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The Role of Skin-Derived Dendritic Cells in CD8+ T Cell Priming Following Immunization with Lentivectors

Karina Furmanov,* Mazal Elnekave,** Dan Lehmann,* Bjorn E. Clausen,† Darrell N. Kotton,‡ and Avi-Hai Hovav*

Although skin dendritic cells (DCs) have been shown to directly present Ag to CD8+ T cells after intradermal immunization with lentivectors, the contribution of the different skin DC subsets to this process remains unclear. Using langerin-diphtheria toxin receptor transgenic mice we demonstrated that ablation of langerhans cells and langerin-expressing positive dermal DCs (Ln+dDCs) did not interfere with the generation of CD8+ T cells by lentiviral vectors. Consistent with these findings, the absence of langerhans cells and LndDCs did not hamper the presentation level of lentiviral-derived Ag by skin DCs in vitro. We further demonstrated that only dDCs and LndDCs were capable of presenting Ag, however, the number of dDCs migrating to the draining lymph nodes was 6-fold higher than that of LndDCs. To study how the duration of DC migration influences CD8+ T cell responses, we analyzed the kinetics of Ag expression at the injection site and manipulated DC migration by excising the injected skin at various times after immunization. A low level of Ag expression was seen 1 wk after the immunization; peaked during week 2, and was considerably cleared by week 3 via a perforin-dependent fas-independent mechanism. Removing the injection site 3 or 5 d, but not 10 d, after the immunization, resulted in a reduced CD8+ T cell response. These findings suggest that dDCs are the main APCs active after intradermal lentiviral-mediated immunization, and migration of dDCs in the initial 10-d period post-immunization is required for optimal CD8+ T cell induction. The Journal of Immunology, 2010, 184: 4889–4897.

CD8+ T cells play an important role in protective immunity against infection with intracellular pathogens. Besides their role in control of primary infections, CD8+ T cells have the capacity to differentiate into heterogeneous long-lived memory cell subsets that provide enhanced protection against secondary infections (1). Because viruses generate robust T cell responses, they represent attractive vaccine vectors for the elicitation of CD8+ T cells (2). Hence, several recombinant viral systems have been tested as putative vaccine vectors, including adenoviral vectors, pox-based vectors, and lentiviral vectors. It has been suggested that the mechanism of immune induction, and thus the quality of the immune responses, depends to some extent on the unique features of each viral vector (3). Therefore, studying the mechanisms used by viral vectors to generate cell-mediated immunity is critical for the development of improved “T cell vaccines.”

Lentivirus-based vectors have been shown to generate robust Ag-specific CD8+ T cell responses after immunization (4–6). This, in part, could be due to the ability of lentivectors to efficiently transduce DCs without compromising their Ag processing and presenting capabilities (7, 8). Lentivectors have major advantages over other recombinant viral vectors, because most hosts lack of pre-existing lentivector-specific immunity, and replication incompetent lentivectors typically elicit only Ag-focused immunity due to expression only of the engineered heterologous gene rather than the expression of endogenous viral genes in the vector (6). In addition, recent studies have shown the elicitation of protective antiviral and anticancer immunity by lentivectors, suggesting that recombinant lentivectors are promising vaccine candidates (9–12).

After injection of lentivector into the skin, DCs migrate from the skin to draining lymph nodes (LNs) and directly present Ag to CD8+ T cells (13). This mechanism, however, is not consistently used for other viruses such as vaccinia and HSV, because those viruses appear to stimulate skin-derived DCs to transport Ag to LNs where the Ag is presented to CD8+ T cells by LN-resident DCs, rather than the skin-derived DCs (14, 15). A possible explanation for these different mechanisms is that lentivectors are not cytolytic and are not altering the Ag-presenting function of the DCs. On the other hand, HSV has a cytolytic effect on DCs that might impair the capability of infected skin-derived DCs to present Ag and thus this function is mediated by LN-resident DCs. Skin DCs can be divided into at least three subsets: langerhans cells (LCs), langerin-positive dermal DCs (LndDCs), and langerin-negative dDCs (16). The precise role of each DC subset in skin infection or immunization remains controversial and appears to depend on the nature of the infectious agent or vector used. There is some evidence that LCs are involved in immunity generated by contact sensitization (17) or tolerance toward a neo-self Ag (18, 19), but not during infection with HSV, influenza, or vaccinia. LndDCs, in contrast, have been found to contribute to immune responses generated after infection with Leishmania major (20), gene gun immunization (21), or contact sensitization (22). More recently, it has been shown that LndDCs present HSV-1 Ags to CD8+ T cells by cross-priming (23). Although it has been

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Abbreviations used in this paper: DBP, dibuthyl phthalate; DC, dendritic cell; dDC, dermal DC; DT, diphtheria toxin; DTR, diphtheria toxin receptor; LC, langerhans cell; LN, lymph node; LndDC, langerin-expressing positive dDC; RLU, relative light unit; RT, room temperature.

