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Epidermal Langerhans Cells Are Dispensable for Humoral and Cell-Mediated Immunity Elicited by Gene Gun Immunization[1]

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Gene gun immunization, i.e., bombardment of skin with DNA-coated particles, is an efficient method for the administration of DNA vaccines. Direct transfection of APC or cross-presentation of exogenous Ag acquired from transfected nonimmune cells enables MHC-I-restricted activation of CD8$^+$ T cells. Additionally, MHC-II-restricted presentation of exogenous Ag activates CD4$^+$ Th cells. Being the principal APC in the epidermis, Langerhans cells (LC) seem ideal candidates to accomplish these functions. However, the dependence on LC of gene gun-induced immune reactions has not yet been demonstrated directly. This was primarily hampered by difficulties to discriminate the contributions of LC from those of other dermal dendritic cells. To address this problem, we have used Langerin-diphtheria toxin receptor knockin mice that allow for selective inducible ablation of LC. LC deficiency, even over the entire duration of experiments, did not affect any of the gene gun-induced immune functions examined, including proliferation of CD4$^+$ and CD8$^+$ T cells, IFN-γ secretion by spleen cells, Ab production, CTL activity, and development of protective antitumor immunity. Together, our data show that gene gun immunization is capable of inducing humoral and cell-mediated immune reactions independently of LC. ***The Journal of Immunology,* 2007, 179: 886–893.**

During the last decade, genetic immunization has gained increasing attention in basic and translational research. Administration of eukaryotic expression plasmids encoding the Ag gene, usually under transcriptional control of a strong viral promoter, has been shown to elicit strong humoral and cell-mediated immune reactions in vertebrates. DNA vaccines have been designed and tested in animal models for a large variety of infectious and noninfectious diseases, including HIV, tuberculosis, leishmaniasis, influenza, malaria, allergy, and cancer (1–5). Among the several techniques of DNA vaccine administration developed to date, gene gun immunization has become highly popular. Basically, this technique makes use of DNA-coated gold microparticles that are propelled onto the skin by a short pulse of pressurized gas (6).

According to generally accepted current concepts, gene gun treatment transfects cells in the uppermost layers of the skin by DNA-coated gold particles directly penetrating into the cytoplasm or nucleus. The major type of professional APC in the epidermis are Langerhans cells (LC)[3] (7). Although constituting only ~1–3% of total epidermal cells, LC with their long and branched processes form a dense and uniform network covering the entire skin surface (8, 9). In addition, the dermal tissue underneath contains a distinct type of dendritic cells (DC), frequently referred to as dermal DC (10). Direct transfection of skin DC leads to Ag gene expression, proteasomal degradation, and MHC-I-restricted presentation of antigenic peptides. Concomitant transfection of nonimmune cells such as keratinocytes provides a source of external Ag that may be endocytosed by DC. Processing of external Ag by the endo/lysosomal pathway would serve MHC-II-restricted presentation of antigenic peptides and subsequent activation of CD4$^+$ T cells. Additionally, cross-presentation of the same Ags on MHC-I molecules may contribute to activation of CD8$^+$ T cells (1, 6). Moreover, gene gun treatment induces maturation of skin DC, including epidermal LC, that have been shown to migrate to draining lymph nodes (LN) and are able to activate Ag-specific CD4$^+$ and CD8$^+$ T cells (11–14).

The contribution of different types of transfected cells to the induction of immune reactions has been dissected by transcriptional targeting of Ag gene expression using cell type-specific promoters. Administration of fascin or dectin-2 promoter constructs by gene gun restricted the expression of the Ag gene to cutaneous DC and induced proliferation and/or IFN-γ production in spleen cells of immunized mice (15, 16). However, the view that directly transfected DC are critical for the induction of CTL has been challenged by studies with DNA vaccines specifically targeting nonimmune cells. Thus, CTL induction with vaccines controlling Ag gene expression by a keratinocyte-specific K14 promoter was as efficient as one with a nonrestricted CMV promoter (17, 18). These

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[3] Abbreviations used in this paper: LC, Langerhans cell; βGal, β-galactosidase; DC, dendritic cell; DT, diphtheria toxin; i.d., intradermally; LN, lymph node; EGFP, enhanced GFP; psi, pounds per square inch.

findings underline the importance of cross-presentation of exogenous Ag on MHC-I molecules, which is a specific feature of professional APC, particularly DC (19) and including LC (20).

