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Rho-Family GTPase Cdc42 Controls Migration of Langerhans Cells In Vivo

Nancy Luckashenak,*1,2 Anna Wähé,*1 Katharina Breit,* Cord Brakebusch, † and Thomas Brocker*

Epidermal Langerhans cells (LCs) of the skin represent the prototype migratory dendritic cell (DC) subtype. In the skin, they take up Ag, migrate to the draining lymph nodes, and contribute to Ag transport and immunity. Different depletion models for LCs have revealed contrasting roles and contributions of this cell type. To target the migratory properties of DCs, we generated mice lacking the Rho-family GTPase Cdc42 specifically in DCs. In these animals, the initial seeding of the epidermis with LCs is functional, resulting in slightly reduced Langerhans cell numbers. However, Cdc42-deficient LCs fail to leave the skin in steady state as well as upon stimulation, as they do not enter the skin-draining afferent lymph vessels. Similarly, also other Cdc42-deficient migratory DC subtypes fail to home properly to the corresponding draining lymph nodes. We used this novel mouse model, in which LCs are locked out, to demonstrate that these cells contribute substantially to priming of Ag-specific CD4 and CD8 T cell responses upon epicutaneous immunization, but could not detect a role in the induction of contact hypersensitivity to various doses of hapten. The Journal of Immunology, 2013, 190: 27–35.

Dendritic cells (DCs) are professional APCs critically important for the initiation of adaptive immunity as well as for the induction of tolerance (1, 2). A number of DC subsets with distinct functions exist within mouse and man (3). Traditionally, DCs of the epidermis or Langerhans cells (LCs) have been used as a model to study the function of migratory DCs (mDCs). However, only recent studies have begun to shed light on the complexity of LC function in cutaneous immunity, indicating that not all mDC subsets are alike. Exactly what role LCs play in the initiation, prolongation, and/or suppression of the varied cutaneous immune responses remains controversial despite a number of mouse models that specifically eliminate or target this cell type (reviewed in Refs. 4, 5). For example, studies using different ablation models of langerin* cells showed normal, reduced, or enhanced contact hypersensitivity (CHS) responses, depending on the LC-depletion model used (6–11). In viral cutaneous immune responses against HSV, the role of LCs seemed to be reduced to Ag transport from epidermis to the skin-draining lymph nodes, as other DC subtypes are responsible for immunity (12, 13). Also, in graft-versus-host–disease, LCs do not play detectable roles in graft rejection (14). Similarly, in cutaneous Leishmania major or Candida albicans infection, LCs are not required for CD8* T cell priming (15, 16), but rather trigger Th17 CD4 T cell responses (16). Taken together, a much more complicated picture than anticipated has begun to emerge with regard to LC biology, and questions remain about their function.

Cdc42 is a member of the highly conserved Rho family of GTPases. Proteins of this family control various cellular processes that are mediated by the actin and myosin cytoskeletons such as migration, polarization, and trafficking of vesicles (17). Cdc42 has been shown in various cell types to regulate actin polymerization, cell polarity, filopodia formation, lamellipodia formation (18), exocytosis, endocytosis, and Golgi to endoplasmic reticulum retrograde transport (19, 20). Despite the numerous functions ascribed to Cdc42, comparatively less data exist describing the function of Cdc42 in hematopoietic cells. Most of the previous studies were conducted using nonhematopoietic cell lines that were either transfected with mutant proteins or in which Cdc42 was chemically inhibited. A role of Cdc42 in DCs has been investigated by inhibiting Cdc42 activity by DNCdc42 in bone marrow–derived DCs (BMDCs). These studies demonstrate roles for Cdc42 in regulating DC morphology (21, 22), podosome formation (23), adhesion (24), endocytosis (25), Ag presentation (22, 24), and interstitial migration (26). Potential caveats of these studies are that DNCdc42 mutant does not inhibit Cdc42 specifically, but also other Rho GTPases (27) and a relatively poor reflection of steady-state in vivo DC subsets by BMDCs.

In this study, we have significantly furthered the knowledge of Cdc42 function by selectively deleting this GTPase in DCs. In this study, we focused on migratory LCs and showed reduced frequencies of LCs with altered morphology and phenotype in the epidermis. Cdc42-deficient LCs failed to migrate from the epidermis to the cutaneous lymph nodes under steady-state or inflammatory conditions. In chimeric mice with migration-deficient LCs, but an otherwise normal hematopoietic compartment, CD4* and CD8* T cell priming was reduced upon epicutaneous immunization, whereas LCs were dispensable for CHS responses.

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; CHS, contact hypersensitivity; DC, dendritic cell; DT, diphtheria toxin; DTA, diphtheria toxin A; EpCAM, epithelial cell adhesion molecule; LC, Langerhans cell; mDC, migratory dendritic cell; MHC I, MHC class I; MHC II, MHC class II; MLN, mesenteric lymph node; wt, wild-type.

