Directly Transfected Langerin\(^+\) Dermal Dendritic Cells Potentiate CD8\(^+\) T Cell Responses following Intradermal Plasmid DNA Immunization

Mazal Elnekave, Karina Furmanov, Itay Nudel, Moran Arizon, Björn E. Clausen and Avi-Hai Hovav

*J Immunol* published online 16 August 2010
http://www.jimmunol.org/content/early/2010/08/16/jimmunol.1001825

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2010/08/16/jimmunol.1001825.DC1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Directly Transfected Langerin+ Dermal Dendritic Cells Potentiate CD8+ T Cell Responses following Intradermal Plasmid DNA Immunization

Mazal Elnekave,* Karina Furmanov,* Itay Nudel,* Moran Arizon,* Björn E. Clausen,† and Avi-Hai Hovav*

Dendritic cells (DCs) play a critical role in CD8+ T cell priming following DNA vaccination. In contrast to other DNA injection routes or immunization with viral vectors, Ag presentation is delayed following needle injection of plasmid DNA into the skin. The contribution of various skin DC subsets to this process is not known. In this study, we show that dermal CD11c+ cells are the most important transgene-expressing cells following immunization. Using langerin- diphtheria toxin receptor mice we demonstrated that langerin+ dermal DCs (Ln+dDCs) were crucial for generating an optimal CD8+ T cell response. Blocking migration of skin cells to the lymph node (LN) ablated immunogenicity, suggesting that migration of dDC subsets to the LN is essential for generating immunity. This migration generated a weak Ag-presenting activity in vivo until day 5 postimmunization, which then increased dramatically. We further found that Ln+dDCs and dDCs were the only DC populations directly presenting Ag to CD8+ T cells ex vivo during the initial 8-d period postimmunization. This activity changed on the following days, when both skin DCs and LN-resident DCs were able to present Ag to CD8+ T cells. Taken together, our in vivo and ex vivo results suggest that activation of CD8+ T cells following intradermal plasmid DNA immunization depends on directly transfected Ln+dDCs and dDCs. Moreover, the type of DCs presenting Ag changed over time, with Ln+dDCs playing the major role in potentiating the initial CD8+ T cell response. The Journal of Immunology, 2010, 185: 000–000.
the time of peak immune response (between weeks 2 and 3 after DNA injection) diminished the magnitude of the CD8⁺ T cell reaction, suggesting that transfer of the Ag from the skin to the LN still takes place during this time (15). More recently, DCs were found to play a critical role in the presentation of plasmid DNA-encoded Ag, an activity that lasted for several weeks after immunization (16). These findings suggest that intradermal (i.d.) plasmid DNA immunization engages unique mechanisms mediated by skin DCs, and our understanding of this process is very limited. Based on these observations, this study was initiated to examine the role of the various skin DC subsets in plasmid DNA-induced CD8⁺ T cell immunity.

Materials and Methods

Abs and reagents

The Abs used in this study were directly coupled to FITC, PE, allophycocyanin, allophycocyanin-Cy7, Pacific Blue, or PerCP-Cy5.5. The following monoclonal Abs were used: anti-CD8α (53-6.7; BD Biosciences, San Jose, CA), anti-IFN-γ (XMG1.2; BD Biosciences), anti-CD103 (2E7; BioLegend, San Diego, CA), anti-CD11c (HL3; BD Biosciences), anti-epithelial cell adhesion molecule (Ep-CAM) (G8.8; BioLegend), anti-CD45.1 (A20; BioLegend), and anti-CD4 (GK1.5; BD Biosciences). Tetrameric H-2Dd complexes folded with the gp120 p18 epitope peptide CD45.1 (A20; BioLegend), and anti-CD4 (GK1.5; BD Biosciences).

Epithelial cell adhesion molecule (Ep-CAM) (G8.8; BioLegend), anti–interleukin-2 (IL-2) (20.11a; BD Biosciences), and anti–IFN-γ (53-6.7; BD Biosciences) Abs were directly coupled to FITC, PE, allophycocyanin-Cy7, Pacific Blue, or PerCP-Cy5.5. The Abs used in this study were directly coupled to FITC, PE, allophycocyanin, allophycocyanin-Cy7, Pacific Blue, or PerCP-Cy5.5. The following monoclonal Abs were used: anti-CD8α (53-6.7; BD Biosciences, San Jose, CA), anti-IFN-γ (XMG1.2; BD Biosciences), anti-CD103 (2E7; BioLegend, San Diego, CA), anti-CD11c (HL3; BD Biosciences), anti-epithelial cell adhesion molecule (Ep-CAM) (G8.8; BioLegend), anti-CD45.1 (A20; BioLegend), and anti-CD4 (GK1.5; BD Biosciences).