Because LC are the most prominent APC in the outermost skin layers, it has been generally assumed that this is the most likely cell type that initiates gene gun-induced immune reactions. LC cover a significant portion of the skin surface and are therefore in an ideal position for both direct transfection by penetrating gold particles as well as uptake, presentation, and/or cross-presentation of Ag that is liberated from transfected nonimmune cells. In addition, one can find dermal DC and other MHC-II$^+$ cells in the deeper layers of skin tissue (21). Importantly, inflammatory processes induced by the gene gun shot have been shown to attract a variety of immune cells (22). Thus, although LC seem to be the most likely candidates, there are several other cell types that could mediate Ag presentation after gene gun immunization.

Hitherto, there was no possibility to discriminate unequivocally the role of LC from that of dermal DC. Recently, however, transgenic knockin mice have been developed that express the human diphtheria toxin receptor (DTR) under transcriptional control of the endogenous Langerin/CD207 promoter (Lang-DTR) (23, 24). Because the affinity of the murine receptor for DT is insufficient to exert toxicity, injection of DT induces selective ablation of Langerin-positive cells in Lang-DTR mice. In the present study, we have used Lang-DTR mice to investigate the dependence of gene gun-induced immune reactions on the presence of LC in the epidermis.

Materials and Methods

Mice and ablation of LC

C57BL/6 mice were obtained from Charles River Laboratories. OT-I, OT-II, Lang-DTR-enhanced GFP (EGFP) (abbreviated below into Lang-DTR) mice, and Lang-EGFP (24) at F9 of a backcross onto B6 background were bred and maintained at the local animal facilities of the Universities of Innsbruck or Salzburg and used for experiments between 6 and 12 wk of age. Animal care and experimentation were conducted in accordance with the European Union guidelines 86/609/EWG for laboratory animal experimentation and approved by the national authorities. For ablation of LC in the epidermis, 1 μg of DT (Sigma-Aldrich) was injected i.p. in 100 μl of pyrogen-free PBS 2 days before immunization. For continuous LC ablation, subsequent DT injections were given at weekly intervals for the entire duration of the experiment.

Immunohistochemistry

Epidermal sheets were prepared from mouse ears as described (25), and stained for MHC class-II-positive cells with PE-conjugated mAb M5-114 (BD Pharmingen). Stained sheets were photographed at $\times$100 magnification on an IX70 fluorescence microscope (Olympus) equipped with a SPOT-2 digital camera (Diagnostic Instruments) device. In some experiments, the density of epidermal LC was determined, by directly assessing intravital EGFP fluorescence, as described above, on the ventral ear skin surface of anesthetized (Lang-DTR $\times$ Lang-EGFP)F_1 mice.

Histology

Gene gun-shot skin specimens were fixed in 4% formaldehyde at 4°C for 1 h and embedded in paraffin. Tissue sections of 2 μm were stained with H&E and photographed on a bright field microscope at $\times$400 magnification (Zeiss).

Flow cytometry

LN cells were isolated from mechanically disrupted LN tissue by collagenase D digestion (Roche; 1 mg/ml in HBSS, 40 min at 37°C, with gentle agitation) and filtered through 70-μm nylon mesh filters. For surface staining, cells were incubated with biotinylated anti-CD11c mAb (clone N418; BD Pharmingen), followed by allophycocyanin-conjugated streptavidin plus PE-conjugated anti-MHC-II mAb (clone M5/114; BD Pharmingen). Staining for Langerin was performed on cells fixed with 4% formaldehyde and permeabilized with 0.1% saponin with anti-CD207 mAb (clone 929F3; Dendritics-AbCys) and allophycocyanin-conjugated anti-rat IgG, followed by anti-MHC-II PE. Cells were recorded on a BD FACSCanto II or a BD FACSArray flow cytometer and analyzed with the BD FACSDiva software (all from BD Biosciences).

RT-PCR

LN were mechanically disrupted in HBSS and lysed in TRIzol (Invitrogen Life Technologies). Total RNA was extracted according to the manufacturer's protocol, and cDNA was produced from 2 μg of RNA with random hexanucleotide primers. PCR primers for a 546-bp fragment from β-galactosidase (βGal) DNA vaccine transcripts were 5′-CGTAATAGCGAA GAGGCC (sense) and 5′-CGGTTTATGCAGCAACGAG (antisense). For a 193-bp fragment from the housekeeping gene acidic ribosomal phosphoprotein PO, GenBank accession number NM_007475.1, transcript primers were 5′-TGCACTCTCGCTTTCTGGAGGGTG-3′ (sense) and 5′-AATG CAGATGGATCAGCCAGGAAGG (antisense). PCR conditions were 1 $\times$ 95°C, 4 min; 35$\times$(95°C, 30 s; 64°C, 45 s; 72°C, 60 s).

