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Langerhans Cells Suppress Contact Hypersensitivity Responses Via Cognate CD4 Interaction and Langerhans Cell-Derived IL-10

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Mice lacking epidermal Langerhans cells (LC) develop exaggerated contact-hypersensitivity (CHS) responses due to the absence of LC during sensitization/initiation. Examination of T cell responses reveals that the absence of LC leads to increased numbers of hapten-specific CD4 and CD8 T cells but does not alter cytokine expression or development of T regulatory cells. CHS responses and Ag-specific T cells are increased in mice in which MHC class II is ablated specifically in LC suggesting that direct cognate interaction between LC and CD4 cells is required for suppression. LC-derived IL-10 is also required for optimal inhibition of CHS. Both LC-derived IL-10-mediated suppression and full LC activation require LC expression of MHC class II. These data support a model in which cognate interaction of LC with CD4 T cells enables LC to inhibit expansion of Ag-specific responses via elaboration of IL-10. The Journal of Immunology, 2009, 183: 5085–5093.

Langerhans cells (LC) have long been considered to be an archetypal tissue-resident dendritic cell (DC). They reside in the epidermis of the skin and acquire local Ag. During inflammation, they migrate to T cell areas in the regional lymph node (LN) and present processed Ag to T cells thereby initiating adaptive immune responses (1). Contact hypersensitivity (CHS) is a mouse model of allergic contact dermatitis, which is one of the most common inflammatory skin disorders and is responsible for considerable morbidity (2). CHS is an ideal assay in which to investigate LC biology because unmanipulated mice are immunized through intact skin, the response is well characterized, and alterations in T cell responses can be correlated with biological function (3). The classic model of LC function has been recently tested by the study of CHS in two types of LC-deficient mice (4, 5).

Langerin-DTR mice were generated by the introduction of the primate receptor for diphtheria toxin (DT) into the endogenous murine langerin locus (6, 7). Langerin is expressed by LC as well as a subset of dermal DC (8–10). DC subsets in the lung, thymus, spleen, and LN also express Langerin (6, 11, 12). Injection of DT ablates all Langerin-expressing DC and results in reduced CHS responses (5–7). However, many of the early studies showing a role for LC in generating cutaneous inflammatory responses were performed before ablation of dermal DC (dDC) was appreciated. Interestingly, at time points when Langerin dermal DC have partially repopulated the dermis but LC are still absent, CHS is unaffected (8). Thus, the absence of LC does not appear to affect CHS. However, if very low dose hapten is used, CHS appears to be reduced in the absence of LC (13). Importantly, Langerin DC found in the dermis are required for optimal CHS responses and fit the proinflammatory role originally ascribed to LC.

Langerin-DTA mice express the active subunit of diphtheria toxin (DTA), not the receptor, as a bacterial artificial chromosome transgene under control of the human Langerin genomic locus (14). Expression is limited to LC leading to their constitutive ablation while leaving all other cells including Langerin dermal DC intact (8). CHS responses in LC-deficient mice were exaggerated due to the absence of LC during the sensitization/priming phase but not the challenge/elicitation phase. Thus, LC actually function to suppress the CHS response. LC-mediated suppression also inhibits rejection of minor-mismatched skin grafts and limits Th1 responses after tick infestation (15, 16).

There have been many investigations into the mechanism of CHS. Mice sensitized with hapten develop CD8⁺ CTLs that secrete IFN-γ and IL-17 (17–19). CTLs induce keratinocyte apoptosis and are required for development of CHS (20). However, the control of CTLs is only partially understood. During CHS, CD4 T cells produce both Th1 and Th2 cytokines (21, 22). CD4 T cells are required for optimal CHS and also regulate CHS responses (17, 23, 24). It has been proposed that CD4 T cells suppress CD8 responses via alterations in Th phenotype or development Foxp3⁺ regulatory cell (Treg) (17, 25–27). Thus, there are a variety of potential mechanisms through which LC could regulate CHS. Herein we examine LC-mediated suppression of CHS by correlating CHS responses with the development of hapten-specific T cells in mice with absent or defective LC.
Materials and Methods