Tetrameric H-2Dd complexes folded with the gp120 p18 epitope peptide CD45.1 (A20; BioLegend), and anti-CD4 (GK1.5; BD Biosciences).

Tetramer analysis

Blood was collected from individual mice in RPMI 1640 medium containing 40 U of heparin per milliliter, and PBMCs were isolated using lympholyte-M (Cedarlane Laboratories, Burlington, NC). Cells were washed with PBS containing 2% FCS and stained for 15 min at room temperature (RT) with H-2Kd/SIINFEKL tetramers or H-2Dd/p18 tetramers. The cells were then stained with anti-CD8α Ab for an additional 15 min at RT and washed with PBS containing 2% FCS. Samples were collected on a LSR II instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Bioimaging of luciferase protein expression

Bioimaging of vector expressing firefly luciferase was done using the In Vivo Imaging System 110 (IVIS-110; Xenogen, Alameda, CA). Mice were anesthetized with ketamine/xylazine mix and injected i.p. with 100 μl of an isotonic salt solution containing 30 mg/ml r-luciferin (Xenogen). Fifteen minutes after luciferin injection, photonic emissions were measured using an IVIS-110 charge-coupled device camera. Luciferase quantification was done using the Living Image software to identify and measure regions of interest.

FITC painting

FITC (Sigma-Aldrich) was dissolved as a 10% (w/v) solution in DMSO (Sigma-Aldrich) and then diluted to 1% (v/v) FITC solution prepared in acetone and dibutyl phthalate (DBP; 1:1). Mice were painted on both sides of the ears 3 d before harvesting the draining LNs.

Pertussis toxin treatment

C57BL/6 and Lang-DTR mice were injected daily with 0.2 μg pertussis toxin (PTX; Sigma-Aldrich) in 20 μl PBS into the base of the ear during the 10 d of the experiment.

In vivo T cell proliferation assay

Splenocytes were obtained from OT-1 CD45.1⁺ mice, washed with PBS, and incubated with same volume of 10 μM CFSE in HBSS for 10 min at 37°C at a final concentration of 5 μM. Labeling was quenched by adding an excess of ice-cold RPMI 1640 complete medium and the cells were washed twice with PBS. CFSE-labeled splenocytes (2 × 10⁶) in 200 μl PBS were transferred into plasmid DNA-immunized B6 CD45.2⁺ mice by i.v. tail injection. Three to 5 d following the cell transfer, the mice were sacrificed and the draining LNs were harvested. The level of CFSE dilution was determined by flow cytometry using anti-CD8α and anti-CD45.1 Abs.

Ag presentation assays

The draining LNs were collected from immunized mice and treated with collagenase type II (1 mg/ml; Worthington Biochemical, Lakewood, NJ) and DNase I (1 mg/ml; Roche, Indianapolis, IN) solution in PBS plus 2% FCS for 20 min at 37°C in a shaker bath. Twenty microfilters of 0.5 M EDTA per 2 ml sample was added to the digested LN 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 according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched CD11c⁺ cells were stained with Abs against CD103, CD11c, Ep-CAM, and in some experiments also CD8α, and then they were subjected to sorting by flow cytometry (FACSria; BD Biosciences). OT-I CD8⁺ T cells or OT-II CD4⁺ T cells were purified by negative selection with the EasyStep mouse CD8⁺ or CD4⁺ T cell enrichment kit, respectively, according to the manufacturer’s instructions (StemCell Technologies, Vancouver, British Columbia, Canada). The purified T cells were incubated with same volume of 5 μM CFSE in HBSS 10 min at 37°C at a final concentration of 2.5 μM. Labeling was quenched by adding an excess of ice-cold RPMI 1640 complete medium, and cells were washed twice with culture medium. CFSE-labeled OT-1 CD8⁺ or OT-II CD4⁺ T cells (5 × 10⁶/well) were incubated with the different DC populations (3 × 10⁵/well) in 96-well U-bottom plates (Nunc, Roskilde, Denmark). The cultures were then incubated for 60 h, the supernatants were collected and stored at −80°C immediately, and the dilution of CFSE fluorescence in the cell fraction was analyzed using a LSR II instrument.

IFN-γ secretion

The level of IFN-γ in the supernatants was measured using an ELISA MAX mouse IFN-γ kit (BioLegend) according to the manufacturer’s in-
structructions. Cytokine levels were determined using standard curves of recombinant cytokines and are expressed as picograms per milliliter.

