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Second-Generation Langerhans Cells Originating from Epidermal Precursors Are Essential for CD8+ T Cell Priming

Mazal Elnekave,* Karina Furmanov,* Yaffa Shaul,* Tal Capucha,* Luba Eli-Berchoer,* Katya Zelentsova,* Björn E. Clausen,† and Avi-Hai Hovav*

In vivo studies questioned the ability of Langerhans cells (LCs) to mediate CD8+ T cell priming. To address this issue, we used intradermal immunization with plasmid DNA, a system in which activation of CD8+ T cells depends on delayed kinetics of Ag presentation. We found that dendritic cells (DCs) located in the skin at the time of immunization have limited ability to activate CD8+ T cells. This activity was mediated by a second generation of DCs that differentiated in the skin several days after immunization, as well as by lymph node–resident DCs. Intriguingly, CD8+ T cell responses were not affected following treatment with clodronate liposomes, immunization of CCR2−/− mice, or local neutralization of CCL20. This suggests that local, rather than blood-derived, DC precursors mediate CD8+ T cell priming. Analysis of DC differentiation in the immunized skin revealed a gradual increase in the number of CD11c+ cells, which reached their maximum 2 wk after immunization. A similar differentiation kinetics was observed for LCs, with the majority of differentiating LCs proliferating in situ from epidermal precursors. By using B6/Langerin–diphtheria toxin receptor chimeric mice and LC ablation, we demonstrated that epidermal LCs were crucial for the elicitation of CD8+ T cell responses in vivo. Furthermore, LCs isolated from lymph nodes 2 wk after immunization contained the immunization plasmid and directly activated Ag-specific CD8+ T cells ex vivo. Thus, these results indicate that second-generation Ag-expressing LCs differentiating from epidermal precursors directly prime CD8+ T cells and are essential for optimal cellular immune responses following immunization with plasmid DNA. The Journal of Immunology, 2014, 192: 000-000.

A s the sole dendritic cells (DCs) located in the epidermis, Langerhans cells (LCs) are thought to be important players in skin-mediated immunity. In agreement with this assumption, in vitro and ex vivo studies demonstrated the ability of LCs to prime naive CD8+ and CD4+ T cells (1, 2). However, studies using transgenic mice, which enable in vivo ablation of LCs, suggested a more limited function for LCs. It was shown that LCs can prime CD4+ Th17 cells under inflammatory conditions, as well as Th2 responses via thymic stromal lymphopoietin signaling (3, 4). LCs also can limit adaptive immune responses by stimulating the differentiation of T regulatory cells (5–7). In contrast to CD4+ T cells, LCs were shown to be dispensable for CD8+ T cell priming in numerous experimental systems (8–11). This activity was mediated by another subset of langerin–positive DCs located in the dermis, termed Ln+dDCs (11, 12). The lack of in vivo evidence for LCs to prime CD4+ Th1-type responses further argues against their contribution to the activation of CD8+ T cells in vivo.

Following infection or immunization, epidermal DCs migrate to the lymph nodes (LNs), whereas the immunodevelopment in the perturbed skin facilitates their repopulation from various sources (13). Under severe inflammatory conditions damaging the epidermis and eliminating large numbers of LCs, these cells can differentiate from bone marrow–derived precursors, either circulating or located in the epidermis (14–16). Repopulation of LCs can also occur from self-renewing epidermal DCs during moderate inflammation, similar to the situation in the steady-state (17, 18). It is possible that LCs freshly differentiating in the presence of local inflammation acquire different immune functions than do terminally differentiated resident LCs that have experienced the immunosuppressive microenvironment in the skin. In agreement with this view, newly recruited, rather than skin-resident, DCs were shown to mediate CD8+ T cell cross-priming following exposure to soluble protein Ag (19). In addition, the nature and quantity of the immunological challenge might also influence the ability of LCs to elicit immunity (20–22). Therefore, it is likely that, under certain conditions, LCs developing in the skin postinfection or postimmunization could contribute to cell-mediated immunity.

In contrast to common infection and immunization models in which CD8+ T cell priming is initiated within hours after inoculation, intradermal immunization with naked plasmid DNA triggered a unique pathway for activating these cells. We demonstrated previously that DC Ag presentation to CD8+ T cells is delayed considerably, starting ∼5 d after the immunization and increasing gradually during the following week (23). Langerin-expressing DCs were found to be crucial for an optimal CD8+ T cell response in this system. Based on the differential repopulation of LCs and Ln+dDCs after in vivo ablation, we proposed that Ln+dDCs were the only langerin-expressing DC subset mediating CD8+ T cell priming (23). Nevertheless, because intradermal administration of plasmid DNA induces local inflammation with the possibility to facilitate...
LC repopulation, we sought to revisit the role of langerin-expressing DCs in this immunization protocol.

Materials and Methods

Abs and reagents

The following mAbs were purchased from BioLegend (San Diego, CA) and used in the study: anti-CD8a (53-6.7), anti-CD103 (2E7), anti-CD11c (N418), anti–Ep-CAM (G8.8), anti-CD45.1 (A20), anti-CD45.2 (104), anti–MHC class II (AF6-120.1), and anti-Ly6C (HK1.4). Anti-langerin Ab (929F3.01) was purchased from Imgenex (San Diego CA). Tetrameric H-2Kb complexes folded with the OVA SIINFEKL epitope were prepared in-house.