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Materials and Methods

Mice

Cdc42flx-Cre mice were generated by breeding CD11c-Cre<sup>+</sup> mice (28) with Cdc42fl<sup>+</sup> mice (29). Mice were analyzed at 6–15 wk of age in this study.

Abs and reagents for flow cytometry

Abs with the following specificities were used in this study: CD11c, I-A<sup>b</sup>, H-2<sup>K<sub>b</sub></sup>, CD11b, CD4, BV5.1/2, CD45.1, CD45.2, CD90.1, BrdU Flow kit (BD Biosciences); CD8, CD103, CCR7, CD40, CD45, IFN-γ, TNF-α (eBioscience); epithelial cell adhesion molecule (EpCAM); CD24, and CD86 (BioLegend); langerin (Dendritics); F480 (Serotec); rabbit anti-β-catenin (Cell Signaling Technology); and goat anti-rabbit (Molecular Probes); dead cell exclusion DAPI (Molecular Probes); or LIVE/DEAD Violet Dead Cell Stain (Invitrogen).

Isolation of DC subsets from lymph nodes, spleen, and thymus

Tissues were digested in a solution of LiberaseCl and DNase I (Roche) in serum-free medium for 20 min at 37°C. Cells were then passed through a 70-μm nylon mesh strainer (BD Biosciences). Cauterizing draining lymph nodes were defined as axillary, brachial, and inguinal unless stated otherwise.

Isolation of LCs

Ears were split into dorsal and ventral halves and then floated on a solution of 0.8% trypsin (Invitrogen) in PBS for 20–30 min. Epidermal sheets were isolated and incubated in complete RPMI 1640 at 37°C for 30 min or incubated in solution of collagenase IV (Worthington Biochemical, Lakewood, NJ) at 37°C for 2 h. Epidermal sheets were then discarded, and the remaining suspension was filtered through a 70-μm nylon mesh strainer. For isolation of mature LCs, epidermal explant cultures were performed in presence of 20 ng/ml GM-CSF for 3 d as previously described (30, 31).

Preparation of epidermal and dermal sheets for microscopy

Split ears were floated on 0.5 M ammonium thiocyanate at 37°C for 20 min. Isolated sheets were then fixed in acetic acid for 5 min, rinsed with PBS, blocked in a solution of 0.25% BSA in PBS with 10% of the appropriate serum, and stained further in blocking solution. Isolated sheets were stained with CD16/32, MHC class II (MHC II) biotin, or APC (BD Biosciences), γδ TCR, langerin PE, or biotin (eBioscience), goat anti-mouse LYVE-1 (R&D Systems), streptavidin Alexa Fluor 555, DAPI (1 μg/ml), rabbit anti-goat IgG or Alexa Fluor 488 (Molecular Probes), or streptavidin-FITC (Southern Biotechnology Associates), and stained ear sheets were either analyzed on an Olympus BX41TRF-5 microscope (Olympus CELL-BND-F software; Olympus) equipped with an F-View II Digital Micro camera (Olympus) or confocal microscopy on a Leica TCS SP5 confocal microscope (Leica Microsystems).

BrdU labeling in vivo

BrdU labeling was performed as described (32). Mice received an i.p. injection of 1 mg BrdU in PBS, and the drinking water was supplemented with 0.8 mg/ml BrdU for 7 d. Epidermis was isolated and digested in a collagenase IV solution as described above. The epidermal cell suspension was then processed using the FITC BrdU Flow Kit (BD Pharmingen).

FITC painting

Mice were combed on the abdomen, and 200 μl 0.5% FITC (Sigma-Aldrich) solution in acetone-dibutylphthalate was applied to the shaved area as described (33, 34). Forty-eight or 96 h later, mice were sacrificed, and axillary, brachial, and inguinal lymph nodes were harvested, and DCs were analyzed.

In vitro migration/whole skin crawl-out assay

The crawl-out assay was performed as originally described with minor modifications (31). Ears were split, and dorsal halves were floated on medium containing 100 ng/ml CCL21 (R&D Systems) for 3 d. Medium and chemokine were replaced daily. Migrated cells were also harvested daily using 1 mM EDTA, stored at 4°C until the end of the assay, and then pooled.

TUNEL assay

Ears were prepared and treated as for the in vitro migration/whole skin crawl-out assay described above. After 2 d, epidermal sheets were isolated by incubation on 0.8% trypsin (Invitrogen) in PBS for 20–30 min (30). Epidermal sheets were isolated and incubated in complete RPMI 1640 at 37°C with constant agitation for 30 min, and the remaining suspension was filtered through a 70-μm nylon mesh strainer. TUNEL positivity was then determined by flow cytometry using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics).