**Serum analysis**

Four weeks after immunization, blood was drawn from the mice and the sera were stored at -80˚C. Ninety-six-well plates (Nunc) were coated overnight at 4˚C with 1 μg OVA protein/well in 0.1 M bicarbonate buffer (pH 9). The plates were washed twice with PBS-0.02% Tween 20 and blocked with PBS-10% FCS (2 h at RT). Subsequently, mouse serum samples diluted serially in PBS were added to the wells for 3 h incubation at RT. This was followed by four washes in PBS-0.02% Tween 20 and the addition of anti-mouse peroxidase-conjugated IgG Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). After incubation for 2 h at RT, the plates were washed five times and 100 μl/well tetramethylbenzidine solution (SouthernBiotech, Birmingham, AL) was added for 5 min followed by the addition of 100 μl tetramethylbenzidine stop solution (SouthernBiotech). Absorption was read at 450 nm using an iMARK microplate reader (Bio-Rad, Hercules, CA).

**Immunofluorescence**

The ears of DNA-immunized and naive mice were excised, formalin fixed, and paraffin embedded. Tissue sections of 5 μM were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol (100, 96, and 80%). Ag retrieval was done for 2.45 min at 125˚C in citrate buffer (pH 6.0). After cooling, the slides were washed with PBS and blocked with CAS Block buffer (Invitrogen) for 20 min. Next, the blocking buffer was replaced with CAS Block buffer containing the primary Ab mouse anti-GFP (Invitrogen; 33-2600) at a 1/100 dilution. Slides remained in the humidified chamber and were incubated at 4˚C overnight. The slides were washed three times with PBS and a secondary Ab Cy2-conjugated goat anti-mouse IgG1k (Invitrogen) was applied for 30 min at RT at a 1/200 dilution. The samples were washed three times in PBS and counterstained with DAPI (MP Biomedicals, Solon, OH; 157574) solution (1 μg/ml PBS) for 30 min at RT in the dark. The samples were then washed twice in tap water and mounted with fluorescent mounting medium (Dako, Glostrup, Denmark; S3623). The confocal pictures were obtained using a Zeiss LSM 710 Axio observer.Z1 with an EC PlanN 10 × 0.3 lens and ×1.5 zoom.

**Statistical analysis**

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

**Results**

**Requirement for Ag persistence following i.d. immunization with plasmid DNA and adenovirus**

We have previously reported that optimal induction of a CD8+ T cell response following i.d. plasmid DNA immunization requires prolonged presentation (during 2 wk) of the Ag to CD8+ T cells (15). Prolonged Ag-presenting activity was also reported in the case of adenovirus (20), and thus we compared immune parameters between these two vectors. BALB/c mice were immunized in the ear pinna with 50 μg of VRC-Env plasmid or with 107 particles of replication-incompetent Ad-env. Three, 5, or 10 d following the immunization the injection sites were excised and the induction of Ag-specific CD8+ T cells was analyzed in the blood 2 wk postimmunization using MHC class I tetramers specific for the p18 immunodominant epitope of the HIV-1 envelope protein. As shown in Fig. 1A, removal of the injection site on days 3, 5, or 10 after immunization resulted in reduced CD8+ T cell responses in plasmid DNA-immunized mice (p < 0.001). Conversely, the adenovirus-induced CD8+ T cell response was not affected by the premature removal of the immunization site. We next tested whether this discrepancy could be explained by the amount of Ag produced in the mice by the two vectors. We immunized another cohort of mice with plasmid DNA or adenoviral vectors encoding the luciferase gene, that is, DNA-luc and Ad-luc, respectively. At various times postimmunization the amount of transgene expressed in the mice was monitored using an IVIS-110. Immunization with DNA-luc and Ad-luc resulted in a similar kinetics of luciferase expression, reaching its maximal level 1–2 d postimmunization and decreased afterward (Fig. 1B). Nevertheless, the magnitude of luciferase expression was considerably higher in DNA-immunized mice than in adenovirus-immunized mice (p < 0.01 on days 1 and 2). These results indicate that unlike adenovirus, induction of CD8+ T cells following plasmid DNA immunization depends remarkably on durable exposure to the Ag. It also suggests that this phenomenon is not due to an insufficient initial Ag load.

**Induction of CD8+ T cells requires direct transfection of CD11c+ cells**

We next sought to characterize the type of transgene-expressing cells responsible for the generation of CD8+ T cells following i.d. plasmid DNA immunization. B6 mice were immunized with DNA vaccine plasmids in which expression of the OVA Ag was driven by the ubiquitous CMV promoter (CMV-OVA), the DC-specific CD11c promoter (CD11c-OVA), or the keratinocyte-specific K14 promoter (K14-OVA). These CD11c-OVA and K14-OVA plasmids were previously shown to express high levels of the OVA transgene following gene gun immunization (21). To visualize the Ag-specific CD8+ T cell population in the peripheral blood samples, we used H-2Kb MHC class I tetramers specific to the SIINFEKL epitope of the OVA protein. As expected, ubiquitous transgene expression by the CMV promoter resulted in a potent induction of