Gene gun immunization

pCI-OVA was constructed by PCR amplification of the chicken OVA coding sequence from plasmid pCDM8-OVA, with primers 5′-CACCGAATTC CATGGGCTCCATCGG-3′ (sense) and 5′-TGTCTCTAGATTAAGGGG AAACACATCTGCC-3′ (antisense) and insertion of the *Eco*RI/*Xba*I-digested fragment into *Eco*RI/*Xba*I sites of pCI (Promega), an expression vector driving eukaryotic transcription by the ubiquitously active CMV promoter. For preparation of DNA vaccines, pCI-OVA and pCI-βGal (26) were purified by use of the Qiagen Endofree kit. Endotoxin contamination was typically $<$0.05 pg/μg DNA, as determined by *Limulus* amebocyte lysate assay (Pyroquant). Gene gun immunization was performed as previously described (27). Briefly, mice received two nonoverlapping shots onto the shaved abdominal skin twice with a 2-wk interval. With each shot, 1 μg of DNA immobilized onto 0.5-mg gold particles was delivered at a pressure of 400 pounds per square inch (psi) with a Helios gene gun (Bio-Rad).

Serum Abs

Serum was prepared from blood samples 1 wk after the last immunization. Ag-specific serum IgG was detected by ELISA with isotype-specific peroxidase-conjugated detection Abs, followed by chromogenic development. IgE was measured by rat basophil degranulation assay, as described elsewhere (28, 29). Ab titers were determined by end point titration and expressed as the dilution factor yielding a response equal to the quantification limit (i.e., mean + 3$\times$ SD of 16 blank values).

ELISPOT

IFN-γ-secreting splenocytes were detected by ELISPOT technique. Spleen cells, 2 $\times$ 10^5 per well, were cultured for 24 h with or without 10 μM immunodominant CTL peptide ($OVA_{257-264}$ SIINFEKL (30)) or 20 μg/ml OVA (Sigma-Aldrich) on 96-well filter-bottom Multiscreen plates (Millipore) that had been coated with anti-IFN-γ mAb (clone AN18; BD Pharmingen). Culture medium was RPMI 1640 supplemented with 2 mM L-glutamine, 50 μM 2-ME, and 1% heat-inactivated FCS. Cytokine spots were detected by use of biotinylated matched pair anti-IFN-γ mAb (clone R46A2; BD Pharmingen), followed by peroxidase-conjugated streptavidin and chromogenic enzyme reaction with 3-amino-9-ethylcarbazol substrate. Spots were counted manually from 600 dots per inch flat bed scans of duplicate wells.

In vivo proliferation assay

Spleen cells from naive OT-I or OT-II donor mice that are transgenic for a TCR with specificity for H2-K^b-restricted $OVA_{257-264}$ (SIINFEKL) or I-A^b-restricted $OVA_{323-339}$, respectively, were stained with 2 μM CFSE (Molecular Probes) for 10 min at 37°C in PBS. After washing, 10^7 cells in 100 μl of PBS were injected i.v. into Lang-DTR mice that had been left untreated or injected with 1 μg of DT 3 days before. One day after injection of cells, mice were gene gun immunized with pCI-OVA, and cells were isolated from draining LN and spleen 3 days later. Cells were stained with PE-conjugated anti-Vβ5.1,5.2 TCR mAb (clone MR9-4; BD Pharmingen) or anti-CD8α (clone 53-6.7; BD Pharmingen), respectively, and analyzed by flow cytometry.

In vivo CTL assay

CTL activity was determined in vivo essentially as described (31), with minor modifications. Briefly, spleen cells from naive C57BL/6 donor mice, depleted from erythrocytes by ammonium chloride treatment, were stained in PBS with CFSE, either at 5 or 0.5 μM. The latter fraction was pulsed in culture medium with 10 μM CTL peptide SIINFEKL for 1 h and combined

with the first fraction at a 1:1 ratio. Of this mixture, a total of 1×10^7 cells was injected i.v. in 100 μl of PBS into recipient mice. After 16 h, recipient splenocytes were analyzed by flow cytometry and percent specific lysis was calculated from the reduction of peptide-pulsed cells relative to nonpulsed reference cells (*left* and *right peaks*, respectively, in Fig. 5*B*).