Generation of bone marrow chimeras

Bone marrow was harvested from femurs and tibiae of Ly5.1 donor mice 6–8 wk of age. RBCs were lysed using the Mouse Erythrocyte Lysing Kit (BD&K D Systems), T cells were depleted using CD90.2 microbeads (Miltenyi Biotec, Gladbach, Germany), and 5 × 10<sup>6</sup> total cells was injected i.v. into lethally irradiated (split dose day −1 and day 0; 600 rad) recipient mice (age 10–12 wk). Following irradiation, and for 5 wk after, chimeric mice were given drinking water supplemented with neomycin trimisulfate (Sigma-Aldrich). Chimeras were analyzed 8–10 wk after bone marrow reconstitution, and chimerism was determined by Ly5.1/Ly5.2 staining in the skin DC populations at the time of sacrifice.

Epicutaneous immunization and T cell proliferation

Chimeric mice were shaved on the left flank and adoptively transferred with 2 × 10<sup>6</sup> CFSE-labeled (5 μM final) OT-I or OT-II T cells. The next day, mice were anesthetized with an i.p. injection of a solution of 10 mg/ml Ketavet (Pfizer, Karlsruhe, Germany) and 2% Rompune (Bayer AG, Leverkusen, Germany) in PBS and 469 μg grade VII OVA (Sigma-Aldrich) in 25 μl PBS was applied to the shaved, hydrated flank (9). The area was then covered with an occlusive patch (DuoDERM Extra Thin; ConvatecTec), mice were sacrificed 6 d later, and draining lymph node cells were analyzed by flow cytometry.

Intracellular cytokine staining

Splenocytes (10 × 10<sup>6</sup>) were restimulated in 1 ml culture medium (RPMI 1640, 10% FCS) with 2 μg STINFEKL or SSIEFARL (both from Polypeptide Group) in the presence of 2 ml GolgiStop (BD Biosciences) for 4 h. Intracellular staining for IFN-γ or TNF-α was performed using the Cytofix/Cytperm kit (BD Biosciences), according to the manufacturer’s protocol.

CHS models

Induction of CHS using a low dose of oxazolone (Sigma-Aldrich) was performed as described (8) with one minor modification. Mice were shaved between the shoulder blades 1 d before immunization (day −1). The next day, 50 μl 0.5% solution of oxazolone (4:1 in acetonelolive oil) was applied to the shaved area (day 0). On day 5, ears were measured and then challenged with 10 μl on each side of one ear with a 0.25% solution of oxazolone (4:1 in acetonelolive oil). Ear swelling was measured 24 h after challenge. CHS induction using a high dose of hapten was performed in the same manner, but sensitization and elicitation were achieved using a 3% solution of oxazolone (4:1 in acetonelolive oil), respectively.

Statistics

Significance was determined using the Student t test and defined as follows: p < 0.05, p < 0.01, and p < 0.001. Bar graphs show average ± SEM from combined experiments and group sizes as indicated in the figure legends.