Mice

Six- to twelve-week-old Langerin–diphtheria toxin receptor (DTR) knockin mice, expressing the human DTR under transcriptional control of the endogenous Langerin/CD207 promoter, and CCR2–/– mice were bred in our animal facility and maintained under specific pathogen–free conditions. The identity of the Langerin-DTR mice used for experiments was always confirmed by genotyping with the following PCR primers: forward, 5′-GCCACCATGAAGCTGCCG-3′ and reverse, 5′-ATACTTATTAGGCGGCCTTTACTTGTACAG-3′. The identity of the CCR2–/– mice was confirmed by genotyping using the following primers: Ccr2F: 5′-TGGGGATAC-TGCTTTAAATGGGGCGCA-3′, Ccr2R: 5′-TCCATTGCTACGGGTCT-3′. OT-I mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at the age of 6–8 wk. C57BL/6 (B6) mice were purchased from Harlan Laboratories (Jerusalem, Israel) and used at the age of 6–8 wk. Research on mice was approved by the Hebrew University Institutional Animal Care and Ethics Committee.

Vectors and immunizations

The CMV-ovA plasmid was a gift from Dr. Maripat Corr (University of California, San Diego, San Diego, CA). The CD11c-OV A was provided by Dr. Joshy Jacob (Emory Vaccine Center, Atlanta, GA). Mice were anesthetized with ketamine/xylazine mix prior to injection into the ear pinna using a 31-gauge needle containing plasmid DNA (50 μg DNA in 80 μl; 40 μl was delivered into each ear). All plasmids used in this study were prepared using the EndoFree Plasmid Giga kit (QIAGEN, Valencia, CA), andplexes folded with the OV A SIINFEKL epitope were prepared in-house.

Isolation and identification of skin DCs

The ears were excised, separated into two halves, and minced with scalps in PBS plus 2% FCS containing collagenase type II (2 mg/ml) and DNase I (1 mg/ml). The processed tissue was transferred into a tube containing 1 ml of the medium and incubated for 30 min at 37°C in a shaker bath. A total of 10 μl 0.5 M EDTA was added per sample, and the incubation continued for 10 min. Following extensive pipetting, the cells were passed through a 70-μm filter, washed twice with PBS plus 2% FCS, and counted. For extracellular staining, the cells were incubated with the relevant panel of Abs for 20 min on ice and then washed with 2 ml PBS plus 2% FCS. Further intracellular staining against langerin was performed using the Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer’s instruction. The samples were collected on a LSR II instrument (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR).

Ag-presentation assay

The draining LNs were collected from immunized mice and treated with collagenase type II (1 mg/ml) and DNase I (1 mg/ml) solution in PBS plus 2% FCS for 20 min at 37°C in a shaker bath. Twenty microliters of 0.5 M EDTA per ml sample was added to the digested LNs and incubated for an additional 10 min. The cells were then washed and filtered. CD11c+ cells were obtained from the digested LNs by positive isolation using MACS Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. The enriched CD11c+ cells were stained with Abs against CD80, CD11c, CD103, Ep-CAM, or MHC II and were subjected to sorting by flow cytometry (FACSria; BD Biosciences). In some experiments, the cells were also stained against CD45.1 and CD45.2. OT-I CD8 T cells were purified by negative selection with the EasySep mouse CD8 enrichment kit (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. OT-I CD8+ T cells (5 × 105/well) were incubated with the different DC populations (1.5 × 105/well) in 96-well U-bottom plates (Nunc, Roskilde, Denmark). The cultures were then incubated for 60 h, and the supernatants were collected and stored at −80°C for IFN-γ analysis. The level of IFN-γ in the supernatants was measured using an ELISA MAX mouse IFN-γ kit (BioLegend), according to the manufacturer’s instructions. Cytokine levels were determined using standard curves of recombinant cytokines and are expressed as pg/ml.

Clodronate liposome treatment

Clodronate was obtained from the clodronate liposomes foundation and encapsulated in liposomes as described earlier (24). To reduce monocytes in the bloodstream and prevent their infiltration into the site of immunization, mice were injected i.p. with 200–400 μl liposome solution every 4 d starting 1 d before immunization.

Generation of chimeric mice

Recipient mice were lethally irradiated by a single dose of 9 Gy; 24 h later, the mice were injected i.v. with 4 × 109 total bone marrow cells from donor mice. For complete reconstitution, mice were rested for ≥8 wk before being included in the experiments.

CCL20 neutralization

Local CCL20 neutralization was performed by repeated intradermal injections of 15 μg anti-mouse CCL20/MIP-3α–neutralizing Ab or the isotype control (R&D Systems) diluted in 40 μl PBS on days 1, 3, 6, and 9 postimmunization.