![FIGURE 1](http://www.jimmunol.org/) The impact of premature removal of the Ag during immunization with plasmid DNA and adenovirus. A, B6 mice were immunized in the ear pinna with VRC-env (50 μg) or 1 × 107 particles of Ad-env, and 3, 5, or 10 d following immunization the ear pinna of the mice was excised. The amplitude of the p18-specific CD8+ T cell population in the peripheral blood of immunized mice as detected on day 14 postimmunization with an H-2Dβp18 tetramer is depicted. Data are shown as the percentages of CD8+ T cells and represent means of five mice per group ± SE. *p < 0.001 compared with the uncut group. B, B6 mice were immunized in the ear pinna with VRC-Luc (50 μg) or Ad-Luc (107 particles) and the levels of luciferase expression were measured. Data present the relative light units values expressed by each group of mice and represent the mean of five mice per group ± SE. RLU, relative light unit.
SIINFEKL-specific CD8+ T cells (Fig. 2A). Restricting OVA expression to only keratinocytes completely abolished the development of Ag-specific CD8+ T cells, whereas limiting the expression to CD11c+ cells resulted in an efficient induction of CD8+ T cells. As shown in Fig. 2A, at the time of peak immune response no significant difference was found in the frequencies of the SIINFEKL-specific CD8+ T cells between CMV-OVA– or CD11c-OVA–immunized mice. Note that in some experiments the levels of tetramer/CD8+ T cells were comparable in CMV-OVA– or CD11c-OVA–immunized mice for each time point tested (data not shown). The reliance of plasmid DNA immunogenicity on CD11c+ expressor cells raises the possibility that the plasmid itself may drain to the local LN after immunization, and therefore could be expressed by LN-resident DCs.

To test this hypothesis, we immunized mice with plasmid DNA and blocked DC migration by injecting the mice daily with PTX at the base of the ear pinna. This treatment led to a considerable reduction in the induction of Ag-specific CD8+ T cells (Fig. 2B). Similar results were obtained when we treated Lang-DTR mice with PTX and simultaneously depleted langerin+ DCs, which were reported to migrate to the LN even in the presence of PTX, although with low frequencies (22). Next, recent work reported that plasmid DNA-derived Ag is presented to CD8+ T cells for >70 d after i.d. administration (16). We thus examined the contribution of CD11c+ and non-CD11c+ cells to this process. B6 mice were immunized with CMV-OVA and CD11c-OVA, and 6 wk after immunization these mice were adoptively transferred with CFSE-labeled CD8+ T cells purified from OT-I mice. Three days later the LNs of both groups were collected and the CFSE dilution in the CD8+ OT-I cells was measured to determine their proliferative activity in vivo. Our analysis indicated that in both immunized groups the Ag was presented to the transferred CD8+ T cells (Fig. 2C). However, immunization with CMV-OVA resulted in higher percentages of dividing cells in comparison with CD11c-OVA–immunized mice (p < 0.01), suggesting that a stronger Ag-presenting activity took place in this group. Finally, we employed an immunofluorescence staining to determine the site of Ag expression in the skin. Mice were immunized with CMV-GFP plasmid and the ear pinna was excised 24 h later for analysis. As demonstrated in Fig. 3, the injection site was swollen due to the injection, and the penetration site was clearly detected in the tissue. Furthermore, expression of the Ag was localized in the dermis, whereas almost no expression was found in the epidermis. Taken together, these data suggest that directly transfected CD11c+ cells in the dermis play a major role in mediating the generation of CD8+ T cells by i.d. plasmid DNA immunization. Even so, expression of the Ag by non-CD11c cells may also contribute to the overall CD8+ T cell response.

**FIGURE 2.** Direct transduction of DCs by plasmid DNA is crucial for the induction of CD8+ T cells. A, Groups of B6 mice were immunized i.d. with CMV-OVA, CD11c-OVA, or K14-OVA plasmids (50 μg), and the induction of SIINFEKL–specific CD8+ T cells was examined in the peripheral blood of each group using H-2Kb/SIINFEKL tetramers. Data are shown as the percentage of CD8+ tetramer+ T cells and represent the means of four to five mice per group ± SE. B, B6 mice or langerin+ cell-depleted Lang-DTR mice were immunized i.d. with CMV-OVA. The mice were treated daily with i.d. injection of PTX to inhibit cell migration. Seven days after the immunization the magnitude of CD8+ T cells was examined in the peripheral blood by using H-2Kb/SIINFEKL tetramers. Data are shown as the percentage of tetramer+CD8+ T cells and represent the means of 4 mice per group ± SE. *p < 0.001 compared with untreated B6 mice. C, Six weeks after immunization, CMV-OVA– or CD11c-OVA–immunized mice were adoptively transferred with CFSE-labeled splenocytes purified from OT-I mice. Sixty hours later, the cervical LNs were collected and the CFSE dilution was assessed by flow cytometry. Results are shown as representative flow plots gating on dividing CD8+ lymphocytes; numbers indicate the percentages of dividing cells and represent the mean of four mice per group ± SE.
Limited Ag-presenting activity in vivo during the first 5 d postimmunization