Tumor challenge

B16F1 melanoma cells (American Type Culture Collection; CRL-6323) and derivatives thereof were cultured in DMEM supplemented with 4 mM L-glutamine, 1 mM Na-pyruvate, 10 mM HEPES, 5% heat-inactivated FCS (PAA Laboratories), and antibiotics, in a humidified atmosphere with 7% CO_2 at 37°C. Cells were stably transfected with pPGK-Geo, a plasmid for eukaryotic expression of a βGal-neomycin-phosphotransferase fusion protein under control of the phosphoglycerate kinase promoter (32). Transfected cells were selected for stable resistance to 1 mg/ml Geneticin G418 in the culture medium. Single-cell colonies were isolated and examined for βGal expression. Clone B16GeoD12 appeared to express the protein stably for several weeks in the absence of selection antibiotic and was therefore used for challenge experiments. For tumor inoculation, cells were washed and detached from culture dishes with Ca^{2+}/Mg^{2+}-free PBS, and 3×10^4 cells in 50 μl of PBS were injected intracutaneously on the shaved abdomen 2 wk after the last gene gun boost, along with groups of naive control mice. Tumor growth was monitored every 2 or 3 days, and relative tumor area was calculated as the product of diameters measured in two perpendicular orientations. Mice were scored positive when tumors reached a size of 25 mm^2, and experiments were terminated when tumors exceeded 100 mm^2.

Statistical analysis

Ab titers and ELISPOT results of different groups were tested for statistical significance by Mann-Whitney rank sum test. Survival curves were compared by Mantel-Haenszel log rank test. Values of $p < 0.05$ were considered statistically significant.

Results

Effects of gene gun treatment on skin LC

According to our standard protocol of gene gun immunization, DNA-coated gold particles are propelled onto the shaved abdominal skin with a pulse of helium gas at a pressure of 400 psi. Preceding experiments have revealed that, under these conditions, the major fraction of gold particles would target the epidermis and a few of them the uppermost layers of the dermis (Fig. 1*A*). In Lang-DTR mice, injection of DT resulted in the complete ablation of epidermal LC and most $CD207^+$ cells in LN for at least 8 days. However, the immune-activating effects of gene gun bombardment might induce accelerated de novo recruitment of LC from a pool of precursor cells. To address this problem, we applied gene gun shots onto the ear skin of (Lang-DTR $\times$ Lang-EGFP)F_1 mice. Periodical examination by intravital fluorescence microscopy revealed that LC were completely ablated by 2 days after DT injection. No $EGFP^+$ LC were detected within the following 7 days, irrespective of whether the skin area was gene gun immunized or left naive (Fig. 1*B*). The same results were obtained with epidermal sheets from Lang-DTR mice after immunohistochemical staining for CD207 (data not shown). As expected, gene gun bombardment efficiently mobilized LC to emigrate from the epidermis in nonablated Lang-EGFP mice. The density of LC in the skin was found unaltered immediately after the gene gun shot, indicating that no mechanical abrasion of epidermal tissue had occurred. Within 1 day, LC density decreased to 10% and stayed low for 7 days. Eleven days after gene gun treatment, LC density had returned to normal levels in all groups (Fig. 1*B*).

DT also ablates the majority of $CD207^+$ cells in cutaneous LN. However, a minor subset of $CD8^+CD207^+$ DC was found to resist DT ablation. It is not clear how these cells relate to skin LC because, after ablation, the LN resident population is restored earlier than epidermal LC, and therefore appears unlikely to be of skin origin (24). To investigate the effect of the gene gun on $CD207^+$ cells in draining LN, we analyzed DT-treated Lang-DTR or untreated wild-type mice at varying time points after gene gun immunization. Approximately 95% of $CD207^+$ LN cells were depleted by DT injection and remained at this level for 1 wk. Gene gun immunization 2 days after DT injection did not significantly alter the frequency of DT-resistant cells during the subsequent observation period of 7 days (Fig. 1*C*).