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demonstrated that skin-derived DCs directly present Ag to CD8+ T cells after intradermal immunization with lentivectors (13), it is unknown which subsets of skin DCs mediate this process. This study was initiated to investigate the contribution of langerin-expressing skin DCs to CD8+ T cell priming by lentivectors.

Materials and Methods

Abs and reagents

The Abs used in this study were directly coupled to FITC, PE, APC, APC-Cy7, or PerCP-Cy5.5. The following mAbs were used: anti-CD62L (MEL-14; eBioscience, San Diego, CA), anti-CD8α (53-6.7; BD Biosciences, San Jose, CA), anti-IFN-γ (XMG1.2; BD Biosciences), anti-CD127 (A7R34; eBioscience), anti-CD103 (2E7; BioLegends, San Diego, CA), anti-CD11c (HL3; BD Biosciences), and anti–Ep-CAM (G8.8; Biologens). SIINFEKL H-2Kb tetramers were purchased from Beckman Coulter (San Jose, CA). CFSE was purchased from Molecular Probes (Invitrogen, San Diego, CA).

Construction and production of dual promoter lentivectors

Lentiviral constructs used the third generation, self-inactivating, replication-incompetent lentiviral backbone vector (pHAGE, gift of Dr. Richard C. Mulligan, Harvard Medical School, Boston, MA), modified for dual promoter-dual transgene expression as we previously published (24). A lentiviral construct for simultaneous dual transgene expression of OVA and eGFP cDNAs (CMV-OVA-UBC-eGFP; hereafter, Lv-OVA; Supplemental Fig. 1) was generated by 5′ NotI and 3′ BamHI ligation of the OVA cDNA into gene expression position 1 (downstream of the CMV promoter), and ligation of the eGFP (Clontech, Mountain View, CA) cDNA into gene expression position 2 (downstream of the human UBC promoter) (24). An additional lentiviral construct for dual transgene expression of both luciferase and eGFP cDNAs (CMV-Luc-UBC-eGFP; hereafter, Lv-Luc; Supplemental Fig. 1) was generated by similar 5′ NotI/3′ BamHI ligation of the cDNA encoding firefly luciferase into gene expression position 1. Vesicular stomatitis virus gp pseudotyped lentivirus was generated by triple transfection of 293T cells with the lentiviral backbone construct together with two helper plasmids encoding the viral genes Gag-Pol-Tat-Rev and VSVG. Cell supernatants containing virus were concentrated by centrifugation (90 min; 48,960 × g). Titers of eGFP-expressing lentiviruses were calculated as “293-transducing units” per milliliters based on flow cytometry of infected 293 cells, and calculated titer of 5 × 10^5 TU/ml were used in all experiments.

Mice and immunizations

Six- to 12-wk-old transgenic knock-in mice expressing the human diphtheria toxin receptor (DTR) under transcriptional control of the endogenous Langerin/CD207 promoter (Lang-DTR) were bred in our facility and maintained under specific pathogen-free conditions. The identity of mice used for all experiments was confirmed by genotyping using the following PCR primers: F-5′-GCCACCATGAAGCTGCTGCCG-3′ and R-5′-ATA-GTTTACGGGGCGCTTTACTTGTACAG-3′. In addition, C57BL/6 (B6), C57BL/6-Pfptm1Sdz (Perforin−), MRL/MpJ-Fas−/− (Fas−), OT-I, and OT-II mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used at the age of 6-8 wk. All animal work was approved by the Hebrew University Institutional Animal Care and Ethic Committee. Mice were anesthetized with ketamine/xylazine mix and a 31-gauge needle was used to inject Lv-OVA or Lv-Luc (5 × 10^6 TU suspended in 40 µl media) into each ear pinna, intradermally. Removal of the ear pinna was performed using sterile scissors after the mice were anesthetized with ketamine/xylazine mix.