In our previous study, we demonstrated ex vivo that considerable Ag presentation levels can be first detected only 10 d after i.d. plasmid DNA immunization (15). To better characterize this process we examined the kinetics of Ag presentation in vivo by two approaches. First, we immunized mice with the CMV-OVA plasmid, and on various days after immunization we adoptively transferred the mice with CFSE-labeled OT-I splenocytes. Three days after transfer the draining LNs were collected and the CFSE dilution was monitored by flow cytometry to calculate the intensity of T cell proliferation resulting from Ag presentation. As demonstrated in Fig. 5A, presentation of the Ag to T cells was low (∼37% of the transferred OT-I cells) during days 2–5 after immunization. Ag presentation increased gradually thereafter, reaching a level of ∼57% between days 4 and 7 and ∼81% on days 8–11. On the second approach we transferred CFSE-labeled OT-I splenocytes into a cohort of naive B6 mice, and 24 h later we immunized them with CMV-OVA plasmid. LNs were collected at various time points after immunization to measure the level of Ag presentation that had accumulated in the mice during this time. We found that the transferred OT-I CD8+ T cells received low stimulation 2 d (∼2%) and 4 d (∼17%) after immunization (Fig. 5B). A considerable presentation of Ag was detected in the immunized mice only 6 d after immunization (∼66%). We next examined the development of Ag-specific CD8+ T cells in the peripheral blood (Fig. 5C). Low frequencies of Ag-specific CD8+ T cells were first detected 5 d after immunization (p < 0.01) and rapidly increased by days 8, 13, and 21. These results suggest that a low Ag-presenting activity is taking place on the first days after immunization. This presentation capacity, however, increased gradually around day 5 and gave rise to a rapid expansion of Ag-specific CD8+ T cells.

Skin-derived DCs directly present Ag to CD8+ T cells

As skin-derived DCs were found to contribute to the induction of CD8+ T cell response following i.d. plasmid DNA immunization, we next examined their involvement in the presentation of the Ag to T cells. Based on our in vivo results, Ag-presenting activity can hardly be detected before day 5. Thus, we painted the CMV-OVA-immunized mice with the CMV-OVA plasmid, and at 24 h the ear pinna was removed and subjected to immunofluorescence analysis. Confocal microscopy of the ear pinna is shown with a 5-μm-thick section using a 10× objective and 1.5× zoom (blue, nuclei stained with the DNA-intercalating dye DAPI; green, anti-GFP Ab). AC, auricular cartilage; DR, dermis; Ep, epidermis.
Instead, we examined T cell activation by evaluating secretion of IFN-γ that occurred following TCR:peptide-MHC ligation. Considerable levels of IFN-γ were found following incubation of the CD8+ T cells with FITC+ DCs (a fraction that contains migratory skin DCs) (Fig. 6B). In contrast, a very low level of IFN-γ was detected in the presence of the FITC^2 DC fraction, consisting of LN-resident and skin DCs that have migrated prior to day 5. Incubation of the CD8^+ T cells with non-DCs failed to activate the cells at all. Importantly, incubating the T cells with DC subsets from nonimmunized mice, or with DC from non–OV A-immunized mice, failed to mediate IFN-γ (data not shown), indicating that this cytokine secretion is Ag-dependent. Next, because B cells were reported to contribute to CD8^+ T cell priming following gene gun immunization (21), we examined their role in naked plasmid DNA immunization in vivo. B cell-deficient mice (μMT mice) were immunized i.d. with CMV-OV A, and the frequencies of SIINFEKL-specific CD8^+ T cells were measured in the blood. As demonstrated in Fig. 4C, the absence of B cells did not affect the generation of Ag-specific CD8^+ T cells as compared with C57BL/6 mice. These data suggest that Ag presentation to CD8^+ T cells is mediated by CD11c^+ DCs, and, more importantly, skin-derived DCs can directly present Ag to T cells.

Time-dependent presentation of the Ag to CD8^+ T cells by skin DCs and LN-resident DCs

Because our data demonstrated that skin DCs present plasmid DNA-driven Ag to CD8^+ T cells, we next sought to examine the contribution of each DC subset to this process. We immunized mice i.d. with CMV-OV A plasmid and on day 5 postimmunization the injection sites were painted with FITC/DBP solution and 3 d later the LNs were collected and CD11c^+ cells were enriched as described earlier. We then FACs sorted the CD11c^+ cell populations based on their FITC staining and expression of surface molecules as follows. To identify the various skin DC subsets in the LN, the FITC^+ population was separated according to the expression of CD103 (Ln+dDCs), and the CD103^2 cells were further segregated into Ep-CAM^2 (dDCs) and Ep-CAM^+ (LCs) (Fig. 7A).