RT-PCR and PCR analysis

The ears, LNs, or sorted DCs were collected at the indicated time points, and total RNA was extracted by TRI Reagent (Sigma-Aldrich). The integrity of the RNA was assessed by agarose gel electrophoresis. Reverse transcription of total RNA was carried out in a 20-μl volume containing 1 μg the total RNA sample (RevertAid H Minus First Strand cDNA Synthesis Kit; Fermentas). Quantitative real-time PCR was performed in an ABI PRISM 7300 System (Applied Biosystems). The reaction was carried out in a 20-μl volume that contained 10 μl TaqMan Gene Expression Master Mix (Applied Biosystems), 1 μl the assay solution containing OVA-specific primers and 6-FAM–labeled probe (Applied Biosystems), and 5 μl diluted cDNA. The following amplification protocol was used: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The
results were analyzed by the ΔΔt method, which reflects the difference in threshold for the OVA gene relative to that of β-actin in each sample. β-actin primers were designed for RT-PCR using the Primer Express program (Applied Biosystems): sense 5'-ATGCCTCCGGGGCTGTA-3', and antisense 5'-CATAGGATCTTTCTGACCAATT-3'. For amplification of the whole OVA gene (1.2 kb) in DCs isolated from the LNs, PCR was performed on total DNA extracted from the cells by TRI reagent, using the following OVA-specific primers: forward 5'-GGGATCCATGGCTC- CATCCGC-3' and reverse 5'-GCTCTAGATTTAAGGGGAAAC-3'.

BrdU labeling

Mice were injected i.p. with BrdU (2 mg/mouse, Sigma, St Louis, MO) on the day of immunization and subsequently received BrdU (0.8 mg/ml) in autoclaved drinking water that was changed every other day. Eleven days after immunization, the ear pinna of B6 mice was excised, processed, and stained with anti-CD45, CD11c, langerin, and Ep-CAM Abs. In another experiment, the ear pinna of chimeric mice was excised 14 d post-immunization, split using forceps, and incubated in a PBS solution containing 0.5% trypsin and 5 mM EDTA for 45 min at 37˚C. The epidermis and dermis were separated, processed, and stained as described above. BrdU+ cells were detected using a BrdU Flow Kit (BD Biosciences), according to the manufacturer’s instructions.

Immunofluorescence staining

Skin tissue was fixed overnight at 4˚C in 4% paraformaldehyde/PBS solution, cryopreserved in 30% sucrose (overnight, 4˚C), embedded in OCT, and cryosectioned into 10-µm-thick sections. The sections were washed three times in PBS, blocked in blocking buffer (5% FCS, 0.1% Triton X-100 in PBS) for 1 h at room temperature, and incubated with primary Ab (rat anti-CD207/Langerin clone: eBioL31 [eBioscience]) overnight at 4˚C. Following three washing steps in PBS, the samples were incubated with a secondary Ab (Cy3-conjugated donkey anti-rat IgG [Jackson Immunoresearch]) diluted 1:100 in blocking buffer) for 1 h at room temperature, washed three times, Hoechst stained, and mounted. As a negative staining control, primary Ab was omitted and replaced by blocking buffer. Signals were visualized, and digital images were obtained using an Olympus BX51 fluorescence microscope. H&E staining was performed on 5-µm paraﬃn sections.

**FIGURE 1.** CD8+ T cell priming following intradermal plasmid DNA immunization relies on a durable presence of Ag-expressing DCs in the skin. B6 mice were immunized in the ear pinna with pACB-OVA (50 µg); the ear pinna of the mice was excised 5, 10, or 15 d following immunization. The amplitude of the OVA-specific CD8+ T cell population in the peripheral blood (A) and LNs (B) of immunized mice, as detected on day 18 postimmunization with H-2Kb/SIINFEKL tetramers. (C) Frequencies of OVA-specific CD8+ T cells in the excised ears at days 0, 5, 10, and 15 postimmunization. Data are shown as the percentages of CD8+ tetramer+ T cells and represent means of four mice/group ± SEM. (D) Twenty-four hours after immunization, the ear pinna was treated with DBP, and 5 or 10 d later the ear pinna was removed or left intact. Tetramer+ CD8+ T cell frequencies were examined in the blood on day 14, as described. (E) Mice were coinjected in the ear pinna with plasmid DNA plus LPS (1 µg) or TNF-α (10 µg), and the ear pinna was excised or left intact. CD8+ T cell responses were examined on day 10, as described. (F) Mice were immunized intradermally with CMV-OVA or CD11c-OVA (n = 5), and the frequencies of Ag-specific CD8+ T cells in the blood were monitored using tetramers, as described above. (G) The ear pinna and cervical LNs were collected from CD11c-OVA–immunized mice at the noted times, and RNA and then cDNA were prepared. The samples were subjected to real-time PCR analysis to determine the expression level of the OVA gene. Data are presented in a logarithmic scale as relative fold increases in the expression of the OVA gene after normalization, according to the expression of mouse 18S, and represent the mean of three mice/time point ± SEM. One representative of two or three independent experiments is depicted. *p < 0.001 versus control group.

**Statistical analysis**

Data are expressed as mean ± SEM. Statistical tests were performed using one-way ANOVA and a Student t test. A p value < 0.05 was considered significant.