Ablation of langerin-expressing cells in vivo

Mice received 1 µg diphtheria toxin (DT) in 100 µl PBS (Sigma-Aldrich, St. Louis, MO) by i.p. injection. For LC depletion mice were treated with DT 5 d before the immunization with Lv-OVA and 21 d subsequent to the first DT injection (after the time of the peak tetramer-specific CD8+ T cell responses) to maintain lower number of LCs in the epidermis. To deplete LCs and LndDCs, mice were treated with DT 7 d and 7 h before the injection of Lv-OVA and then every 7 d to maintain the ablation of the noted cells for the duration of the study. Similar administration of DT into wild-type B6 mice did not influence the magnitude and kinetics of the examined immune responses. Notably, initial experiments using administration of DT even more frequently (every 5 d, for example) resulted in considerable weight loss and rapid death of the immunized mice; thus the 7-d dosing frequency was selected for further study.

Tetramer and phenotypic analysis of the T cells

Blood was collected from individual mice in RPMI 1640 medium containing 40 U heparin per milliliter, and PBMCs were isolated using Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada). Cells were washed with PBS containing 2% FCS and stained for 15 min at room temperature (RT) with H-2Kb/SIINFEKL tetramers. The cells were then stained with anti-CD8α and anti-CD62L Abs for an additional 15 min at RT, washed with PBS containing 2% FCS. In certain experiments, single-cell suspensions were prepared from spleens of individual animals in PBS and 2% FCS and the staining was performed as described previously, with the exception of an additional staining with anti-CD127 Ab. Samples were collected on a LSR II instrument (BD Biosciences) and analyzed using the FlowJo software (Tree Star, Ashland, OR).

Splenocyte stimulation and intracellular cytokine staining

Splenocytes were harvested from individual mice 5 wk after the immunization, and RBCs were lysed by using ACK buffer. The cells were then washed with PBS and 2% FCS, counted, and resuspended (4 × 10^6 cells per tube) in RPMI 1640 medium (Sigma-Aldrich), supplemented with 10% FCS, 0.2% NaHCO3, 20 U/ml penicillin, 20 µg/ml streptomycin. The cells were incubated with Golgi Plug (2 µl/ml), anti-CD28 (2 µg/ml), anti-CD49d (2 µg/ml), and SIINFEKL peptide (12 µg/ml). Unstimulated cells were incubated with all the above reagents, except for the peptide. The cells were incubated at 37°C for 6 h and then washed with PBS and 2% FCS, stained with anti-CD8α Abs for 15 min. Permeabilization was performed overnight with Cytofix/Cytoperm solution (BD Biosciences). Cells were washed 1× Perm/Wash buffer (BD Biosciences) and then stained with Abs specific to the IFN-γ cytokine. After an additional washing step with 1× Perm/Wash buffer, the samples were collected on a LSR II instrument and analyzed using FlowJo software.

Bioimaging of luciferase protein expression

Bioimaging of in vivo firefly luciferase gene expression was performed using the Roper Chemiluminescence Imaging System model LN/CCD-1300EB (Roper Scientific, PrincetonInstrument, Trenton, NJ). Mice were anesthetized with ketamine/xylazine mix and injected i.p. with 500 µl isotonic salt solution containing 30 mg/ml D-Luciferin (Xenogen, Alameda, CA). Ten minutes after luciferin injection, photon emissions were measured and raw data were analyzed using the MetaView software to assess photon flux in regions of interest in each mouse.

ELISA

Four weeks after immunization, blood was drawn from the mice by retro-orbital bleeding, and the sera were saved at −80°C. The 96-well plates (Nunc, Naperville, IL) were coated overnight at 4°C with 1 µg OVA protein/well in bicarbonate buffer. The OVA coating solution 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 peroxidases-conjugated IgG, IgG1, or IgG2C Abs (Jackson ImmunoResearch Laboratories, West Grove, PA). After incubation 2 h at RT, plates were washed five times and 100 µl/well of TMB solution (Southern Biotechnology Associates, Birmingham, AL) was added for 5 min, followed by the addition of 100 µl TMB stop solution (Southern Biotechnology Associates). Absorption was read at 450 nm using the iMARK microplate reader (Bio-Rad, Hercules, CA). To measure IFN-γ levels in the supernatant of T-cell DC cultures, we used the ELISA MAX mouse IFN-γ kit (Biolegend), according to the manufacturer’s instructions.

Painting with FITC

FITC was dissolved as a 10% (w/v) stock solution in DMSO (Sigma-Aldrich) and working solutions of 1% (v/v) FITC were prepared in acetone and dibuthyl phthalate (DBP) (1:1). Mice were painted on both sides of the ears with 25 µl 1% FITC solution immediately following immunization with Lv-OVA. In some experiments the ear pinna was treated with acetone/DBP (1:1) solution.