The different DC subsets were then incubated with OT-I CD8^+ T cells for 60 h, and the supernatants were collected from the cultures to measure IFN-γ secretion by ELISA. As shown in Fig. 7B, secretion of IFN-γ was found when the T cells were cocultured with dDCs and Ln^+dDCs. No secretion was measured in the

FIGURE 5. Kinetics of in vivo Ag presentation following i.d. plasmid DNA immunization. A, B6 mice were immunized i.d. with CMV-OV A plasmid (50 μg), and on day 2, 4, or 8 postimmunization the mice were adoptively transferred i.v. with 2 × 10^6 CFSE-labeled OT-I splenocytes. Three days later the LNs were harvested from the immunized mice to analyze the proliferation of the transferred CD8^+ T cells by flow cytometry. B, Twenty-four hours before the immunization with CMV-OV A plasmid, B6 mice were transferred with 2 × 10^6 CFSE-labeled OT-I splenocytes. LNs were collected from the mice 2, 4, or 6 d after the immunization to quantify the expansion of the transferred OT-I CD8^+ T cells. Results are shown as representative flow plots gated on dividing CD8^+ lymphocytes; numbers indicate the percentages of dividing cells. C, A large cohort of B6 mice was immunized i.d. with CMV-OV A plasmid (50 μg), and the presence of SIINFEKL-specific CD8^+ T cells was monitored in the blood at the indicated time points. Data are presented as tetramer^CD8^+ T cells and represent the mean of per mice per time point ± SE.

FIGURE 6. Skin DCs directly present Ag to CD8^+ T cells. A, Five days after immunization with CMV-OV A, the ear pinna of B6 mice was painted with FITC/DBP solution, and 3 d later the draining LNs were pooled from five mice. CD11c^+ cells were enriched and then sorted into non-CD11c, FITC^− CD11c^+ , and FITC^+CD11c^+ subpopulations. B, The various CD11c^+ cell subsets were immediately incubated with purified OT-I CD8^+ T cells and supernatants were collected 60 h later to quantify the concentration of INF-γ by ELISA. Data are representative of two independent experiments. C, B6 and μMT mice were immunized i.d. with CMV-OV A. The percentages of SIINFEKL-specific CD8^+ T cells in the peripheral blood were examined every week with H-2Kb/SIINFEKL tetramers and represent the means of five mice per group ± SE.
presence of LCs. Interestingly, low levels of IFN-γ were found also in wells containing FITC^2CD11c^+ cells, a population that consists of LN-resident and skin DCs that have migrated to the LN prior to day 5. We next examined the capacity of Ln^dDCs to cross-present plasmid DNA-encoded Ag, as previously suggested during HSV-1 infection. For this purpose we incubated the purified DC subsets with CD4^+ T cells isolated from OT-II mice and measured secretion of IFN-γ into the supernatant. LN-resident DCs and dDCs were included in the assay since the LN-resident DCs have well-documented cross-presenting capacity (24), whereas dDCs lack this ability (12, 25). Our results indicated that whereas LN-resident and dDCs were able to efficiently prime CD4^+ T cells, Ln^dDCs failed to do so (Fig. 7C). Because Ag-presenting activity proceeds in the mice after days 5–8 (15), we examined the capacity of the noted DC subsets to present Ag to CD8^+ T cells on day 12 postimmunization. We used an alternative sorting strategy, in which LNs were collected from the immunized mice, enriched for CD11c^+ cells, and stained with anti-CD8^a, CD11c, CD103, and Ep-CAM Abs. LN^-resident DCs were identified by the expression of CD11c and CD8, whereas the CD11c^-CD8^2 fraction was separated based on CD103 expression (Ln^dDCs), and further according to Ep-CAM expression; that is, CD103^-Ep-CAM^- (dDCs) and CD103^-Ep-CAM^+ (LCs) as before (Fig. 8A). In contrast to the results obtained on day 8, all skin DC subsets were able to induce IFN-γ secretion by OT-I CD8^+ T cells. Moreover, LN-resident DCs were also capable of activating the T cells in higher levels than the skin DC subsets. Taken together, these findings suggest that the contribution of the various DC subsets to Ag presentation change over time. While Ln^-dDCs and dDCs play the primary role in stimulating Ag-specific CD8^+ T cells during the first week postimmunization, LN-resident DCs have a higher presenting capacity later on.