Mice

Langerin-DTA (14), Langerin-Cre (28), I-Aβ-flox (29), and IL-10-flox (30) mice have been previously described. Rag1−/− mice were obtained from The Jackson Laboratory. All experiments were performed with 6- to 10-wk-old age- and sex-matched mice. Mice were housed in microisolator cages and fed autoclaved food and acidified water. The University of Minnesota institutional care and use committee approved all mouse protocols.

Antibodies

Abs to the following targets were used: muLangerin (929F3, Dendritics), CD4, CD8, CD11c, CD11b, CD40, CD44, CD62L, CD69, CD80, CD86, CD90.2, CD103, B7DC, ICOS, ICOS-L, OX40-L, 4-1BB, I-A/E, FoxP3, Fas-L (BioLegend) CD90.1, B7H1, and BrdU (eBioscience), 2-4G2 (anti-FcR) was purified from hybridoma supernatants, as previously described (14).

Contact hypersensitivity

Mice were sensitized on day 0 by epicutaneous application of 25 μl of 0.5% DNFB (2,4-dinitro-fluorobenzene, Sigma-Aldrich) in acetone:olive oil (4:1) or vehicle alone onto dry shaved abdominal skin. All mice used in a given experiment were derived from the same mating pairs (i.e., siblings) to minimize the possibility of small changes in background affecting CHS. Groups of four to eight experimental and three vehicle control mice were used in all experiments. Lower concentrations of DNFB were used as noted. On day 5, baseline ear thickness was measured with an engineer micrometer followed by challenge with 10 μl of 0.2% DNFB to both sides of one ear. Ear thickness was measured daily after challenge and data were expressed as the ear size at 24 h minus the baseline thickness. CHS to FITC used 0.5% FITC diluted in acetone/dibutylphthalate (1:1) for sensitization and challenge.

Flow cytometry

Single-cell suspensions of dermis, LN, and spleen were obtained and stained as previously described (14). Live/dead discrimination was obtained using propidium iodide or ethidium monoazide (Invitrogen). To evaluate cytokine expression, cells were cultured for 5 h with phorbol myristic acid and ionomycin, including monensin during the last 2 h of culture as described (31). The intracellular cytokine staining was performed using BD Bioscience kit (BD Biosciences) according to the manufacturer’s directions. For BrdU incorporation, BrdU was continuously administered in drinking water at a concentration of 0.8 mg/ml for 4 days before sacrifice. A modified BrdU staining protocol was used for analysis of BrdU and Foxp3. Lymphocytes from the skin-draining inguinal, axillary, and brachial lymph nodes were isolated and surface stained, followed by fixation in eBioscience FixPerm for 30 min. Cells were then stained for BrdU following the BrdU Flow Kit manufacturer’s instructions (BD Pharmingen). Detection of CCR4, E-selectin, and P-selectin were performed as described (32, 33). Samples were analyzed on a FACS Calibur or LSR-II flow cytometers (BD Biosciences). Data were analyzed with FlowJo software (TreeStar).

Immunofluorescence

Eight micrometer-thick cryostat sections were made from frozen tissue samples and mounted on poly-L-lysine coated slides. After 10 min of cold acetone fixation, the sections were air-dried and stored in a freezer. Sections were stained and mounted as described (15). Epidermal sheets were prepared by affixing ears to slides using double-sided adhesive tape (3M), followed by incubation in 10 mM EDTA in PBS for 2 h at 37°C and physical removal of the dermis, as previously described (28). Images were captured using a microscope (DM5500; Leica) with digital system and LAS AF software (version 1.5.1).