**Results**

**Plasmid DNA–mediated CD8+ T cell responses require extended presence of Ag-expressing DCs in the immunized skin**

Administration of naked plasmid DNA into the skin is an immunogenic approach to prime potent CD8+ T cell responses (25). To elicit cellular immunity, B6 mice were immunized intradermally with plasmid encoding OVA under control of the CMV promoter (CMV-OVA). In agreement with previous data (23), excision of the immunization site 5 or 10 d after immunization considerably decreased the magnitude of the CD8+ T cell response in the blood and the LNs (Fig. 1A, 1B). We next examined the frequencies of OVA-specific CD8+ T cells at the site of immunization, the ear skin, at various times after immunization. As demonstrated in Fig. 1C, in agreement with the blood and LN results, the percentages of these cells increased significantly during the 2 wk postimmunization. This suggests that local events taking place in the skin during this period of time are critical for efficient immune induction. We recently reported that Ag presentation to CD8+ T cells in this system is exceptionally delayed, because considerable presentation was monitored only 5 d after immunization (23). To examine whether a slow migration of skin DCs to the LNs explains the delayed kinetics of Ag presentation to T cells, we artificially accelerated DC migration by applying DBP onto the immunization site 24 h after immunization (Fig. 1D). Unexpectedly, CD8+ T cell responses were decreased significantly, despite the forced migration of skin DCs, and excision of the injection site
further reduced immunogenicity. We next immunized mice with plasmid DNA combined with LPS or TNF-α to facilitate the maturation of DCs receiving the plasmid (Fig. 1E). Again, these treatments lowered the magnitude of activated CD8+ T cells in comparison with immunization with plasmid DNA alone. In addition, removal of the immunization site 5 d after the immunization completely abolished CD8+ T cell priming in all experimental groups.

To further examine the role of DCs, we restricted Ag expression to DCs by immunizing mice with plasmid DNA encoding the OVA Ag under the control of the CD11c promoter (CD11c-OVA). As we previously reported, expression of the Ag by DCs generated efficient CD8+ T cell responses similar to those obtained by the ubiquitous CMV promoter (Fig. 1F) (23). In addition, akin to immunization with the CMV promoter, excision of the ear pinna ablated T cell responses after immunization with the CD11c-OVA plasmid (data not shown). Using real-time PCR to quantify Ag expression, we found a very high amount of OVA mRNA in CD11c+ cells 1 d after immunization, which decreased gradually thereafter. Nevertheless, considerable expression levels were still detected in the skin for ≥3 wk postimmunization (Fig. 1G). Similar kinetics of expression were also measured in the draining LNs (Fig. 1G). Collectively, these data suggest that DCs with the capacity to express plasmid-encoded Ag must be present in the immunized skin for ≥10 d to prime optimal CD8+ T cell responses.

Ag presentation to CD8+ T cells is mediated by newly differentiated skin DCs

Following infection or immunization, a portion of the DCs leave the skin and migrate to the draining LNs, whereas newly differentiating DCs repopulate the site of infection/immunization (13). In plasmid DNA–immunized mice, the extended dependence on the presence of the immunization site suggests that DCs differentiating in the skin subsequent to the time of immunization might play an essential role in CD8+ T cell priming. To address this issue, we first examined whether skin DCs have the capacity to activate CD8+ T cells following plasmid DNA immunization. The skin of CMV-OVA–immunized mice was treated with DBP on day 7 postimmunization; 3 d later, the cervical LNs were collected, enriched for CD11c+ cells, and stained with Abs against CD11c and MHC class II molecules. Skin DCs were then FACs sorted (CD11cintermediateMHCIIhigh) and cocultured with OVA-specific CD8+ OT-I T cells, and the levels of IFN-γ in the supernatants were measured 60 h later to determine T cell activation by DCs (Fig. 2A). Of note, cell proliferation cannot be evaluated because DCs purified following intradermal DNA immunization failed to drive proliferation of OT-I T cells ex vivo (23, 26). As demonstrated in Fig. 2A, skin DCs were able to induce a potent secretion of IFN-γ, suggesting that these cells are involved in CD8+ T cell priming. We next sought to examine the Ag-presenting capabilities of DCs located in the skin at the time of immunization (primary DCs) and those that are present in the skin later on (secondary DCs). To differentiate between these two populations, we populated the skin with an FITC solution 24 h before the immunization to label DCs present at the immunization site, which represent primary DCs. Because a considerable degree of Ag presentation can be observed in the mice 1 wk after immunization (23), we treated the injection site with DBP on day 7 and purified migratory DCs from the LNs 3 d later (Fig. 2B). As depicted in Fig. 2B (upper panel), gating on skin-derived DCs (CD11c+CD8−) enabled us to identify and FACs sort FITC+ primary DCs, as well as FITC− DCs. LN-resident DCs