Results

Cdc42flx-Cre mice exhibit defects in the epidermal DC compartment

In Cdc42flx-Cre mice, spleen and thymic DCs were present at normal frequencies and numbers despite the fact that the Cdc42 gene, message, and protein were virtually absent (Supplemental Fig. 1). Also, other cells of the hematopoietic compartment did not show alterations in cell number or phenotype (data not shown). Strikingly, the CD11c<sup>+</sup>, MHC II<sup>H</sup> mDC population of the cutaneous lymph nodes showed significantly reduced frequencies and total cell numbers in these mice (Fig. 1A). Upon closer examination of this cutaneous mDC population, it was apparent that even though langerin<sup>+</sup> dermal DCs (langerin<sup>+</sup>, CD103<sup>+</sup>) were present at normal frequencies in Cdc42flx-Cre mice, the epidermal LC population (langerin<sup>+</sup>, CD103<sup>+</sup>) was virtually absent (Fig. 1B). Moreover, LCs in Cdc42flx-Cre mice exhibited a ~17-fold
FIGURE 1. Initial characterization of DC populations of the cutaneous draining lymph nodes and epidermis in Cdc42flx-Cre mice. Skin-draining lymph node DCs were analyzed based on the expression of MHC II and CD11c. The resident DC population (R1) and the mDC population (R2) were defined as shown (A). Statistics in dot blots indicate frequencies of the respective cell population. Shown data are combined from seven independent experiments with a total of \( n = 11 \) mice/group. mDCs of the cutaneous draining lymph nodes were stained with anti-CD103 and anti-langerin to define the dermal and epidermal DC populations (B). Statistics in dot blots indicate frequencies, and bar graphs show total numbers of the respective cell types from two independent experiments (\( n = 4 \)) with similar results (B) Epidermal sheets were stained with anti-MHC II (red) Ab and visualized by microscopy (C, top panel). Insets highlight the altered, more rounded morphology of Cdc42flx-Cre LCs, which is further indicated by arrowheads. Images were acquired at original magnification \( \times 20 \). Scale bar, 100 \( \mu \)m. LCs were enumerated by counting five fields per epidermal sheet per mouse. Bar graph shows one representative experiment out of three with similar results. FACS analysis of single-cell suspensions from epidermis and dermis (Figure legend continues)
decrease in total number in cutaneous lymph nodes, whereas langerin+ dermal DCs showed an ∼4-fold reduction as compared with control mice (Fig. 1B). The numbers of CD103+ langerin+DCs were also ∼3-fold reduced (Fig. 1B). In contrast, LCs were present in epidermal sheets isolated from Cdc42flx-Cre mice, although their frequency was about half of Cre control mice (Fig. 1C, top panel). The reduction of LCs in the epidermis could also be confirmed by FACS analysis on epidermal cell suspensions (Fig. 1C, bottom panel). However, FACS analysis of dermal cell suspensions could not reveal a reduction of dermal DC subpopulations (Fig. 1C, bottom panel), whereas EpCAM+ LCs in dermal layers from Cdc42flx-Cre mice were present at reduced frequencies (Fig. 1C, bottom panel, Dermis). In addition to a reduced number, the Cdc42flx-Cre LCs clearly displayed an altered or more rounded morphology typical of stimulated LCs (Fig. 1C, top panel, insets) (35). In LCs sorted from the epidermis of Cdc42flx-Cre mice, levels of CD42 message were virtually absent (Supplemental Fig. 1B, 1C).

To determine if the rounded morphology of Cdc42flx-Cre LCs reflected a more activated phenotype, we analyzed LCs from the epidermis by flow cytometry. The expression of many cell-surface markers was comparable between LCs from Cre controls and Cdc42flx-Cre mice including that of MHC II and CD40 (Fig. 1D, Supplemental Fig. 2), but the expression of other markers such as langerin, β-catenin, and CD45 was substantially reduced (Fig. 1D). The expression of some markers was even slightly elevated on Cdc42flx-Cre LCs such as CD86 and MHC class I (MHC I), suggesting perhaps that the cells may be more stimulated in the steady state (Fig. 1D, Supplemental Fig. 2). When LCs were matured during the course of an epidermal sheet culture (30, 31), we observed even greater differences in cell phenotype. Cdc42flx-Cre LCs upregulated MHC I, MHC II, CD40, and CD86 normally, if not better, as compared with control cells, but could not increase cell-surface expression of CD24 and CCR7 to a similar extent as LCs from control mice (Fig. 1D, bottom panel). As CCR7 is necessary for LC migration to cutaneous draining lymph nodes (36), an inability to properly upregulate this chemokine receptor may partially explain the absence of LCs in cutaneous lymph nodes of Cdc42flx-Cre mice.

As shown previously, resident LCs continuously proliferate in the epidermis of adult mice potentially contributing to LC renewal in the steady state (37). To test if the proliferative capacity of Cdc42flx-Cre LCs was altered, we performed in vivo BrdU-labeling assays (32) and found that Cdc42flx-Cre LCs actually displayed a slightly reduced BrdU incorporation rate than Cre control LCs, which, however, was statistically not significant (Fig. 1E).

\[ \text{Cdc42flx-Cre LCs are unable to leave the epidermis or transport Ag to the draining lymph nodes} \]

We next examined if LC migration could be induced under stimulatory conditions such as during a cutaneous FITC-painting assay. Also, upon application of the FITC solution, only very few FITC+ cells were detected in the lymph nodes of Cdc42flx-Cre mice as compared with controls (Fig. 2A, top left panel). Of those present, a significantly reduced percentage and number of cells were identified as LCs (Fig. 2A, top right panel). However, the differences observed for CD103+langerin+ dermal DCs were not statistically significant. To analyze whether langerin+ dermal DCs were also affected, we analyzed lymph nodes at an earlier time point, when migration of this DC subset seems to peak (38). At this earlier 48-h time point, we detected 5–7-fold reduced numbers of CD103+langerin+ dermal DCs in draining lymph nodes (Fig. 2A, bottom panel). These data indicate that Cdc42 controls migratory capacities of both LCs and dermal DCs. To examine the migration of LCs more closely, we next used an ear-explant assay as a model of CCL21-directed in vitro migration (31). However, similar to what we observed in vivo, Cdc42flx-Cre LCs exhibited an ∼30-fold reduction in their ability to migrate in vitro (Fig. 2B, left panel). In addition, analysis of the remaining cells in the skin revealed that LCs remain in the epidermal layer, as nearly no langerin+ cells reach the dermis (Fig. 2B, right panel).