Ag presentation assays

Draining LNs were collected from immunized mice 2 or 3 d post-immunization and treated with collagenase type II (1 mg/ml, Worthington Biochemical, Lakewood, NJ) and DNase I (1 mg/ml, Gibco, Carlsbad, CA). Cells were incubated in RPMI 1640 medium containing 2% FCS for 20 min at 37°C in a shaker bath. A total of 20 µl EDTA 0.5 M was added to the digested LNs and the incubation allowed to proceed for an additional 10 min. The cells were
then washed and filtered. CD11c<sup>+</sup> cells were obtained from the digested LNs by positive isolation using MACS Microbeads, according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). The enriched CD11c<sup>+</sup> cells were stained with Abs against CD103, CD11c, and Ep-CAM and then subjected to sorting by flow cytometry (FACSAria; BD Biosciences). OT-I CD8<sup>+</sup> T cells were purified by negative selection with Easystep mouse CD8<sup>+</sup> T cell enrichment kit according to the manufacturer’s instructions (StemCell Technologies, Vancouver, British Columbia, Canada). The purified OT-I CD8<sup>+</sup> T cells were incubated with same volume of 5 mM CFSE in HBSS 10 min at 37˚C, for a final concentration of 2.5 μM. Labeling was quenched by adding excess of ice-cold RPMI 1640 complete medium and cells were washed twice with culture medium. CFSE-labeled OT-I CD8<sup>+</sup> T cells (5 x 10<sup>5</sup>/well) were incubated with each indicated DC population (3 x 10<sup>5</sup>/well) in 96-well U-Plates (Nunc). The cells were then incubated for 60 h and the dilution of CFSE fluorescence was analyzed by the LSR II instrument.

**Processing of skin tissue for flow cytometry analysis**

The ear pinna was excised, washed with 70% ethanol for 1 min, and then with PBS. The skin was separated into two halves, incubated with dispase/collagenase solution (1.2 U/ml) for 30 min at 37˚C, and the epidermis and dermis sheets were separated and minced to little pieces. After 30 min of incubation with collagenase/DNase solution (1 mg/ml) at 37˚C, the tissue fragments were washed, centrifuged, and filtered. Staining with Abs was performed as described previously.

**Statistical analysis**

Data were expressed as means ± SEMs (SE). Statistical tests were performed using one-way ANOVA and the Student t test, and a p value < 0.05 was considered significant.

**Results**

**Ablation of LCs does not alter CD8<sup>+</sup> T cell responses**

To analyze the role of LCs in immune induction after immunization with lentivectors, we used the Lang-DTR mouse model that allows conditional ablation of langerin-expressing cells in vivo by the administration of DT. DT was injected into the mice 5 d before the immunization and an additional injection was given 21 d later to maintain depleton (Fig. 1A). This depletion strategy was used because 5 d after the DT injection, the majority of Ln<sup>+</sup>dDCs returned, whereas most of the LC population remained absent beyond the time of the peak CD8<sup>+</sup> T cell response (Fig. 3A, 3B).

The DT-treated and untreated mice were immunized with 5 x 10<sup>5</sup> TU Lv-OVA and induction of OVA-specific CD8<sup>+</sup> T cell responses was monitored by staining PBMCs with anti-CD8α Abs and SIINFEKL/H-2K<sup>b</sup> MHC class I tetramers. As shown in Fig. 1B, depletion of LCs did not alter the kinetics and magnitude of the SIINFEKL/tetramer-specific CD8<sup>+</sup> T cells. The function of these cells was also examined by restimulating splenocytes collected 4 wk after the immunization with the SIINFEKL peptide for 6 h, followed by an intracellular cytokine staining analysis. This analysis shows that production of IFN-γ by CD8<sup>+</sup> T cells was also comparable between the DT-treated and untreated groups (Fig. 1C).

To examine the effect of LC ablation on Ab production, we collected sera from the mice 4 wk after the immunization and measured OVA-specific Ab titer by standard ELISA. Our results indicate that in both groups of mice OVA-specific IgG levels, as well as the IgG subtypes IgG1 and IgG2C levels were similar (Fig. 2). These findings suggest that LCs are not the skin DC subset mediating the induction of cellular and humoral immunity after intradermal immunization with lentivectors.