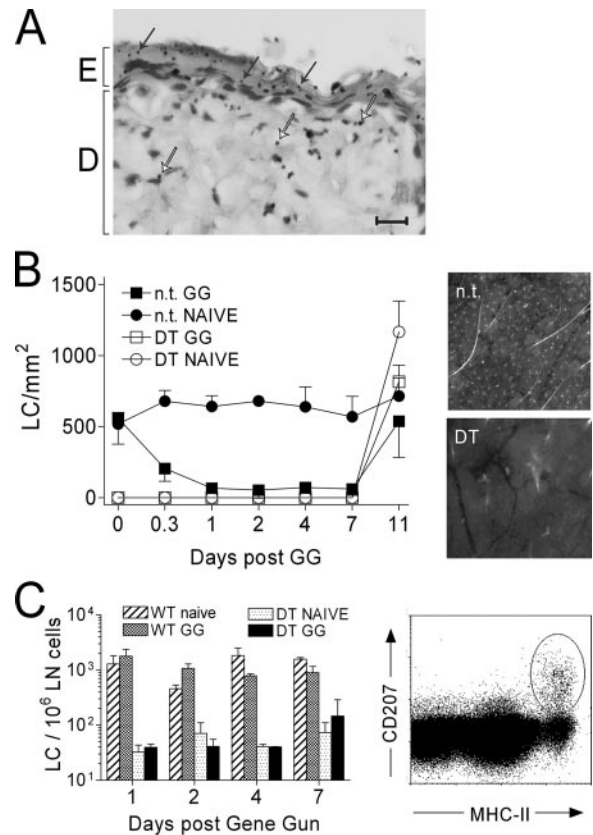

FIGURE 1. *A*, Histological analysis of gene gun-treated skin. Gold particles (1.6 μm) were shot onto shaved abdominal skin at a helium gas pressure of 400 psi. Paraffin sections were stained with H&E and photographed at $\times$400 magnification. E, epidermis; D, dermis. Gold particles targeted to the epidermis (filled arrows) or dermis (open arrows) are indicated. Bar, 10 μm. *B*, Density of LC in (Lang-DTR $\times$ Lang-EGFP)F_1 mice. Mice were left untreated (n.t.) or injected with 1 μg of DT (DT) on day -2. On day 0, left ears were gene gun immunized (GG); right ears were left naive. Anesthetized mice were inspected at the indicated points of time by fluorescence microscopy at $\times$100 magnification. Viable LC were identified by their EGFP fluorescence and morphology. Data are means $\pm$ SD of six to nine randomly selected square fields of 0.04 mm^2 from each of three mice per group. *Right panel*, Representative intravital microscopy photographs taken at day 4 after DT treatment; $\times$100 magnification. Photographs were slightly contrast intensified to visualize the nonfluorescent skin surface. *C*, Frequency of LC in draining LN. B6 wild-type (WT) or DT-treated Lang-DTR mice (DT) were immunized on the left ears by a single gene gun shot onto each the dorsal and ventral side 2 days after DT injection, whereas the right ears were left naive. At the indicated points of time, left and right auricular LN were separately isolated and analyzed for $MHC\text{-}II^+CD207^+$ cells. Data are means $\pm$ SD of two mice per condition and time point. *Right panel*, Representative dot plot of flow cytometric analysis of a naive wild-type LN showing the gate used for the identification of LN LC.

Gene gun immunization in the presence or absence of LC induces proliferation of Ag-specific $CD4^+$ T cells with equal efficiency

First, we examined whether gene gun immunization would still be able to induce proliferation of Ag-specific $CD4^+$ T cells in draining LN of LC-depleted mice. Lang-DTR mice were treated with

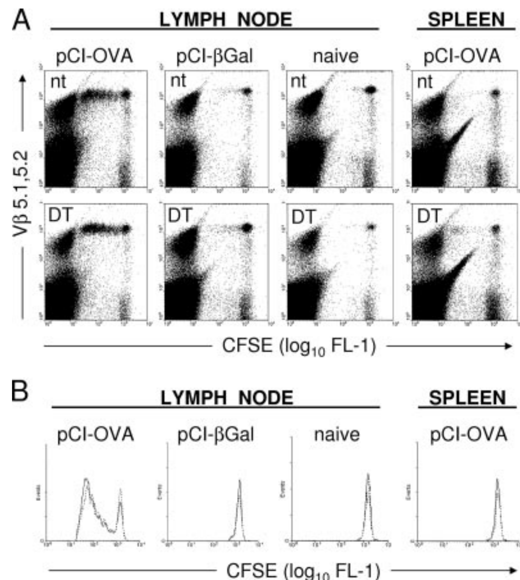

FIGURE 2. Gene gun immunization induces T cell proliferation in the absence of LC. Lang-DTR mice were either left untreated (nt) or injected with DT and gene gun immunized 2 days later with pCI-OVA or pCI-βGal or left naive. Proliferation of CFSE-labeled OT-II donor splenocytes isolated from draining LN or spleen was analyzed by flow cytometry 3 days after immunization. Cells were stained with PE-conjugated anti-Vβ5.1,5.2 mAb and gated on viable lymphocytes. *A*, Dot plots shown are representative of four experiments. *B*, Histograms of double-positive cells shown in *A*. Solid line, DT treated; dotted line, untreated.