To find out if mDCs of other tissues were also affected in Cdc42flx-Cre mice, we next analyzed mesenteric lymph nodes (MLN), which drain the small intestine. In this study, CD11c+MHC IIHI mDCs were also reduced, although not to an extent that was statistically significant (Fig. 2C). A more detailed analysis of CD11c+MHC IIHI cells in MLN for expression of the markers CD103 and CD11b (39) revealed statistically highly significant reductions of both CD103+ DC populations in MLN of Cdc42flx-Cre mice (Fig. 2D). Taken together, our data indicate that mDCs are reduced in cutaneous as well as MLN of Cdc42flx-Cre mice.

**LCs do not enter the dermal lymphatics under inflammatory conditions and effectively self-destruct in the epidermis in situ**

We isolated and stained the dermis for langerin in combination with LYVE-1 and MHC II to visualize cord formation within the dermal lymphatics (40) to gain a better understanding of the location of the block in migration. Cord formation was detected exclusively in control dermal sheets, suggesting that either Cdc42flx-Cre LCs do not enter the lymphatics or that the cells do not even leave the epidermis (Fig. 3A). The second scenario is more likely, given the fact that we were virtually unable to detect langerin+ cells in the dermis of Cdc42flx-Cre mice at the end of the assay.

All Cdc42-deficient LCs were trapped within the epidermis, as patches of LCs formed clusters that contained whole cells and what appeared to be cell debris (Fig. 3B and data not shown). These trapped Cdc42flx-Cre LCs showed several morphological alterations with appearance of smaller fragments of varying subcellular size, which could be due to disruption of cellular integrity. To exclude apoptosis in response to inflammation, we performed a TUNEL assay on LCs in the epidermis during the course of a crawl-out assay. We observed no statistically significant difference in TUNEL-positive cells between Cre and Cdc42flx-Cre LCs (Fig. 3C), suggesting that the cells are not undergoing apoptosis.
FIGURE 2. Cdc42flx-Cre LCs fail to migrate under inflammatory conditions. A 0.5% solution of FITC was applied to the shaved abdomen of mice. Draining axillary and inguinal lymph nodes were removed 48 and 96 h later and stained with the indicated Abs for flow cytometry analysis (A). Dot plots show analyses at 96 h, and bar graphs indicate total numbers at both 48 and 96 h. Statistics show frequencies of FITC+MHC II+ cells and are pooled data from three independent experiments (n = 5) with similar results (A). Dorsal halves of split ears were floated on media containing 100 ng/ml CCL21 for 3 d. Migrated cells were pooled, stained, and analyzed by flow cytometry (B, left panel). LCs were defined as CD11c+, MHC II+, langerin+, and CD103+. Numbers above the bar graph indicate total LC numbers from two independent experiments (n = 4) with similar results (B, right panel). After 48 h, epidermis and dermis were analyzed for remaining DC subsets as described for Fig. 1C, lower panel (B, right panel). MLN DCs were analyzed based on the expression of MHC II and CD11c (C). DCs (CD11c+MHC II+ cells) were further analyzed for expression of CD103 and CD11b (D). Numbers indicate frequencies of the respective gates and are highly significant for all three populations (D, p ≤ 0.0005). Statistics are from two independent experiments (n = 8 [Cre] and n = 4 [Cdc42flx-Cre]) with similar results. **p < 0.01.
Roles for LCs in T cell priming in vivo

We had an animal model at hand in which LCs, although present in the epidermis, could not leave these upper layers of the skin. As a consequence, Cdc42-deficient LCs could not serve to transport skin-derived Ag to the skin-draining lymph nodes, nor could they participate in priming of Ag-specific T cells. We therefore re-evaluated the effect of LCs on epicutaneous immune responses (9) in vivo. To study selectively the contribution of Cdc42-deficient LCs, we performed this assay using Ly5.1→Cre and Ly5.1→Cdc42flx-Cre bone marrow chimeras. It has been already established that lethal irradiation and bone marrow reconstitution spares the epidermal LC compartment while leading to the replacement of virtually all other DC subsets including dermal langerin+ DCs (41, 42). Furthermore, migratory defects of Cdc42-deficient DCs were preserved in chimeras (data not shown). Ly5.1→Cre and Ly5.1→Cdc42flx-Cre chimeras were generated and epicutaneously immunized after reconstitution and confirmation of chimerism (data not shown). As shown in Fig. 4A, 6 d following Ag application, OT-I T cells showed a high amount of proliferation in Ly5.1→Cre mice. In contrast, in Ly5.1→Cdc42flx-Cre chimeras OT-I proliferation was clearly reduced, but this was apparent only when the number of cell divisions were taken into account (Fig. 4A). Interestingly, the percent of OT-I that proliferated was roughly equal in both groups. However, the number of cell divisions (≥5 cell divisions) achieved per cell was significantly higher in Ly5.1→Cre chimeras (Fig. 4A). This result appears to contrast a previous report (9) describing that dermal langerin+ cells would mediate this response to protein immunization on the flank. To exclude this, we determined the skin chimerism of our animals after sacrifice and found that our mice contain ≈98% wild-type (wt) langerin+ dermal DCs (not shown).