**Ln<sup>+</sup>dDCs are not essential for lentivector-induced immunity**

Although LCs were not found to generate immune responses in various viral and bacterial experimental infection models, recent studies have suggested that Ln<sup>+</sup>dDCs do play a role in T cell activation (20, 22). We thus asked whether Ln<sup>+</sup>dDCs contribute to the generation of Ag-specific immune responses after intradermal administration of lentivectors. Mice were administered with DT 7 d and 7 h before the immunization, and every 7 d postimmunization. This strategy leads to the elimination of both LCs and Ln<sup>+</sup>dDCs in the skin and LNs of the mice (Fig. 3A, 3B). To identify the various skin DC subsets in the LN, the FITC<sup>+</sup> population was separated according to the expression of CD103 (LN<sup>+</sup>dDCs), and the CD103<sup>−</sup> cells were further segregated into Ep-CAM<sup>−</sup> (dDCs) and Ep-CAM<sup>+</sup> (LCs). Ablation of Ln<sup>+</sup>dDCs did not influence the kinetics and magnitude of the SIINFEKL/tetramer-specific CD8<sup>+</sup> T cells, or their capacity to produce IFN-γ after in vitro restimulation (Fig. 4B, 4C). OVA-specific Ab production (total IgG, IgG1, or IgG2C) was also similar between DT-treated and untreated mice (Fig. 5). Taken together, our data indicate that the capacity of intradermally delivered lentivectors to prime CD8<sup>+</sup> T cells does not require the presence of Ln<sup>+</sup>dDCs or LCs in mice.

**Ag presentation is not impaired by the absence of LCs and Ln<sup>+</sup>dDCs**

To further investigate the role of skin DCs in immune induction after intradermal immunization with Lv-OVA, we analyzed the Ag-presenting activity in the immunized mice. Because LCs and Ln<sup>+</sup>dDCs were found to be dispensable for generating immunity in vivo, we tested whether their ablation would influence the level of Ag presented to CD8<sup>+</sup> T cells. Lang-DTR mice were treated with DT 7 d and 1 d before the immunization with Lv-OVA, and the ears were immediately painted with DBP/ FITC solution to identify skin-derived DCs migrating to the draining LNs. Two days after the immunization, the draining LNs from DT-treated (n = 5) and untreated mice (n = 5) was collected, and the CD11c<sup>+</sup>...
cell population was enriched. Administration of DT resulted in a complete ablation of CD11c<sup>+</sup>FITC<sup>+</sup>CD103<sup>2</sup>Ep-CAM<sup>+</sup> LCs from the pool of FITC<sup>+</sup> skin DCs that have migrated to the LNs (Fig. 3A). In addition, we observed a significant reduction in the number of migratory CD11c<sup>+</sup>FITC<sup>+</sup>CD103<sup>+</sup> Ln<sup>+</sup>dDCs (Fig. 3A). No noticeable change was found in the dDCs cells (CD11c<sup>+</sup>FITC<sup>+</sup>CD103<sup>2</sup>) arriving in the draining LNs. Consistent with these results, there was a profound decrease in the FITC<sup>+</sup>CD11c<sup>+</sup> cell population, which migrated to the LNs as a result of the DT treatment [Fig. 6A, (25)]. This decrease, however, was more pronounced in the CD11c<sup>bright</sup> cells, whereas the CD11c<sup>medium</sup> cell population was hardly affected (Fig. 6A). Careful analysis reveals that the expression level of CD11c on LCs (CD11c<sup>+</sup>FITC<sup>+</sup>CD103<sup>+</sup>Ep-CAM<sup>+</sup>) and Ln<sup>+</sup>dDCs (CD11c<sup>+</sup>FITC<sup>+</sup>CD103<sup>2</sup>) was brighter than that of dDCs (Fig. 6B). We then FACS-sorted the FITC<sup>+</sup> and FITC<sup>−</sup> DCs from the enriched CD11c<sup>+</sup> cell population. The different DC subsets were incubated with CFSE-labeled CD8<sup>+</sup> T cells, and 60 h later the cultures were analyzed to measure CFSE dilution as a read-out of CD8<sup>+</sup> T cell proliferation. Fig. 6C demonstrates that considerable reduction in the CFSE level was observed only when the labeled OT-I CD8<sup>+</sup> T cells were incubated with the FITC<sup>+</sup> CD11c<sup>+</sup> cell fraction. Furthermore, CFSE dilution was more robust in DT-treated mice in which the FITC<sup>+</sup>CD11c<sup>+</sup> fraction contains mainly dDCs. Incubation of the OT-I CD8<sup>+</sup> T cells with the FITC<sup>−</sup>CD11c<sup>+</sup> cell population generated a weak reduction in the CFSE levels in both DT-treated and untreated mice. This weak response could be due to residual skin-derived DCs that failed to be labeled by the FITC. Finally, we also demonstrated that non-CD11c cells lack the capacity to present
Ag to the CD8⁺ T cells. These findings suggest that langerin-expressing DCs are not necessary for optimal Ag presentation to CD8⁺ T cells during intradermal immunization with lentivectors.

dDCs and Ln⁺dDCs, but not LCs, are capable of presenting Ag to CD8⁺ T cells in vitro