Discussion

In this study, we demonstrated that following i.d. plasmid DNA immunization, directly transfected skin DCs are the primary mediators of CD8^+ T cell priming. Within the skin DC population, Ln^-dDCs and dDCs were the main subsets involved in direct presentation of the Ag to CD8^+ T cells. The impact of ablation Ln^-dDCs on the generation of CD8^+ T cells was dramatic during the first week of immunization. This, however, changed by the second week after the immunization, a time during which LN-resident DCs were the major APC subset in the draining LN. These results are consistent with a recent study that reported a change in the composition of DC populations mediating Ag presentation following infection with HSV-1 (12). Nevertheless, in contrast to the HSV-1 system where initial presentation by LN-resident DCs was followed by presentation by skin DCs, we identified an important role for skin DCs (particularly Ln^-dDCs) in the first steps of initiating CD8^+ T cell responses following i.d. plasmid DNA immunization.

FIGURE 7. The contribution of the various skin DC subsets to Ag-presenting activity. A, B6 mice were immunized in the ear pinna with CMV-OVA and 5 d postimmunization the ear pinna was painted with FITC/DBP solution. Three days later the LNs were collected from 10 mice, pooled, and enriched for CD11c^+ cells. The cells were then sorted into different DC subsets based on their FITC labeling and expression of the CD103 and Ep-CAM molecules as demonstrated. B, The various DC subsets were incubated with purified OT-I CD8^+ or OT-II CD4^+ T cells, and supernatants were collected 60 h later to measure INF-γ secretion by ELISA. *p < 0.05; **p < 0.001 compared with the T cells only.

FIGURE 8. Ag presentation during the second week postimmunization. A, B6 mice were immunized in the ear pinna with CMV-OVA, and 12 d after the immunization the LNs were collected from 10 mice, pooled, and enriched for CD11c^+ cells. The enriched cells were then sorted into different DC subsets based on their expression of the CD8, CD103, and Ep-CAM molecules as demonstrated. B, The various DC subsets were incubated with purified OT-I CD8^+ T cells, and supernatants were collected 60 h later to measure INF-γ secretion by ELISA. *p < 0.001 compared with the OT-I T cells.
The potent capacity of Ln\textsuperscript{d}DCs to present Ag ex vivo does not always correlate with their ability to prime CD8\textsuperscript{T} cells in vivo. For example, ablation of Ln\textsuperscript{d}DCs during immunization with lentivectors (a system in which T cell priming is mediated by skin DCs) had no effect on lentivector-induced immunity, despite the superb capability of Ln\textsuperscript{d}DCs to present lentiviral-derived Ags (11). Furthermore, the contribution of Ln\textsuperscript{d}DCs to HSV-1–specific immunity seems to be minimal, as LN-resident CD8\textsuperscript{T} DCs are thought to mediate this process (9, 26). On the other hand, Ln\textsuperscript{d}DCs were shown to be involved in the early priming of CD8\textsuperscript{T} T cells following infection with Leishmania major (27), as well as during contact hypersensitivity responses (28). Collectively, these observations may indicate that, as a relatively small DC population of the skin, the contribution of Ln\textsuperscript{d}DCs to the overall Ag-presenting activity might be masked by the activity of larger DC subsets, such as dDCs and LN-resident DCs in the context of the lentiviral system, as we have recently shown (11). In our system, Ag-specific CD8\textsuperscript{T} T cells were detected as soon as 5 d after immunization, a time during which the absence of Ln\textsuperscript{d}DCs resulted in a diminished tetramer response. We thus speculate that Ln\textsuperscript{d}DCs play an important role in presenting Ag during the first 5 d, an activity that could increase the Ag-specific T cell pool for a further expansion by the larger DC subsets. It has been shown that unmethylated CpG sequences directly induce the activation and maturation of plasmacytoid DCs (pDCs) (29), suggesting a potential role for pDCs in plasmid DNA-induced immunity. However, pDCs are excluded from the skin, whereas our data suggested that immune induction depends on migration of skin DCs to the LNs (30). Thus, pDCs may have limited contribution during the first week of immunization, and this, however, may change later on when skin DCs already reach the LNs and spread the Ag or plasmid.