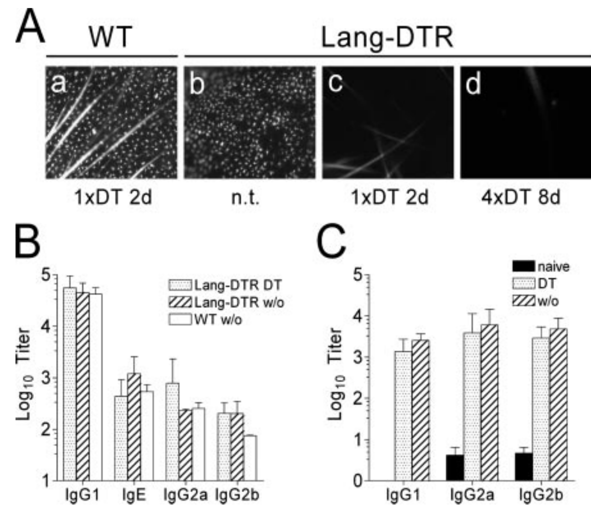

FIGURE 3. *A*, LC ablation after DT treatment. Wild-type (WT) C57BL/6 mice 2 days after a single DT injection (*a*); Lang-DTR mice without DT treatment (n.t.) (*b*), 2 days after a single DT injection (*c*), or 8 days after the last of four weekly injections of DT (*d*). Epidermal sheets were stained for MHC-II. Microphotographs were taken at ×100 magnification. Data are representative of six experiments. *B*, Serum Ig isotypes 1 wk after two gene gun immunizations with pCI-OVA given at a 14-day interval. Lang-DTR mice were either left untreated (w/o) or LC depleted by weekly injections of DT (DT). Wild-type C57BL/6 mice (WT) without DT treatment were immunized for control. *C*, IgG isotypes 3 wk after a single gene gun immunization with pCI-βGal in Lang-DTR mice that had been left untreated (w/o) or injected with DT 2 days before immunization. *B* and *C*, Data represent means ± SD of groups of four mice each.

DT to deplete the epidermis of LC, and 3 days later, injected with CFSE-labeled OVA-TCR transgenic OT-II spleen cells. Next day, mice were gene gun immunized with pCI-OVA. Three days later, $V\beta5^+$ OT-II T cells had vigorously proliferated in draining LN of LC-depleted mice when immunized with pCI-OVA (Fig. 2*A*). Histogram analysis revealed that the velocity of proliferation and the fraction of dividing cells were virtually identical with that observed in nondepleted Lang-DTR mice (Fig. 2*B*). In contrast, OT-II T cells that had homed to the spleen did not proliferate in either group of mice. Also, no proliferation was seen in naive mice or mice immunized with pCI-βGal as an irrelevant DNA vaccine (Fig. 2).

LC depletion does not influence gene gun-induced Ab production

Having established that LC were not required to stimulate Ag-specific proliferation of $CD4^+$ T cells, we next investigated whether gene gun immunization was able to induce Ab production in the absence of LC. Injection of DT into Lang-DTR mice induces LC ablation that lasts for about 1 wk, after which time LC start to reappear slowly in the epidermis (23, 24). However, 1 wk may be too short a time period for the development of efficient humoral immunity. Therefore, we tested whether the state of epidermal LC ablation can be extended by repetitive injections of DT. Epidermal sheets of Lang-DTR mice were completely devoid of MHC-II^+ cells by 2 days after a single i.p. injection of 1 μg of DT, whereas the same treatment had no effect in C57BL/6 wild-type mice. When Lang-DTR mice were injected with 1 μg of DT four times at weekly intervals, we could not detect any overt side effects on the overall health state of these mice. Moreover, epidermal sheets analyzed 8 days after the last DT injection were still completely free of LC (Fig. 3*A*).

These findings enabled studies on long-term effects of gene gun immunization in LC-depleted mice. To investigate Ab production, Lang-DTR mice were kept devoid of LC by weekly injections of DT over the entire duration of the experiment. Groups of untreated Lang-DTR and wild-type mice were included for comparison. Two days after the first DT injection, mice were gene gun immunized with pCI-OVA and boosted 2 wk later, again by gene gun. When analyzed 1 wk after the boost, we found similarly high titers of OVA-specific IgG1 and IgE in both groups, as well as in DT-treated wild-type mice immunized in the same way (Fig. 3*B*). Likewise, the titers of IgG2a and IgG2b were similar in all groups. However, these isotypes were two orders of magnitude lower than IgG1. Therefore, we also analyzed the humoral response to gene gun immunization with pCI-βGal, a vaccine that had proven to induce a more balanced response with higher levels of Th1-associated isotypes in earlier studies (our unpublished observations) (16). A single gene gun immunization in LC-depleted mice was sufficient to induce high titers of IgG1, IgG2a, and IgG2b 3 wk later. For all three isotypes, we did not observe any statistically significant differences to mice that had not received DT before immunization (Fig. 3*C*).