**FIGURE 2.** DCs differentiating in the skin after the immunization present Ag to CD8+ T cells. (A) Mice were immunized intradermally with CMV-OVA, and the injected skin was treated with DBP 7 d after. After 3 d, LNs were collected, enriched for CD11c+ cells, and skin DCs (CD11cintermediateMHCIIhigh) were FACs sorted and cocultured with OVA-specific OT-I CD8+ T cells for 60 h to quantify the concentration of IFN-γ by ELISA. (B) The ear pinnae of B6 mice were painted with FITC solution and immunized 24 h later with CMV-OVA. In some experiments, the immunized pinna was treated with DBP 7 d after immunization. At 10 d postimmunization, the cervical LNs were pooled from 8–14 mice, enriched for CD11c+ cells, and stained with anti-CD11c and CD8 Abs. (C and D, upper panels) Gating strategy used to identify and purify primary skin DCs (CD11c+CD8− FITC+), secondary skin DCs (CD11c+CD8− FITC−), and LN-resident DCs (CD11c+CD8−). (C and D, lower panels) The noted DC subsets were incubated with OT-I CD8+ T cells, and supernatants were collected 60 h later. (E) Twenty-four hours prior to immunization with CMV-OVA, the ear skin was painted with FITC; 7 d postimmunization, the skin was repainted with TRITC/DBP solution. Three days later, the cervical LNs were pooled from 21 mice, enriched for CD11c+ cells, and stained with anti-CD11c and CD8 Abs. Primary skin DCs (CD11c+CD8− FITC+), secondary skin DCs (CD11c+CD8− TRITC+FITC−), and double-negative skin DCs lacking FITC or TRITC labeling (DN-DCs) were purified and cultured with OT-I cells, as described above. Results are representative of at least two independent experiments.

*p < 0.05 versus IFN-γ secretion in cultures of LNs and secondary DCs plus T cells.
were also purified based on the expression of both CD11c and CD8. To test the Ag-presenting ability of the various DC subsets, we incubated them with OVA-specific CD8⁺ OT-I T cells and measured IFN-γ production in the supernatants after 60 h. Significant IFN-γ levels were observed in cultures containing DCs and T cells in comparison with T cells or DCs alone (p < 0.0001). Further, secondary DCs (FITC⁺) were able to induce higher IFN-γ levels than were primary DCs (p < 0.05), suggesting that this subset has superior Ag-presenting capability, because not all of the FITC⁺ cells represent skin DCs that have experienced the Ag. In addition to skin DCs, LN-resident DCs contributed to T cell priming at similar levels as did secondary DCs. With the idea to mimic more adequately the situation in vivo, we repeated the experiment without the DBP treatment (Fig. 2D). In contrast to DBP-treated mice, the frequencies of primary (FITC-labeled) DCs were much lower in LNs isolated from the mice 10 d after immunization (Fig. 2D, upper panel). These primary DCs failed to drive efficient OT-I CD8⁺ T cell activation, whereas secondary DCs did so effectively (Fig. 2D, lower panel). Moreover, LN-resident DCs were also capable of activating CD8⁺ T cells. Finally, we used an additional strategy to differentiate between primary and secondary skin DCs. Similar to the above experiments, the immunization site was labeled with FITC 24 h before immunization; at day 7 postimmunization the skin was painted with a TRITC/DBP solution, and CD11c⁺ cells were enriched from LNs 3 d later. As demonstrated in Fig. 2E, in agreement with the above findings (Fig. 2D) primary FITC⁺ skin DCs represent a very minor population of migratory DCs in the LNs. Moreover, secondary TRITC⁺ DCs, but not primary FITC⁺ DCs, were able to present Ag to OT-I cells (p < 0.001). Lower presentation levels were also observed by double-negative (FITC-TRITC⁻) skin DCs, suggesting that DCs differentiating in the skin immediately after the immunization and migrating prior to day 7 also contribute to CD8⁺ T cell priming. Taken together, these data suggest that DCs differentiating in the skin beyond the time of immunization have the capacity to present Ag to CD8⁺ T cells.

Blood-derived skin DC precursors are dispensable for CD8⁺ T cell priming

We next thought to examine the origin of secondary DCs in the immunized skin. Under inflammatory condition, CCR2 was shown to mediate the recruitment of circulating precursors of the three main skin DC subsets: LCs, dermal DCs, and Ln⁺dDCs (14, 27, 28). Thus, we used CCR2⁻/⁻ mice for intradermal immunization with plasmid DNA. Interestingly, despite the absence of CCR2, the mice successfully developed intact CD8⁺ T cell responses compared with wild-type B6 controls (Fig. 3A). We next treated the mice with clodronate liposomes throughout the experiments to eliminate monocytes that might differentiate into DCs in the immunized skin. Again, although the treatment efficiently reduced the frequencies of MHC class II⁺ Ly6C⁺ cells in the skin (Supplemental Fig. 1A), it had no impact on the elicitation of CD8⁺ T cells (Fig. 3B). We then blocked the chemotactic activity of CCL20 using a neutralizing Ab (Supplemental Fig. 1B). CCL20 is the ligand of CCR6, which is important for the infiltration of circulating LC precursors into inflamed skin (29). Despite successful local depletion of inflammatory cells, the absence of CCL20 activity did not affect the CD8⁺ T cell response in comparison with an appropriate isotype control (Fig. 3B). These data suggest that secondary skin migratory DCs, which contribute to efficient Ag presentation and activation of CD8⁺ T cells, do not originate from circulating precursors.