The dermis-restricted expression of the Ag correlates well with the lack of immunogenicity seen in K14-OVA–immunized mice. It is not yet clear why the CMV promoter-driven Ag is expressed mainly in the dermis, and we speculate that it is due to the injection technique we employed. In any case, such an expression pattern can explain the important role of dDCs and Ln\textsuperscript{d}DCs in mediating plasmid DNA-induced immunity. Recent work demonstrated the capability of Ln\textsuperscript{d}DCs to cross-present exogenous Ag following HSV infection (12). Furthermore, these cells were found to be developmentally related to CD8\textsuperscript{T} DCs, which are known for their cross-presenting capacity (24). Consistently, in our system the late presentation of the Ag by CD8\textsuperscript{T} DCs (LN-resident DCs) to CD4\textsuperscript{+} and CD8\textsuperscript{T} T cells is probably due to their capacity to present and cross-present exogenous Ag derived from migratory skin DCs, respectively. Ln\textsuperscript{d}DCs, on the other hand, efficiently presented Ag on MHC class I molecules, whereas a weak MHC class II presentation was detected. This suggests that Ln\textsuperscript{d}DCs were directly transfected with the plasmid DNA and that these cells were still migrating from the injection site to the LN between days 5 and 8. In agreement, during this time dDCs, which have been reported to lack cross-presenting capacity (12, 25), also migrated to the LN and presented Ag to CD8\textsuperscript{T} T cells via a direct presentation pathway. This may explain the reduced T cell response found when the injection site was excised up to 10–15 d after the immunization (15), since directly transfected DCs are still migrating during this time. It is also in agreement with the comparable CD8\textsuperscript{T} T cells frequencies found in CMV-OVA– and CD11c-OVA–immunized mice. Nevertheless, we cannot exclude the possibility that some of the presentation by Ln\textsuperscript{d}DCs was mediated by cross-presentation, since the lack of presentation to CD4\textsuperscript{+} T cells could be explained by a preferential class I presentation of exogenous Ag by this DC subset.

Although a limited Ag-presenting activity was detected prior to day 5 elicited low CD8\textsuperscript{T} T cell activation, the main Ag presentation and T cell activation took place only during the second week following immunization. It is still unclear why plasmid DNA-induced CD8\textsuperscript{T} cell responses rely on such slow kinetics of DC migration. A possible explanation for this phenomenon is that some of the directly transfected skin DCs may undergo apoptosis due to the immunization and thus limit the number of DCs capable of presenting Ag in the LN. In this regard, it has been shown that by inhibiting apoptosis of DCs during i.d. DNA vaccination it is possible to prolong their life span and enhance immunogenicity (31). It is also possible that the low percentages of CD8\textsuperscript{T} T cells elicited early after the immunization protect DCs from apoptosis and enhance their function, promoting by this activity the subsequent development of CD8\textsuperscript{T} and CD4\textsuperscript{T} T cell responses (32). Alternatively, the slow generation of CD8\textsuperscript{T} T cells could be explained by the lack of sufficient CD4 help, a process that is crucial for CD8\textsuperscript{T} T cell priming following plasmid DNA immunization (16). In such a scenario, directly transfected skin DCs migrating before day 5 may preferentially present Ag via MHC class I, which could be inefficient to prime potent CD8\textsuperscript{T} T cell response due to the absence of activated CD4\textsuperscript{T} T cells. This could change on the following days by the arrival of new DCs that may have captured exogenous Ag in the skin or in the LN and present the Ag on MHC class II as we demonstrated.

The reliance of plasmid DNA immunogenicity on DCs as transgene-expressing cells is surprising. A previous study using the exact same plasmids (K14-OVA and CD11c-OVA) for gene gun immunization found that expression of the transgene by keratinocytes, and not by DCs, is crucial for immune induction (21). This suggests that the administration route of plasmid DNA dictates the mechanisms engaged by the immune system for generating immunity. Gene gun-mediated immunity differs from that induced by i.d. immunization in several ways. For example, removal of the injection site 3 d after gene gun immunization did not impair cell-mediated immunity (33), whereas during i.d. administration this completely abolished immunity (15). Additionally, maximal T cell response can be found 5 d after gene gun immunization (34), whereas in i.d. immunization the response is just initiated at this time. Furthermore, in vivo ablation of Ln\textsuperscript{d}DCs during gene gun immunization did not result in a reduced T cell response, quite the opposite from the important role of this DC subset in our system. It is possible that these differences are due to the relatively extensive local tissue damage induced by gene gun as compared with needle immunization. As a result, a local inflammatory response might facilitate Ag presentation and immune induction. It is also plausible that in gene gun immunization soluble unprocessed Ag could drain to the LN (35), and thus skin DCs might be dispensable for immune induction.

In conclusion, in this study, we presented the unique mechanisms activated by plasmid DNA for generating immunity following i.d. immunization. Understanding the immunological pathways involved in DNA vaccination will be crucial for improving the immunogenicity of this modality for future use in clinical trials.

Acknowledgments
We thank Dr. Dan Lehmann, Guy Brachya, Elad Horowitz, and Alexander Rouvinski for technical assistance. We also thank Maytal Bivas-Benita and Geoffrey O. Gillard for critical reading of this manuscript.

Disclosures
The authors have no financial conflicts of interest.

References