Next, we performed analyses of intracellular cytokine production to determine effector functions, but could not determine differences. Both Ly5.1→Cre as well as Ly5.1→Cdc42flx-Cre chimeras contained a similar percentage of IFN-γ+ OT-I cells (Fig. 4A). We next repeated this same experiment with OT-II TCR-transgenic T cells, during which we detected an even more severe T cell expansion and proliferation defect in Ly5.1→Cdc42flx-Cre mice (Fig. 4B). However, also OT-II cells did not differ in their capacities to produce IFN-γ or TNF-α (Fig. 4B), indicating that the priming in Ly5.1→Cre as well as Ly5.1→Cdc42flx-Cre chimeras was not qualitatively different regarding effector functions of primed cells. Together, these data show that migratory LCs substantially contribute to efficient CD4 and CD8 T cell priming upon epicutaneous immunization.

LCs are not required for an optimal CHS response to low-dose or high-dose hapten

The role of LCs in inducing and/or suppressing CHS remains controversial. With regard to CHS, we realized that we had a unique model with which to study this complicated immune response. Unlike the diphtheria toxin (DT) receptor– or DTA-mediated LC–ablation mouse models, our Ly5.1→Cdc42flx-Cre chimeras still contain LCs, thereby precluding any potential DT-induced inflammatory and/or developmental tolerance issues. Furthermore, we showed that LCs in these chimeras fail to leave the epidermis and efficiently prime T cells in vivo (Figs. 2 and 4, respectively).

We began to examine the CHS response in Ly5.1→Cdc42flx-Cre chimeras by first using a low-dose regimen of hapten treatment as described (8). Ear swelling measured 24 h after challenge showed that Ly5.1→Cdc42flx-Cre chimeras mounted a normal ear swelling response when compared with the Ly5.1→Cre chimera controls (Fig. 5). This would indicate that epidermal LCs are not required for an optimal CHS response under these conditions,
because in this model LCs are unable to transport Ag to the draining lymph nodes (Fig. 2). When we treated mice with a more conventional high dose of hapten, we again observed normal CHS responses in Ly5.1→Cdc42flx-Cre chimeras as compared with Ly5.1→Cre chimera controls (Fig. 5), suggesting that LCs do not play a role in this cutaneous immune response. Taken together, in stark contrast to what has been reported, these results argue against both a stimulatory and/or regulatory role for LCs in the context of CHS (6, 8, 10, 11).

Discussion

In this report, we have used the Cdc42flx-Cre mouse model as a tool to gain a better understanding of the role of Cdc42 in an in vivo DC subset as well as to address the function of LCs in mediating cutaneous immunity. We found that Cdc42 is required for the maintenance of normal LC numbers in the epidermis, suggesting that this molecule plays a role in LC homeostasis. In fact, a number of studies support a role for Cdc42 in cell survival. However, the absence of Cdc42 in LCs did not reduce their proliferative capacities to statistically significant levels and it therefore remains unclear, if this tendency to proliferate less actually contributed to reduced LC numbers in the steady state (Fig. 1E). In epithelial cells, cell survival is regulated by a signaling cascade involving Par6 and GSK-3β (43), both of which are known to function in conjunction with Cdc42 to regulate cell polarity (17, 44). Cdc42 was also found to be required for cell survival induced by E-cadherin homophilic interactions via activation of Stat3 (45). As LCs are dispersed throughout the epidermis in a distinct pattern maintained by E-cadherin–mediated adhesion to neighboring keratinocytes (46, 47), it is plausible that this interaction plays a role in their survival in the steady state.

The more rounded appearance of Cdc42flx-Cre LCs in the epidermis in the steady state was reminiscent of stimulated LCs (35). Although we did detect for some cell-surface molecules such as CD86 and MHC I a slightly higher level of cell-surface expression in Cdc42flx-Cre LCs, the cells were not overtly more mature. It is more likely that the rounded morphology of Cdc42flx-Cre LCs reflects poor maintenance of E-cadherin–mediated adhesions to keratinocytes. In Cdc42flx/K5 Cre mice, in which Cdc42 is targeted for deletion in keratinocytes, it was shown that Cdc42 was required for maintenance of the level of the intracellular signaling molecule β-catenin and, as a direct consequence, also the level of E-cadherin (29). We did not assess the level of cell-surface E-cadherin in our cells, but intracellular β-catenin levels were reduced in Cdc42flx-Cre LCs (Fig. 1D), and perhaps this is reflected in both reduced E-cadherin expression and subsequent adhesion.