Kinetics of DC subsets accumulating in the skin following intradermal immunization with plasmid DNA

To better understand the origin of secondary DCs, we analyzed the type of DCs accumulating in the skin after immunization. Because langerin-expressing cells were found to affect the elicitation of CD8⁺ T cells (23), we focused our analysis on these cells. Following injection of plasmid DNA, a gradual increase in the frequency of MHC class II- expressing cells was observed up to day 15, after which this population decreased considerably during the subsequent 2 wk (Fig. 4A). Another subset of cells expressing both MHC class II and CD11c followed the differentiation of the above population, suggesting that some MHC class II⁺ cells might differentiate into CD11c-expressing DCs (Fig. 4A, 4B). Next, we analyzed the differentiation of langerin-expressing DCs by staining the cells with Abs against the following markers: CD45, MHC class II, CD11c, langerin, Ep-CAM, and CD103 (Supplemental Fig. 3) (11). As expected, langerin-expressing cells constituted only a small fraction of the total CD11c⁺ DCs (Fig. 4C). The majority (>75% at day 15) of langerin-expressing cells were LCs, based on the expression of langerin and Ep-CAM, but not CD103, and they displayed similar differentiation kinetics as did CD11c⁺ DCs. A smaller population of Ln⁺dDCs (CD11c⁺langerin⁺CD103⁻) was found in the immunized skin, as well (Fig. 4C). In contrast to LCs, the number of Ln⁺dDCs increased on day 3 and remained relatively stable during the next 2 wk. In addition to these two

**FIGURE 3.** Blood-derived skin DC precursors are dispensable for CD8⁺ T cell priming. (A) B6 and CCR2⁻/⁻ mice were immunized with CMV-OVA in the ear pinna, and the magnitude of OVA-specific CD8⁺ T cells was examined in the blood in the following weeks using H-2Kb/SIINFEKL tetramers. Data are shown as the percentages of CD8⁺ tetramer⁺ T cells and represent the means of five mice/group ± SEM. (B) Twenty-four hours before the immunization with CMV-OVA, B6 mice were treated i.p. with clodronate liposomes or empty liposomes as a control (n = 5/group), and the treatment continued every 4 d throughout the experiment. Ag-specific CD8⁺ T cell responses were measured in the blood, as described above. (C) Following intradermal immunization with CMV-OVA, the chemokine CCL20 was neutralized locally in the immunized ear by intradermal administration of anti-CCL20 Ab on days 1, 3, 5, and 7 postimmunization. As a control, the immunized mice received treatment using an isotype control Ab. The impact of CCL20 neutralization on OVA-specific CD8⁺ T cells was examined, as described, and represents the mean of four mice/group ± SEM. Results are representative of at least two independent experiments.
well-known langerin-expressing subsets, a third DC subset expressing CD11c and langerin, but not CD103 or Ep-CAM, was detected, as previously reported (11). The numbers and kinetics of this population were comparable to Ln+dDCs (Fig. 4C). Because LCs represent the major langerin-expressing DC subset accumulating in the skin of plasmid DNA–immunized mice, we further tested whether they differentiated from epidermal precursors or from bone marrow–derived cells. For this purpose, we adoptively transferred lethally irradiated CD45.2 B6 mice with bone marrow purified from congenic CD45.1 B6 mice. Eight weeks after reconstitution, only radioresistant LCs (CD11c+langerin+Ep-CAM+) in the skin were of host origin, whereas the remaining skin DCs were of donor origin (Supplemental Fig. 2A). The mice were then immunized intradermally with plasmid DNA, and LCs were analyzed as described above, and the numbers of LCs originating from host (CD45.2+) or donor (CD45.1+) cells were determined. Data are the mean of four ears/time point ± SEM. (E) At 14 d postimmunization, the epidermis and dermis of the ears of CD45.1→CD45.2 chimeras were separated, processed, and stained to identify the localization and origin of LCs at the immunization site. Representative FACS plots are presented demonstrating the presence of host-derived (CD45.2+) and donor-derived (CD45.1+) LCs (CD11c+MHCII+Ep-CAM+langerin+) in the epidermis and dermis. (F) H&E and immunofluorescence staining of the ear skin 14 d postimmunization (blue, nuclei stained with the DNA intercalating dye Hoechst; red, anti-langerin Ab). One representative of two or three independent experiments is depicted. The arrowheads represent langerin-expressing cells in the epidermis.

* p < 0.001, host-derived LCs versus donor-derived LCs. AC, Auricular cartilage; Dr, dermis; Ep, epidermis.