We next examined the capacity of each DC subset migrating from the skin to present Ag to CD8⁺ T cells. B6 mice were immunized intradermally with Lv-OVA and the ear pinna was painted with FITC solution. Three days after the immunization, LN cells were pooled from 10 mice, enriched for CD11c⁺ population and stained with Abs against CD11c, CD103, and Ep-CAM. The cells were then sorted into dDCs, LCs, and Ln⁺dDCs population according to the gating strategy described in Fig. 7A. This analysis indicates that the majority of skin DCs migrating to the LNs were dDCs, whereas significantly lower numbers of Ln⁺dDCs and LCs were found (Fig. 7B). After incubation with CFSE-labeled OT-I CD8⁺ T cells, a considerable reduction in the CFSE level was observed in the presence of dDCs or Ln⁺dDCs (Fig. 7C). No proliferation of CD8⁺ T cells was seen with LCs or LN-resident DCs. To verify whether Ln⁺dDCs present Ag to CD8⁺ T cells via cross-priming or direct priming, we tested their capacity to activate OT-II CD4⁺ T cells. As demonstrated in Supplemental Fig. 2, Ln⁺dDCs failed to activate CD4⁺ T cells ex vivo, whereas dDCs were the only DC subset capable of presenting Ags to CD4⁺ T cells. We further tested whether the various skin DCs are efficiently transduced with the lentiviral vector after intradermal immunization. Mice were immunized with 5 × 10⁶ TU Lv-OVA/eGFP, exposed to acetone/DPB solution, and 3 d later the enriched CD11c⁺ cell population was analyzed for GFP-positive cells by flow cytometry. Expression of GFP was seen in dDCs, Ln⁺dDCs, and LCs (Fig. 7D).

Moreover, it seems that the three DC subsets were efficiently transduced, because the percentage of GFP⁺ cells in each DC subset was similar to the percentage of the particular DC subset in the total migratory DC population. These results therefore suggest that after immunization with lentivectors, dDCs are the largest DC subset with APC capabilities migrating to the LNs. Ln⁺dDCs are also capable of presenting lentiviral-derived Ag to CD8⁺ T cells by cross-priming; however, because their number is very low, the contribution of Ln⁺dDCs to the overall Ag presentation may be dispensable.

Kinetics of Ag expression at the injection site

The level and duration of Ag expression is thought to influence immune induction, and thus we analyzed the kinetics of Ag expression at the immunization site. B6 mice were immunized with 5 × 10⁶ TU Lv-Luc and the magnitude of luciferase expression at the injection site was measured in vivo at various times post-immunization. Interestingly, 4 d after the immunization insignificant levels of luciferase expression was detected at the injection site.
injected skin of the immunized mice (Fig. 8A, 8B). However, 2 wk after the immunization, the expression of luciferase increased considerably, and 7 d later the expression was greatly reduced. We also examined the mechanism of this Ag clearance by immunizing perforin−/− and Fas−/− transgenic mice with Lv-Luc. As shown in Fig. 8C, the level of luciferase expression in perforin−/− mice was considerably higher than that measured in B6 and Fas−/− mice 3 wk after the immunization (p < 0.01). These results demonstrate the unique kinetics of Ag expression in the skin after administration of lentivectors. They also suggest that Ag clearance of Lv-derived transgenes is mediated by a perforin-dependent, Fas-independent pathway.

Excision of the immunization site alters the kinetics and function of CD8+ T cells

It has been shown that after immunization with lentivectors, DCs present Ag to CD8+ T cells for more than 3 wk postimmunization (13). We asked whether such durable Ag-presenting activity requires prolonged migration of skin-derived DCs from the injection site. To manipulate migration of skin-DCs, we injected mice in the ear pinna with 5 × 10^6 TU Lv-OVA in the ear pinna and excised the ear pinna immediately exposed to acetone/DPB solution (1:1). Three days later the draining LNs were collected and enriched for CD11c+ cells using magnetic beads. The cells were then stained with anti-CD11c, CD103, and Ep-CAM Abs and analyzed by flow cytometry. Data presented are the percentages of GFP-positive LCs, Ln+ dDCs, and dDCs in the draining LNs.