The near-complete absence of LCs in the cutaneous as well as mDC subsets in MLN of Cdc42flx-Cre mice was surprising given that the splenic DC compartment of these mice is fairly normal (Figs. 1A, 1B, 2C, 2D and Supplemental Fig. 1A, top panel). This suggests that Cdc42 is differentially required for the localization of specific mDC subsets, although the mechanism(s) by which this is achieved remains unknown. This migration defect could be explained by the fact that Cdc42flx-Cre LCs showed only weak upregulation of CCR7 as compared wt mice (Fig. 1D), as this receptor is required for LC migration from the skin under both noninflammatory and inflammatory conditions (36). In addition, cross linking of CCR7 by CCL19 has been shown to activate Cdc42 in BC1 cells, a growth factor–dependent DC culture derived from murine splenocytes (48). Therefore, it is possible that CCR7 signaling, necessary for LC migration, is impaired in the

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Cdc42flx-Cre LCs fail to trigger optimal CD8 and CD4 T cell responses. Cdc42flx-Cre and Cre mice were lethally irradiated and received Ly5.1 congenic bone marrow. Eight to 10 wk later, chimeras received 2 × 10^6 CFSE-labeled, purified OT-I (A) or OT-II (B) Thy1.1^{+} T cells by i.v. injection. The next day, grade VII OVA was applied epicutaneously and the area covered with an occlusive patch. Draining axillary, brachial, and inguinal lymph nodes were harvested 6 d later. Visualization by flow cytometry of proliferated T cells was achieved by staining cell suspensions with anti-Thy1.1 (CD90.1) and either anti-CD8 (A) or CD4 Abs (B). Cell numbers of OT-I (A) and OT-II (B) cells in nodes were enumerated (right panels) according to the gating strategy shown in the dot plots. Proliferation of T cells was determined by measuring dilution of CFSE (bottom panels). Results shown are from one representative experiment out of three (n = 3–5). Intracellular cytokine analyses were performed after in vitro restimulation for 4 h with the respective peptides and gating on either CD90.1^{+}CD8^{+} OT-I cells (A) or CD90.1^{+}CD4^{+} OT-II cells (B) (gating not shown). Overlays show unstimulated (filled histograms) and peptide-stimulated (open histograms) cells. Results shown are from one representative experiment out of two (n = 4–5). *p < 0.05, **p < 0.01, ***p < 0.001.
Both of these models result in the toxin-mediated death of either the DT receptor or the active DTA.

Five days later, the mice were challenged on the ear with 0.25 or 1% oxazolone to elicit the low-dose or high-dose response, respectively. Swelling was measured as the difference in ear thickness between days 5 (prechallenge) and 6. Results shown are from one representative experiment out of three with similar results. Each dot represents an individual mouse.

Absence of Cdc42 in Cdc42flx-Cre LCs. However, deficiency to upregulate CCR7 was not observed in Cdc42-deficient BMDCs (26), possibly reflecting inherent differences between in vitro–generated and in vivo DCs.

However, our observation that under inflammatory conditions Cdc42flx-Cre LCs show morphological alterations in the epidermis in situ (Fig. 3B) further complicates the situation. Again, the mechanism by which this takes place is currently unknown, but it may be a combination of factors that result in this phenotype. As reported previously (26), DCs deficient for Cdc42 may simply become entangled within the extracellular matrix. Eventually, this entanglement may lead to the cells’ mechanical self-destruction via a continued effort to migrate. As a result, in the absence of Cdc42 LCs fail to leave the epidermis.