Secondary LCs are crucial for optimal CD8+ T cell priming in vivo

Using Langerin-DTR mice, we previously demonstrated a role for Ln+dDCs in CD8+ T cell priming (23). As shown in Fig. 5A, treating mice with DT 7 d before the immunization had no effect on the magnitude of the CD8+ T cell response, whereas repeated administration of DT diminished the reaction considerably. This approach is commonly used to ablate LCs (pretreatment with DT) or both LCs and Ln+dDCs (repeated DT treatments) during an
experiment (6, 7, 30). Based on this knowledge, we reasoned that Ln\(^\text{d}\\)dDCs were important for activating CD8\(^+\) T cells following intradermal administration of plasmid DNA (23). Nevertheless, it is becoming increasingly clear that, under inflammatory conditions, LCs rapidly repopulate the skin (15, 16), as was also observed in our experiments (Fig. 4C, 4D). Therefore, the repeated administration of DT ablated primary LCs and Ln\(^\text{d}\\)dDCs, as well as secondary LCs. To re-examine this issue, we generated bone marrow chimeras by reconstituting lethally irradiated Langerin-DTR mice with bone marrow from CD45.1\(^+\) B6 wild-type mice. At 8 wk after reconstitution, only the radioresistant epidermal LCs expressed the DTR; other langerin-expressing cells did not. Following immunization with plasmid DNA and ablation of langerin-expressing cells by repeated DT injections, we found a considerable reduction in the magnitude of CD8\(^+\) T cells (Fig. 5B). Ablation of host-derived LCs, but not LCs originating from donor cells, was confirmed at the immunization site (Supplemental Fig. 4). Of note, similar to Langerin-DTR mice, the reduction in CD8\(^+\) T cell responses was observed beyond 10 d postimmunization and correlated with the accumulation of LCs in the tissue. These data suggest that, although primary LCs residing in the epidermis at the time of immunization are dispensable, secondary LCs originating from epidermal precursors during inflammation are required for CD8\(^+\) T cell priming in vivo. We also examined the reverse chimeras, in which bone marrow from Langerin-DTR mice was transferred into lethally irradiated B6 wild-type mice (Fig. 5C). In these animals, epidermal LCs are not affected by the DT treatment, whereas other langerin-expressing cells are eliminated. A reduction in the CD8\(^+\) T cell response also was observed in these chimeras, indicating that bone marrow–derived langerin-expressing DCs (see Fig. 4D) also contribute to efficient CD8\(^+\) T cell priming.

**Plasmid-transfected epidermal LCs activate CD8\(^+\) T cells ex vivo**

We next examined the ability of epidermal LCs to present Ag to CD8\(^+\) T cells ex vivo. To differentiate between LCs originating from epidermal precursors and the remaining skin-derived DCs, we generated bone marrow chimeras by reconstituting lethally irradiated CD45.1 mice with CD45.2 congenic bone marrow. Eight or fourteen days after immunization, LNs were collected, enriched for CD11c\(^+\) cells, and stained with Abs against CD11c, CD8, CD45.1, and CD45.2. Skin DCs (CD11c\(^+\)CD8\(^-\)) were sorted further using FACS to obtain epidermal LCs (CD45.1\(^+\)) and all other skin-derived DCs (CD45.2\(^+\)) (Fig. 6A). We first examined whether the sorted DC populations contained the injected plasmid by amplifying the OVA gene from total DNA prepared from the cells harvested 14 d postimmunization. Our analysis indicated that CD45.1\(^+\) LCs were the only skin DCs harboring the plasmid, whereas the OVA gene could not be detected in CD45.2\(^+\) skin DCs (Fig. 6B). We noticed that LN-resident DCs also contained the OVA plasmid, although to a lower extent than LCs. We next cocultured the sorted DCs with OT-I CD8\(^+\) T cells for 60 h and measured the secretion of IFN-\(\gamma\) in the supernatant as an indication of the degree of T cell activation. As shown in Fig. 6C, 8 d after immunization, presentation of the Ag to CD8\(^+\) T cells was mediated only by skin DCs, without any contribution by epidermal LCs. In contrast, 14 d after immunization, activation of CD8\(^+\) T cells was mediated by epidermal LCs and skin DCs, along with LN-resident DCs that also participated in Ag presentation. This pattern of ex vivo Ag presentation by LCs correlates well with the kinetics of CD8\(^+\) T cell priming in the absence of LCs (Fig. 5B), because ablation of LCs had no impact on the magnitude of the CD8\(^+\) T cell response until day 10. Collectively, our data suggest that, following plasmid DNA immunization, plasmid-harboring epidermal LCs reach the LNs and are critically involved in CD8\(^+\) T cell priming during the second week after immunization. In addition, other blood-derived skin DCs are presenting Ag to CD8\(^+\) T cells, most likely in a mechanism involving cross-priming.

**Discussion**

We studied the role of langerin-expressing DCs in CD8\(^+\) T cell priming following intradermal administration of plasmid DNA. In this immunization setting, a potent activation of CD8\(^+\) T cells is initiated only 5 d postimmunization (23) by DCs that were not present in the skin at the time of immunization. Although there is no explanation for this kind of delayed kinetics of Ag presentation, it demonstrates that, under certain conditions, LCs are crucial for the priming of CD8\(^+\) T cells in vivo. The finding that LCs isolated from skin-draining LNs contain the Ag-encoding plasmid and are able to activate CD8\(^+\) T cells ex vivo strongly suggests that directly transfected LCs present Ag to CD8\(^+\) T cells in vivo. This is in line with the reported inability of murine LCs to cross-present Ag in vivo (3, 11, 12). This interpretation is further supported by the failure of plasmid DNA to induce immunity when Ag expression is restricted to keratinocytes using the K14 promoter (23).