We next tested whether the excision of the ear pinna affected the development of the CD8+ T cells into memory cells. Fig. 9B demonstrates that the removal of the injection site on day 3 resulted in high expression of the central memory-associated molecule CD62L on the surface of the SIINFEKL-specific CD8+ T cells in the blood. A similar pattern of expression was not seen in mice whose ear pinna was excised on day 5 or 10 postimmunization. Consistent with the results in the blood, the total number of SIINFEKL-specific CD8+ T cells in the spleen was lower when the injection site was removed 3 or 5 d postimmunization (Fig. 9C). Nevertheless, the number of central memory cells, according to the dual expression of CD127 and CD62L on SIINFEKL-specific CD8+ T cells, was similar across the different groups (Fig. 9D). Thus, our findings suggest that although lentivectors induced prolonged Ag presentation by DCs,
Expression of CD62L on CD8+ tetramer+ T cells in the peripheral blood of Lv-OVA immunized mice as detected with an H-2Kb/SIINFEKL tetramer. Data are showed as the percentage of CD8+ tetramer+ T cells and represent the means of three mice per group ± SE. B, Expression of CD62L on CD8+ tetramer+ T cells in the PBMCs of the immunized mice. C, Total numbers in the spleen of SIINFEKL-specific CD8+ T cells, and (D) the number of splenic CD62L+CD127hi SIINFEKL-specific CD8+ T cells in Lv-OVA immunized mice. The data are the means of three mice per group ± SE.

<10 d of migration are necessary for skin DCs to generate optimal CD8+ T cells responses. In addition, removal of the Ag early after the immunization leads to a rapid progression of the CD8+ T cells into the memory compartment.

Discussion

In this study, we provide evidence that LCs and Ln+ddCs do not contribute to CD8+ T-cell priming or Ab production in vivo after intradermal immunization with lentivectors. Because Ag-presenting activity in this setting is reported to be mediated by skin-derived DCs (13), it is likely that classical langerin-expressing negative dDCs are the skin DCs that directly present the lentivector-derived Ag to CD8+ T cells. Addressing the role of each skin DCs subset in vivo is possible by DT administration, because Ln+ddCs were reported to re-populate the skin more rapidly than LCs (26–29). Based on this information, we demonstrated that in the absence of LCs and Ln+ dDCs, the migratory skin-derived DCs can efficiently present Ag to CD8+ T cells. Furthermore, we demonstrated that dDCs migrating from the skin can present Ag to CD8+ T cells in vitro. Surprisingly, despite the dispensability of Ln+ddCs in our in vivo studies, these cells were capable of presenting Ag in vitro as efficiently as dDCs. This inconsistency could be explained by the observation that the number of dDCs in the draining LNs was considerably higher than the number of Ln+ddCs. Thus, an efficient priming of CD8+ T cells can be achieved even in the absence of the Ln+ddCs. It has been demonstrated recently that Ln+ddCs present HSV-1 Ags to CD8+ T cells by cross-priming (23). We found that this DC subset is capable of presenting lentiviral Ag to CD8+ T cells but not CD4+ T cells early after the immunization. Thus, in agreement with Bedoui et al. our data strongly suggest that Ln+ddCs present lentiviral-derived Ags by cross-priming. This may also explain the prolonged Ag presentation observed after immunization with lentivectors. It has been suggested that non-DC professional APCs, such as B cells, can contribute to T cell activation during gene gun genetic immunization (30). Our data suggest that this is not the case in our system as CD11c+ LN cells failed to present Ag to CD8+ T cells. This finding is in agreement with recent work demonstrating the inability of CD19+ cells to prime naive CD8+ T cells ex vivo after lentivector administration (6).

During administration of lentivectors, Ag expression at the injection site showed relatively delayed kinetics in comparison with other vectors, which we tested previously. We have shown that after DNA immunization luciferase expression in the skin peaked 1–2 d after the immunization (31). Similar kinetics of luciferase expression was shown after intradermal immunization with adenovector (A.H. Hovav, unpublished data). We cannot explain yet the reason for the delayed expression of lentivector-derived Ag, and it might relate to the effect of the virus on the viability of the infected cells. Incubation of skin tissue with lentivectors was reported to transduce many types of cells in the tissue (32). Nevertheless, we could not detect the type of cells expressing lentiviral-encoded Ag in the skin early after the immunization by immunochemistry, most likely due to the low level of Ag expressed in the mice at this time (Fig. 6). He and colleagues have shown that Ag-presenting DCs can be found in the LNs as soon as 24 h postlentivector immunization (13). We also found expression of the Ag by the various skin-DCs in the LNs 3 d after the immunization. In any case, the unique pattern of Ag expression observed after lentivirus injection seems to be very effective in inducing immunity probably due to the high capability of lentivirus to transduce DCs. In contrast to immunization with naked plasmid DNA (31), the kinetics of Ag clearance in the setting of lentivectors correlated with the emergence of Ag-specific CD8+ T cells. These cells are likely to mediate clearance of the Ag expressed in the skin, as we demonstrated that this process is mediated by the perforin-pathway.