Gene gun-mediated activation of $CD8^+$ T cells in the absence of LC

To investigate the dependence on LC of gene gun-induced activation of $CD8^+$ T cells, we repeated the experiment shown in Fig. 2 with OT-I donor cells and found that LC were also dispensable for the proliferation of OVA-specific $CD8^+$ T cells. Three days after immunization with pCI-OVA, proliferating OT-I cells could be observed in draining LN of LC-depleted mice. This effect was Ag specific, because no such proliferation was found in naive mice or mice immunized with pCI-βGal (Fig. 4*A*).

These results prompted us to investigate $CD8^+$ T cells at a more functional level. Lang-DTR or C57BL/6 wild-type mice, either

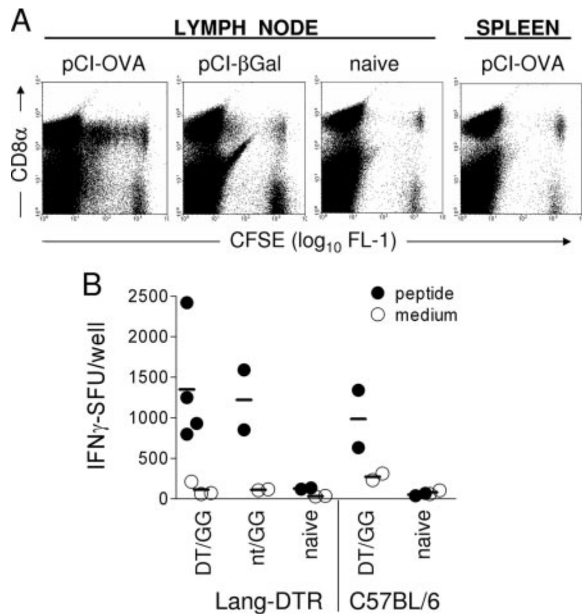

FIGURE 4. LC-independent activation of $CD8^+$ T cells after gene gun immunization. *A*, Proliferation of CFSE-labeled OT-I spleen cells in Lang-DTR mice that had been depleted of LC 3 days before gene gun immunization with pCI-OVA or pCI-βGal as an irrelevant Ag. Naive mice were included for control. Recipient LN or spleens were analyzed 3 days after gene gun. Cells were stained with PE-conjugated anti-CD8 mAb and analyzed by flow cytometry. Cells are gated on viable lymphocytes. Dot plots shown are representative of four experiments. *B*, IFN-γ-secreting spleen cells analyzed by ELISPOT technique. Wild-type (C57BL/6) or Lang-DTR mice, either left untreated (nt) or LC depleted by weekly injections of DT, were gene gun immunized with pCI-OVA (GG) twice at a 2-wk interval. One week later, spleen cells were isolated and restimulated in vitro with MHC-I-restricted $OVA_{257-264}$ peptide or medium. Data show IFN-γ-secreting spot-forming units (SFU)/10^6 cells of individual animals; bars represent means of each group.

untreated or under continuous DT treatment, were gene gun immunized twice with pCI-OVA. Two weeks later, spleen cells were found to secrete IFN-γ in response to the H-$2K^b$-restricted CTL peptide SIINFEKL, but not medium. The frequency of IFN-γ-positive cells was completely unaffected by LC ablation, as demonstrated by its similarity to that found in DT-treated wild-type or untreated Lang-DTR control mice (Fig. 4*B*).

Gene gun immunization induces CTL activity with similar efficiency in LC-depleted and wild-type mice

The appearance of IFN-γ-producing cells in response to restimulation with a MHC-I-restricted peptide points to a functional differentiation of effector CTL. Therefore, we examined whether gene gun-immunized mice were able to eliminate CTL peptide-loaded target cells in vivo. Lang-DTR mice, either untreated or under continuous LC ablation by weekly DT treatment, were immunized twice with pCI-OVA at a 2-wk interval. Another 2 wk later, mice were injected with a 1:1 mixture of peptide-pulsed and nonpulsed C57BL/6 splenocytes that were CFSE stained at different brightness. Mice were able to clear ~80% of peptide-pulsed target cells overnight, irrespectively of whether LC had been ablated or left intact. Wild-type C57BL/6 mice that had been immunized in the same way showed a similar capacity to eliminate peptide-pulsed target cells. In contrast, naive mice of both strains were unable to eradicate the target cells (Fig. 5).