Ag-specific in vitro T cell restimulation assay

Mice were painted on belly with 25 μl of 0.5% DNFB diluted in aceton: olive oil. Five days later, the painted skin and the draining lymph nodes were harvested and processed. Skin was minced and digested with collagenase and hyaluronidase as described (14). Cells were further purified using Ficol gradient separation 670 g for 30 min and labeled with CFSE (Invitrogen) according to the manufacturer’s directions and used as responders. For stimulators, Rag1−/− CD90.1 spleens were harvested and haptenated with 5 nM DNBS (2,4-dinitrobenzenesulfonic acid, Sigma-Aldrich) for 30 min at 37°C. Cells were washed and irradiated with cesium-irradiator with 2500 rad. Four × 105 responders were cocultured with 2 × 10^5 stimulators in 200 μl complete RPMI 1640 in CO2 incubator for 4 days. After culture, the cells were harvested and analyzed by flow cytometry. The supernatant cytokine content was determined using Luminex.

Multiplex cytokine analysis

Cell culture supernatants were analyzed for following cytokines: IL-4, IL-5, IL-10, IL-13, IL-17, and IFN-γ using Bio-Rad custom made Luminex kit on the basis of the manufacturer’s recommended protocol. TGF-β was assessed using Panomics single-plex kit. The samples were run and analyzed using Bio-Rad Luminex 200 system.

Statistics

Statistical comparisons between groups were made with a Student’s two-tailed t test.

Results

Langerin-DTA mice develop exaggerated CHS responses

We have previously reported increased CHS responses in Langerin-DTA mice that had been generated and maintained on the FVB background (14). To assess whether this phenotype was related to the background strain of the mice, we backcrossed Langerin-DTA mice a total of nine generations onto C57BL/6 mice. These mice consistently developed exaggerated CHS responses to DNFB (Fig. 1, A and B). The degree and timing of the increased ear swelling in Langerin-DTA mice on the C57BL/6 background was similar to that observed on the FVB background. Similar CHS results were also obtained using the hapten FITC (Fig. 1C). Finally, we examined CHS responses at different doses of
DNFB (Fig. 1D). The degree of ear swelling in both wild-type (WT) and Langerin-DTA mice decreased with lower concentrations of DNFB. However, CHS responses were exaggerated in Langerin-DTA mice at both normal (0.5%) and lower (0.3%) doses. We do not observe decreased CHS in the absence of LC at very low hapten dose (0.1%) as has been reported by others (13). Thus, the increased CHS responses that develop in the absence of LC are not isolated to a single strain, hapten, or dose. All subsequent experiments used C57BL/6 mice and the standard 0.5% DNFB regimen.

**Langerin-DTA mice develop increased numbers of hapten-specific T cells**

To understand how LC limit the development of CHS responses, we first focused on alterations in T cell populations in secondary lymphoid tissues. Analysis of bulk T cell populations from LN and spleen showed no alteration in numbers of CD4 or CD8 cells. Expression of CD69, CD44, and CD62L were similar in naive or sensitization were stimulated ex vivo with PMA/ionomycin and analyzed for expression of IFN-γ by intracellular flow cytometry. Live cells were gated on CD4 (top) or CD8 (bottom) and unpainted (top) skin from WT (left) and Langerin-DTA mice (right) were examined by immunofluorescence for the presence of CD4 (green) and CD8 (red) cells. Nuclei were highlighted by 4-6-diamidino-2-phenylindole staining (blue). Bar represents 100 μm. C. The number of infiltrating T cells was determined by flow cytometry from collagenase treated DNFB-painted or unpainted skin (as indicated). T cell number/cm² is shown for Langerin-DTA (■) and WT (□) (∗, p < 0.05). D. As in A, cells were isolated from dermis 5 days after DNFB painting and analyzed for IFN-γ expression. Representative experiments from at least three repeats are shown.