As we had a unique model in which to study the function of LCs in cutaneous immunity, we determined the requirement for LCs in stimulating an immune response following epicutaneous protein immunization. Although perhaps not directly biologically relevant to a specific disease model, epicutaneous immunization has potentially broad applications in the area of needleless vaccine development. This method of immunization is currently under investigation as an adjuvant to injected vaccines for diseases such as traveler’s diarrhea and pandemic influenza (http://www.intercell.com) (9). Determining the specific role of each cutaneous DC subset would certainly improve the efficacy of such vaccines by helping to identify specific targets and strategies. As suggested by a previous study, we showed that LCs are required for an optimal CD8+ and CD4+ T cell response to epicutaneously applied protein Ag (Fig. 4) (9). Furthermore, for CD8+ T cells, the absence of LC participation in epicutaneous protein immunization resulted in a qualitative defect in T cell proliferation, as the same percentage of OT-I T cells proliferated in both Cre and Cdc42flx-Cre chimeras, but the number of divisions per cell was also strikingly reduced in Cdc42flx-Cre chimeras (Fig. 4). The obvious outcomes of this are a reduced number of effector and perhaps memory T cells, although both of these models boast no DT-induced alterations in the skin environment, alteration or compensatory mechanisms remain a possibility. Also, recent findings in Batf3−/− mice, which lack langerin+ dermal DCs but have normal numbers of LCs, show normal CHS responses (51). In our Cdc42flx-Cre mice, LCs are present at a reduced frequency (Fig. 1C), but they are simply unable to leave the epidermis (Fig. 2), effectively removing them from the equation. Therefore, we have been able to examine the role of LCs in CHS in the face of a nonmigratory LC population and have determined that this cell type plays no significant role (Fig. 5). We have also ruled out the possibility that LCs serve as Ag transporters in the context of CHS, as we observed severely impaired transport of cutaneously applied FITC to the skin-draining lymph nodes in Cdc42flx-Cre mice (Fig. 2A), but still achieve normal CHS responses (Fig. 5). This is not surprising given a recent report demonstrating that, exclusively, the langerin+ CD103+ dermal DC population propages the immune response to a keratinocyte-restricted Ag (52). The authors elegantly show that this dermal DC population is even able to acquire keratinocyte-associated Ag independently of LCs by a currently unknown mechanism.

Although not diminished, the CHS response in our Cdc42flx-Cre chimeric mice does seem to be somewhat elevated, suggesting eventually a regulatory role for LCs in this response. However, even with a high dose of hapten, we were unable to observe a significant increase in swelling, as has been described in the DTA LC–deficient mouse model (10, 11). This discrepancy may be explained by differences in our mouse models and systems employed to study CHS. To study CHS, we use bone marrow chimeras in which wt, congenic bone marrow is grafted into irradiated Cre or Cdc42flx-Cre hosts, leaving predominantly the flox-Cre LC population intact while resulting in the replacement of all other DC subsets by wt precursors (41, 42). The studies showing enhanced CHS in the face of non–fully functional or completely absent LCs did not employ bone marrow chimeras. It could be argued that the dermal and other DC populations replenished following bone marrow engraftment are not the same as those normally present. The inflammation and cell death induced following irradiation may result in a functionally distinct dermal DC population that does not produce as robust a response as normal. Under these conditions, perhaps a regulatory role for LCs in CHS is therefore masked in our model system.

In summary, we have described a role for the Rho-family GTPase Cdc42 for the first time, to our knowledge, in an in vivo DC subset. We showed that Cdc42 is required for normal LC phenotype and morphology in vivo. Furthermore, using this mouse model, we have definitively described a role for LCs in the propagation of the immune response following epicutaneous immunization while demonstrating that these cells play no apparent role in CHS.
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Disclosures
The authors have no financial conflicts of interest.

References
Figure S1

A. Spleen

- Cre
- Cdc42flx-Cre

CD11c+ MHCII+ conventional DCs spleen

B. Thymus

- Cre
- Cdc42flx-Cre

CD11c+ MHCII+ conventional DCs thymus

B. Spleen CD11c+ MHCII+ cells DNA PCR

- Cre
- Cdc42flx-Cre

LCs DNA PCR

C. Spleen CD11c+ MHCII+ cells mRNA

- Cre
- Cdc42flx-Cre

LCs mRNA

D. Cre Cdc42flx-Cre

- Cdc42
- Actin
Figure S1. Dendritic cell populations of spleen and thymus in Cdc42flx-Cre mice and efficiency of the model system. Conventional dendritic cells (F480⁻, PDCA1⁻, CD11c⁺, MHC II⁺) in the spleen and thymus of Cdc42flx-Cre and Cre mice (A). Splenic DC (F480⁻, CD11c⁺, MHCII⁺) and Langerhans cells (CD45⁺, MHCII⁺, CD24⁺) were sorted and DNA was isolated. Equal amounts of total DNA were used in a PCR reaction to detect the different Cdc42 alleles. A sample obtained from a Cdc42flx mouse in absence of Cre was included as a PCR control for the floxed allele (B). Cdc42 message was determined by Real Time RT-PCR in sorted splenic DCs and Langerhans cells from Cdc42flx-Cre and Cre mice. Relative message levels were obtained by normalization to Ubiquitin C message levels (C). Residual protein in Cdc42flx-Cre splenic DCs was determined by Western blot of sorted cells. Actin served as a protein loading control (D).
Figure S2. Mature Cdc42flx-Cre Langerhans cells upregulate MHC I and classic co-stimulatory molecules but exhibit differences in the expression of a number of other cell surface molecules. Immature and mature Langerhans cells (CD45⁺, MHCII⁺, Langerin or CD24⁺) were stained for flow cytometry with the indicated antibodies.