Following immunization with plasmid DNA, expression of the Ag is observed in the skin for \(>3\) wk. This holds true when the Ag is expressed under the control of both the ubiquitous CMV and the DC-specific CD11c promoters (23) (Fig. 1G). The durable expression by CD11c\(^+\) cells in the skin is surprising because, during
inflammation, Ag-expressing DCs are thought to migrate rapidly to draining LNs to prime naive T cells. Because extracellular plasmids are rapidly degraded after immunization, it is unlikely that infiltrating DCs will take up plasmid upon arrival in the skin. In agreement, skin-derived DCs (not LCs) isolated from the LNs 14 d after immunization do not contain the plasmid (Fig. 5D, 5E). A possible explanation for the extended Ag expression in the skin is transfection of both LCs and epidermal LC precursors with the plasmid. In this regard, although the plasmid was administered intradermally, the injection volume generated high pressure in the thin ear skin, thus increasing its possibility of reaching the epidermis. This conclusion is supported by our observation that secondary LCs originating from host precursors contain the plasmid. Interestingly, LNs-resident DCs (CD11c+CD8α+) also harbor plasmid 14 d postimmunization, albeit a lower amount, which could be explained by the ability of activated skin-derived LCs to express CD8 after entering the LNs (31).

In contrast to intradermal immunization with naked plasmid DNA, administration of the plasmid via a gene gun induced rapid T cell responses in which Ln"dDCs, rather than LCs, were essential for CD8α T cell priming (32). Nevertheless, following gene gun immunization, large numbers of migratory skin DCs were observed in the LNs for ~2 wk postimmunization (33). This suggests that skin DCs have the potential to present Ag for a long period of time, whereas local signals induced in the skin greatly impact the ability of LCs to present Ag. Indeed, activation of CD8α T cells in gene gun immunization depends on cross-presentation, a function that cannot be executed by murine LCs. This might also explain why our attempts to facilitate the maturation and migration of skin DCs by coadministration of LPS and TNF-α failed to enhance, and rather reduced, immunogenicity. Because CD8α T cell priming in our system relies on direct presentation by LCs whose numbers increased gradually after immunization, the presence of LPS or TNF-α might induce a premature emigration of such LC precursors, thus preventing their proliferation and future use as APCs. Interestingly, a recent study using a cutaneous graft-versus-host disease model demonstrated that LCs were not capable of priming CD8α T cells or of inducing their migration into the skin. Indeed, LCs licensed CD8α T cells to become effector cells with the ability to induce skin injury (34). Nevertheless, although effector CD8α T cells are elicited by plasmid DNA, the absence of LCs reduces their numbers considerably, thus indicating that LCs prime CD8α T cells directly in our system.

Preventing the skin infiltration of circulating DC precursors by various approaches failed to reduce plasmid DNA–induced CD8α T cell responses, suggesting that newly immigrating langerin-expressing DCs are dispensable for CD8α T cell priming. Although it has never been reported, we cannot exclude that some LC precursors might enter the epidermis via a CCR2- or CCR6-independent pathway. Nevertheless, these results are in line with the reduced frequencies of CD8α T cells observed in DT-treated B6→Langerin-DTR chimeric mice, in which LCs that have differentiated from epidermal precursors are ablated and, hence, cannot prime CD8α T cells. However, a reduction in CD8α T cell responses also was observed in Langerin-DTR→B6 chimeras, in which Ln"dDCs, but not host-derived LCs, are ablated by DT. Notably, depletion of Ln"dDCs that were located in the skin at the time of immunization cannot explain this result, because we demonstrated that primary DCs failed to present Ag to CD8α T cells. This alleged inconsistency can be explained by a recent observation that the epidermis of chimeric mice contains increased numbers of LysM+ LC precursors that are originating from bone marrow cells (15). These LysM+ cells initially lack expression of langerin and Ep-CAM, but they upregulate both markers during their differentiation into LCs and, thus, are susceptible to DT-mediated killing. Moreover, upon DT treatment, the frequencies of LysM+ LC precursors increased even more in the skin of chimeric mice. In agreement with this finding, we detected bone marrow–derived LCs in chimeric mice following immunization (Fig. 4D, Supplemental Fig. 4), and ablation of epidermal LCs by DT facilitated the differentiation of bone marrow precursors (Supplemental Fig. 4). Thus, Langerin-DTR→B6 chimeras represent an artificial situation in which epidermal LCs consist of a considerable portion of bone marrow–derived LCs that are ablated by the DT treatment, leading to impaired CD8α T cell priming.

In conclusion, our study demonstrates that LCs have the ability to elicit cell-mediated immunity in vivo. We propose that newly differentiating LCs following immunization can potentiate CD8α T cell priming when initial Ag presentation by other skin DCs is insufficient. From a therapeutic point of view, intradermal plasmid DNA immunization represents an attractive vaccination strategy to harness LCs for developing cell-mediated immunity.

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