**DNFB-specific T cells in Langerin-DTA and WT mice are phenotypically similar**

CHS to DNFB has been shown to generate a mixed Th1/Th2 response (23). LC could potentially promote Th2-type responses such that an exaggerated Th1 response would develop in their absence. This hypothesis predicts a decreased production of Th2 cytokines in Langerin-DTA mice. To test this, we examined the cultured supernatants from DNBS-stimulated cells using multiplex cytokine analysis. We observed a 2-fold increase in amounts of IFN-γ and IL-17 in Langerin-DTA cultures (Fig. 4A). Importantly, levels of IL-13 and IL-10, which
are associated with Th2-type responses, were increased com-
pared with naive controls but were similar in WT and Langerin-
DTA mice. TGF-β1 was not increased compared with negative 
controls and we did not observe detectable amounts of IL-4 or 
IL-5. Thus, the absence of LC does not alter the production of 
Th2-type cytokines.

Intracellular cytokine analysis of divided cells, revealed modest 
numbers of CD4 cells expressing IFN-γ and very few expressing 
IL-17 (Fig. 4B). Approximately 30% of CD8 cells expressed 
IFN-γ and very few expressed IL-17 (Fig. 4C). This suggests that 
IFN-γ is largely derived from CD8 cells. The percentage of cells 
expressing IFN-γ or IL-17 was similar in WT and Langerin-DTA 
mice. In addition, the granzyme-B expression was unaltered in 
Langerin-DTA mice. Thus, although the number of Ag-specific 
cells is increased in the absence of LC, the primarily Th1/Tc1 
response to DNFb is unaltered.

Foxp3+ Treg are unaltered in Langerin-DTA mice

We next sought to determine whether Foxp3 Treg cells partici-
pated in LC-mediate suppression of CHS. Bulk analysis of CD4+ 
cells from naive and sensitized WT showed equal percentages 
(Fig. 5A) and numbers (data not shown) of Foxp3+ cells in WT 
and Langerin-DTA mice. Skin homing Treg can be identified in 
the skin-draining LN based on expression of Foxp3, CD103, and 
P- or E-selectin (32). As with the total Foxp3 population, the 
percentages of these cells in skin-draining LN was similar in WT 
and Langerin-DTA mice (Fig. 5, B and C).

The homeostasis of skin-tropic Treg cells depends on their 
expression of the chemokine receptor CCR4 (33). In skin-drain-
ing LN, LC produce the CCR4 ligand CCL22, and this may help 
attract CCR4+ Treg cells, and promote their survival and/or 
proliferation in vivo. However, Langerin-DTA mice had a
normal frequency of CCR4+ Treg cells in the skin-draining LN (Fig. 5C, left). Treg cell proliferation as measured by incorporation of BrdU was equivalent to that seen in WT mice (Fig. 5C, right) indicating that Treg homeostasis is normal in the absence of LC.

There are several potential mechanisms other than Foxp3 Treg that could participate in LC-mediated regulation. We did not observe an increase in IL-10 or TGFβ-producing T cells (Fig. 4A) suggesting that Tr1 and Th3 are not affected by the absence of LC. In addition, numbers of other DC subsets (data not shown) as well as surface expression of costimulatory molecules were similar in WT and Langerin-DTA mice (Supplemental Fig. 1a).4

4 The online version of this article contains supplemental material.
LC-mediated suppression requires LC expression of MHC class II (MHC-II)