Removal of the injection site early after the immunization (days 3 or 5) resulted in reduced CD8+ T cell responses, suggesting that migration of skin DCs is still taking place during this time. Although excision of the ear pinna on day 3 immediately affected the CD8+ T cell response, the impact of day 5 excision was seen only 2 wk later. This suggests that up to day 5 a sufficient number of dDCs has arrived in the draining LNs, providing efficient presentation of the Ag for the next several days. We recently reported that removal of the ear pinna 3, 5, and 10 d after intradermal immunization with plasmid DNA resulted in immediate reduction in the level of Ag-presenting activity and low frequencies of Ag-specific CD8+ T cells (31). This may indicate that the viability of
skin DCs (mainly dDCs) mediating Ag presentation after lentivector immunization is higher than of cells in plasmid DNA immunized mice. A relatively rapid reduction in the number of dDCs was also reported during HSV skin infection, a virus that is known by its cytolytic capability (15). We hypothesize that during lentiviral immunization, migratory dDCs survive and present Ag in the LNs for a longer period, because the viability and functionality of these cells are not hampered by the lentivector as in the case of HSV. This characteristic of lentivectors may also explain the robust Ag-presenting activity measured by He et al. 3 wk after the immunization (13); whereas we demonstrated that the excision of the ear pinna on day 10 did not affect the kinetics or the phenotype of the CD8 T cell response. Prolonged Ag presentation is thought to shape the antiviral immune response (33, 34). Durable Ag-presenting activity was also observed during immunization with adenovector long after the Ag was cleared and found to generate long-term immunity (35). In addition, there is evidence that durable Ag exposure contributes to the generation of long-lived memory CD8 T cells (36). Still, it is possible that Ag-presenting activity was reduced due to the removal of the injection site on day 10 postlentivector immunizations, but this probably had minimal impact on the CD8 T cell responses. Interestingly, premature removal of the injection site resulted in a rapid differentiation of CD8 T cells into memory cells, an observation we observed also after plasmid DNA immunization (31). Excision of the ear pinna eliminates the Ag reservoir and therefore it might reduce antigenic stimulation and facilitate memory formation as suggested previously (37, 38). Alternatively, it is possible that effector and memory CD8 T cells may receive different signals early after immunization that determine the fate of their differentiation.

Recently, it has been reported that after gene gun immunization, Ln dDCs were required for optimal production of IgG2c in the acute phase, whereas LCs were required for maximal IgG1 responses (21). This dependency of Ab production on langerin-expressing cells was not observed in our system. In addition, ablation of LCs and Ln dDCs in gene gun immunized mice resulted in enhanced numbers of IFN-γ-producing T cells, probably because Ln dDCs act as a negative regulator of Th1 immune responses (21). Again, we could not detect significant differences in the capability of the T cells to produce IFN-γ, suggesting that the nature of an immunogen controls the function of skin DCs in immune induction.

Our findings indicate that langerin-expressing skin DCs are dispensable for intradermal lentivector-induced immune responses in vivo, suggesting that dDCs are the major APCs in this model. We also propose that DC migration from the injection site takes place in the initial 10 d in the process of generating optimal CD8 T cell responses. This study provides novel information regarding the immune mechanisms used by lentiviral vectors after s.c. immunization.

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Disclosures

The authors have no financial conflicts of interest.

References


**Supplementary Figure 1**

![Supplementary Figure 1](image)

**Figure S1: Schematic illustration of lentiviral vectors employed in the study** Both vectors feature a third generation self-inactivating, replication incompetent lentiviral vector. A deletion in the 3' long terminal repeat (3'ΔLTR) region allows for inactivation of the LTR viral promoter following transduction of target cells. Expression of ovalbumin (OVA) or luciferase is driven by the cytomegalovirus (CMV) promoter whereas eGFP gene is driven by the human ubiquitin C (UBC) promoter.
Supplementary Figure 2

**Figure S2**: Activation of CD4+ T cells by skin DCs following intradermal immunization with Lv-OVA. B6 mice were immunized intradermally with Lv-OVA in the ear pinna and the immunization sites were painted with FITC. Three days after the immunization the draining LN were pooled from 10 mice, CD11c+ population was enriched and the cells were sorted according to the expression of the CD11c, CD103 and Ep-CAM markers in the FITC+ populations as described earlier. The purified DC subsets (3×10^4 cells/well) were then co-cultured with OT-II CD4+ T cells (5×10^4 cells/well) for 60 hrs, and the levels of IFN-γ secreted to the supernatant were measured by ELISA. * P< 0.005, dDC versus T cells.