absence of LC. Wt or Langerin-DTR mice injected with DT on day –3 (n = 5–6) were sensitized on the abdomen with 2% Oxazolone. Five days later, draining LN T cells were purified from sensitized mice and transferred into wt recipients which were challenged on the right ear with 0.5% Oxazolone 1 day later. N represents control mice receiving T cells from nonsensitized (naive) wt donors. Swelling was measured as the difference between the painted and nonpainted ear. One representative experiment of three is shown; points represent individual mice. Ear swelling responses were compared using a repeated measures ANOVA; wt vs DTR + DT; p = <0.05 in all three experiments.

**Epidermal LC, and not LN-resident Langerin^+^ DC, are required for the initiation of CHS**

Although the majority of LC reside in the epidermis of the skin, Langerin^+^ cells have also been identified at other sites, including secondary lymphoid organs (15, 24, 29). These Langerin-expressing populations are also depleted upon injection of DT (15, 24), but Langerin^+^ LN DC reappear 2 wk after DT treatment (Fig. 4A), corroborating a recent report that not all lymphoid tissue Langerin^+^ DC are derived from epidermal LC (30). To confirm that the reduction in CHS was specifically due to a loss of epidermal LC, CHS experiments were repeated in Langerin-DTR mice injected...
with DT for 4 wk. At this timepoint, the epidermis is still mostly empty (15), but the LN-resident Langerin\^/H11001 DC pool has been re-populated (Fig. 4A). Fig. 4B shows that CHS is still reduced in mice specifically lacking epidermal LC, supporting a requirement for cutaneous LC for efficient initiation of the T cell response. The slightly enhanced ear swelling in CHS mice depleted of LC compared with Fig. 1A reflects low levels of epidermal repopulation that may have occurred by 4 wk (15).

Discussion

It is unclear why the immune system requires so many phenotypically distinct populations of DC, and whether each subset plays a distinct role in the induction and regulation of immunity and tolerance. The skin provides a tractable system in which to address this issue, because topical Ag is directly targeted to skin DC, and can be easily tracked in draining LN. Early studies on DC function defined LC as prototypic immature tissue DC. These data lead to the concept of the LC paradigm: LC detect pathogens and allergens that penetrate the epidermal barrier and present this information to naive T cells in skin-draining LN. This paradigm has recently been challenged by work with skin viral or protozoan pathogens, which indicated that other DC subsets are more important than LC in initiating T cell responses to cutaneous Ags (10–12). However, an important caveat to these experiments is that the epidermis is destroyed as a result of infection (HSV), or parasites are injected into the dermis (Leishmania), thus potentially bypassing the need for LC. Furthermore, it is clear that LC are potent stimulators of T cells, and have been proposed to prime robust...
vaccine responses following cutaneous immunization (31). Therefore, to specifically determine the role of LC compared with dermal DC in skin immunity, we generated the Langerin-DTR mouse in which LC could be specifically and inducibly depleted upon a single systemic injection of DT (15).

In this study, we have extended our observation that trinitrochlorobenzene-specific responses are reduced in the absence of LC (15), and show that LC are also required for optimal T cell responses to the hapten Oxazolone. In the absence of LC, T cells are suboptimally primed in skin-draining LN, principally due to inefficient transport of Ag from the epidermis (Fig. 3). Kissenpfennig et al. (24) recently published a similar Langerin-DTR model in which they reported no requirement for LC during the initiation nor elicitation of CHS responses to dinitrofluorobenzene. Although we can confirm that LC do not contribute to the elicitation of the disease (Fig. 1D), the contradictory requirement for LC for the initiation of the response may be explained by differences in hapten and in mouse background (C57BL/6 vs 129), although we have now reported reduced CHS in response to two different haptens (trinitrochlorobenzene (15) and Oxazolone). Furthermore, the group sizes used for the dinitrofluorobenzene experiment (n = 4; Ref. 24) may have been too small to statistically separate the suboptimal responses found in our experiments, in particular, at higher doses of hapten.

LC may transport viral peptides to draining LN where they transfer the Ag to resident CD8+ DC that subsequently stimulate the CD8 T cell response (13, 32). In our model, FITC is inefficiently transported to LN in the absence of LC, supporting a role for LC in trafficking cutaneous Ags from the skin. At present, we cannot differentiate between direct presentation of Ags to naive T cells by LC in LN T cell areas vs transfer of Ag to a LN-resident DC population as described by Carbone and coworkers (13). We were surprised to find that the reduction in FITC+ MHCIImed cells in mice lacking LC was already evident 48 h postpainting, because it has been proposed that dermal DC migrate within 48 h of activation, but that LC require 96 h for peak accumulation in draining LN (24, 28).

In contrast, it has been suggested that the primary function of LC may be a regulatory role, either upon arrival in draining LN, or in situ in the skin. In the Langerin-DTR mice, we did not find a role for LC in controlling effector T cells, and depletion before challenge had no effect on the magnitude or the duration of the CHS response (Fig. 1). Previous data, in which topical application of steroids was used to deplete LC before hapten challenge, suggested a regulatory role for LC during the elicitation phase of CHS (33). However, steroids would nonspecifically alter the skin microenvironment, which could therefore explain the contradiction with results from Langerin-DTR mice (Fig. 1 and Ref. 24).

More recently a transgenic mouse model was published in which LC are constitutively absent due to transgenic expression of the toxic DTA subunit from the human Langerin promoter (25). In these mice, CHS responses appear to be deregulated in the absence of LC, resulting in enhanced ear swelling, which Kaplan et al. (25) propose is due to aberrant priming of the T cell response in draining LN. This difference to our data could be due to the fact that, because LC are constitutively absent in the Langerin-DTA mice, development of the T cell repertoire may be altered in some way, possibly due to failing (peripheral) tolerance mechanisms (34) resulting in hyperresponsiveness to certain Ags. This hypothesis may be further supported by recent findings that circulating DC homing into the thymus contribute to clonal deletion of self-reactive T lymphocytes (35). Furthermore, transgenic expression of DTA from the human Langerin-promoter may not exactly replicate expression of endogenous murine Langerin; in particular, lymphoid-organ-resident Langerin+ DC are not absent in the Langerin-DTA mice (25). This does not, however, explain the enhanced vs reduced CHS as we have clearly addressed in Fig. 4B.

The role of LC in skin immunity has become increasingly unclear over the last few years. We have shown that LC are required for efficient transport of topical Ag to draining LN, and that this result in suboptimal priming of the CHS response. However, whether LC directly present Ag to naive T cells, or whether the priming step is amplified by transfer of cutaneous Ags to LN-resident DC, is not known. The relative importance of LC in directly priming the T cell response will likely depend upon the route of Ag delivery (topical compared with intradermal or s.c.) and whether the epidermis is left intact e.g., following infection. He et al. (36) recently demonstrated that skin DC present viral Ags following cutaneous vaccination with a recombinant lentiviral vector, unfortunately, however, LC and dermal DC were not distinguished in this study. Our data highlight the apparent redundancy between LC and dermal DC in priming hapten-specific T cell responses. Thus, it remains unclear why these distinct populations of DC have been maintained in the skin throughout evolution, and whether they play distinct roles in skin immunity or tolerance. Ongoing investigations will determine whether functional differences between these subsets become apparent during other skin immune responses, such as presentation of epidermally derived proteins, or after infection.

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Disclosures
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