Because we observed an increase in proliferation of both CD4 and CD8, we next examined whether interaction between LC and CD4/T cells is required for LC-mediated suppression of CHS. We bred mice that express Cre recombinase selectively in epidermal LC (Langerin-Cre) (28) to mice in which the gene of interest had been floxed (29). The floxed allele was created on a H-2b background (Langerin-Cre) (28) to mice in which the gene of interest had been floxed (29). The floxed allele was created on a H-2b background (28). As with Langerin-DTA mice, Langerin-Cre mice ablated floxed genes only in LC with very high efficiency (28). Epidermal sheets from Langerin-Cre I-A\(^{b}\) mice had been fully activate/condition DC for optimal elaboration of cytokines (38). Expression of CD40 and costimulatory molecules was unchanged on LC from Langerin-Cre mice compared with littermates. However, expression of FasL, was absent on LC from Langerin-Cre mice incubated with blocking Abs to CD40 lose FasL expression (28, 39). Moreover, LC from WT mice incubated with blocking Abs to CD40 lose FasL expression (28). Although CHS responses were significantly increased, the enhancement of ear swelling was somewhat less than that observed with Langerin-DTA mice. This is consistent with the absence of FasL on LC from Langerin-DTA mice (Fig. 7, E), demonstrating that IL-10 mediated suppression and MHC-II-dependent suppression do not act synergistically but are in the same pathway.

Ligation of CD40 by CD40L expressed by T cells is required to fully activate/condition DC for optimal elaboration of cytokines (38). Expression of CD40 and costimulatory molecules was unchanged on LC from Langerin-Cre mice incubated with blocking Abs to CD40 lose FasL expression (28, 39). Moreover, LC from WT mice incubated with blocking Abs to CD40 lose FasL expression (28). This is consistent with the absence of FasL on LC from Langerin-Cre I-A\(^{b}\) mice. Because these LC cannot interact CD4 T cells, they do not receive CD40 stimulation from CD40L.
(CD154) on CD4 T cells and remain unactivated. Interestingly, FasL is not expressed by dermal DC and may represent another mechanism in addition to LC-derived IL-10 by which LC limit Ag-specific responses.

Discussion
We have demonstrated that LC-mediated suppression of CHS responses requires cognate interaction with CD4 T cells to limit the number of hapten-specific CD4 and CD8 T cells via an IL-10-dependent mechanism. The absence of LC does not alter cytokine
expression by hapten-specific T cells or impair the development and homeostasis of Treg.

The fact that Langerin-Cre I-AβΔLIR mice recapitulate the exaggerated CHS responses and increased numbers of hapten-specific cells seen in Langerin-DTA mice argues that direct LC-CD4 interaction is an obligate step in LC-mediated regulation. Furthermore, increased CHS in Langerin-Cre IL-10ΔLIR mice demonstrates that optimal LC-mediated suppression requires LC-derived IL-10 and suggests that increased CHS in IL-10ΔLIR mice (40) is at least partially dependent on LC-derived IL-10. IL-10 can directly inhibit T cell function, induce a state of anergy/unresponsiveness, promote Treg development, and decrease APC activation (37). As we did not detect any alterations in Treg development or in DC populations, we favor the hypothesis that LC-derived IL-10 acts directly on effector T cells and propose the following model. LC arrive in the skin-draining LN 3–4 days after hapten application, which is well after skin-resident Langerin+ dDC (and quite likely other dDC) have transported haptenated Ags to the LN and initiated adaptive responses (41, 42). Upon cognate interaction with CD4 cells, presumably via CD40-CD40L interaction, LC express IL-10, which inhibits hapten-specific T cells. In the absence of LC-CD4 T cell interaction, LC are not stimulated to secrete IL-10. This results in a failure to suppress expansion of hapten-specific cells thereby leading to the development of exaggerated CHS. The absence of any effect on bulk T cell population in Langerin-DTA mice argues that LC-mediated suppression is Ag-specific and does not affect bystander cells.

Although we have demonstrated a role for LC-derived IL-10, other regulatory molecules are also likely to help dampen CHS responses. FasL is selectively expressed by LC that have cognate CD4 T cell interaction (Fig. 7 and Ref. 39) and could reduce effector responses by directly inducing apoptosis of hapten-specific T cells (43). In vitro LC negatively regulate T cell activation via cell-cell contact (44). However, Fas-FasL interactions appear to be redundant as CHS is unaffected in lpr and gld mice (20), though the absence of Fas-FasL interactions in all cell types may obscure the role of FasL on LC. Because we detect Ag-specific T cells based on their ability to proliferate, we cannot use the DNFB-restimulation assay to determine whether cells in Langerin-DTA undergo reduced levels of apoptosis. TGFβ is a potent immunoregulatory cytokine secreted by LC and could suppress CHS responses. We have generated Langerin-Cre TGFβΔTR mice but LC do not develop without autocrine/paracrine TGFβ, which precludes the use of these mice in functional assays (28). Other LC-specific molecules such 2–3-indolamine-deoxygenase, could also participate (45).

The presence of a population of CD4 T cells that regulate CHS responses has been suggested by increased CHS responses in CD4-depleted mice (21, 22, 24, 46). It has been speculated that these cells represent Foxp3+ Treg and/or Th2 cells that balance the predominantly Th1 CHS response (23, 25). Data for Treg-mediated suppression comes from studies in which CHS responses can be suppressed by adoptive transfer of either Foxp3 transduced cells or Treg from orally tolerized mice (26, 27, 47). Transgenic over-expression of RANKL by keratinocytes dramatically increases the number of Treg in skin draining LN (48). Because LC express RANK, the receptor for RANKL, we anticipated that elimination of LC in Langerin-DTA mice would lead to the development of many fewer Treg. However, we observed normal Treg development and homeostasis.

Cytokine analysis of DNFB-sensitized mice revealed increased IFN-γ and IL-17 in the absence of LC. These were primarily produced by CTL. Increased quantities of these effector cytokines are consistent with increased CHS in LC-deficient mice. We observed virtually no expression of IFN-γ or IL-17 by CD4 T cells and instead found modest levels of IL-13 and IL-10. A role for Th2 cells in CHS regulation derives from observations that CD4 cells in hapten-treated mice produce primarily IL-4, IL-5, IL-10, and little IFN-γ (17). Interestingly, LC-deficient mice immunized by gene gun develop reduced IgG1 and enhanced IgG2a Ab production (49). However, the levels of IL-13 were similar in WT and Langerin-DTA mice indicating that Th2 responses develop normally. We did not detect IL-4 or IL-5 expression as has been seen previously after DNFB sensitization (17) possibly due to differences in genetic background. In addition, CHS responses were increased in Langerin-DTA mice to both DNFB, which generates a Th1-type response and to FITC which has been reported to generate Th2-type responses (50). Thus, though Treg and Th2 cells may participate in limiting the extent of CHS responses in intact mice, LC-mediated suppression appears to operate via an alternative mechanism.

Langerin-DTR mice in which LC and other Langerin+ DC are ablated by injection of DT do not develop enhanced CHS even at time points after DT administration, when Langerin+ dermal DC have partially repopulated the dermis but LC are still largely absent (6–8). A potential compensatory response to the constitutively empty epidermal DC niche or expression of DTA cannot explain increased CHS responses in Langerin-DTA mice because Langerin-Cre I-AβΔTR and Langerin-Cre IL-10ΔLIR mice both develop enhances CHS. In addition, heterozygous-flox Langerin-Cre mice have normal CHS responses indicating that genes present on the bacterial artificial chromosome transgene or expression of Cre itself does not affect CHS. The discrepancy between Langerin-DTA and DTR mice could reflect the still depleted number of Langerin+ dDC at the time of CHS sensitization or that repopulating Langerin+ dDC have properties distinct from those present in the steady-state. Alternatively, because Langerin-DTA, Langerin-Cre I-AβΔTR, and Langerin-Cre IL-10ΔTR mice have defective/absent LC from birth, we cannot exclude that MHC-II/IL-10 dependent LC-mediated suppression may function during ontogeny. Future inducible ablation systems that are more LC-specific should help resolve the acute role of LC in skin immune responses.

In summary, we have described a novel mechanism of immune-noregulation in the skin. LC-mediated suppression may function to drive the resolution of cutaneous immune responses and could also inhibit responses against skin commensal organisms and benign environmental Ags.

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Disclosures

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