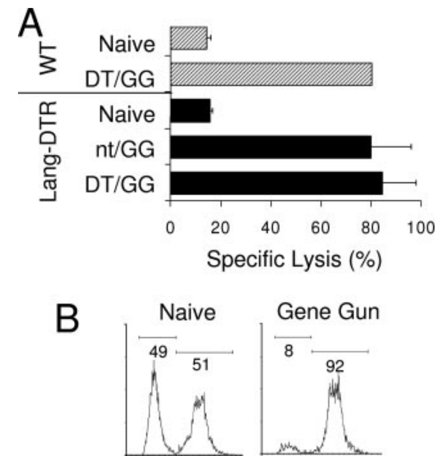

FIGURE 5. LC-independent induction by gene gun of Ag-specific cytolytic activity. *A*, Specific lysis of $OVA_{257-264}$ peptide-pulsed syngenic target cells injected into Lang-DTR or wild-type mice (WT). Mice were left untreated (nt) or LC depleted (DT) by weekly injections of DT. Starting 2 days after the first injection of DT, mice received two gene gun immunizations with pCI-OVA at a 2-wk interval. Cytolytic activity was analyzed by in vivo CTL assay 2 wk after the boost. Data represent means ± SD of two to four mice per group. *B*, Representative histograms of in vivo CTL assays of naive (*left*) or gene gun-immunized mice (*right*). *Left peaks* are peptide pulsed; *right peaks* are nonpulsed reference cells. Histograms were gated for $CFSE^+$ cells. Numbers below marker bars represent percentage of $CFSE^+$ cells.

Establishment of protective antitumor immunity after gene gun immunization is independent of LC

Next, we were interested to determine whether the observed CTL activity might have relevance for the protection of mice against tumor growth. For this purpose, we established a model of intradermally (i.d.) transplantable melanoma. This model is based on B16 cells stably transfected to express a βGal-neomycin phosphotransferase fusion protein (Geo) as a surrogate tumor Ag. A single cell clone, B16GeoD12, was isolated that proved to express the transgene without apparent loss of activity over several weeks of cell culture in the absence of selection antibiotic (data not shown). When inoculated i.d. with this clone, C57BL/6 mice developed progressively growing tumor lesions. However, mice that had been gene gun immunized against βGal were completely resistant to this tumor. Similarly, three of four Lang-DTR mice that were kept devoid of LC for the entire period of immunization and additional 2 wk after tumor inoculation did not accept the tumor. In the only positive mouse of this group, tumor growth was 20 days delayed with respect to naive mice (Fig. 6).

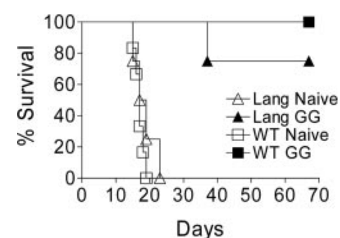

FIGURE 6. Induction of protective antitumor immunity by gene gun immunization. Lang-DTR mice permanently depleted of LC by weekly injections of DT (Lang) or C57BL/6 wild-type mice (WT) were either left naive (naive, open symbols) or immunized twice with pCI-βGal at a 2-wk interval (GG, filled symbols). Two weeks after the boost, mice were inoculated with B16-GeoD12 and tumor growth was monitored. Tumors were scored lethal when reaching a size of 100 mm^2. Groups: Lang-DTR, four mice each; WT, six mice naive and five mice immunized.

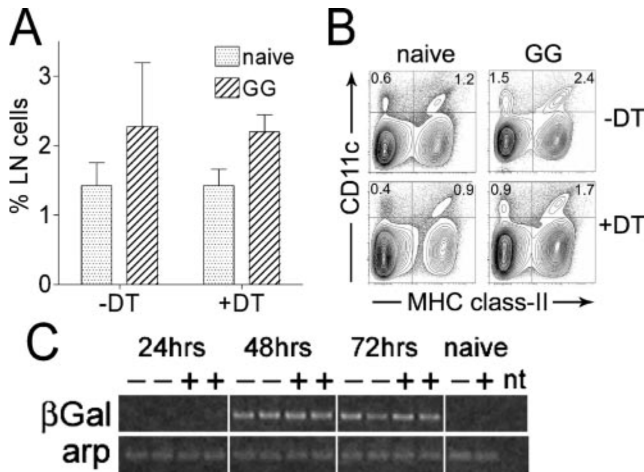

FIGURE 7. Frequency of CD11c$^+$MHC-II$^+$ cells and dynamics of vaccine transcription in draining LN. *A*, Lang-DTR mice were left untreated (−DT) or injected with 1 μg of DT (+DT) 2 days before gene gun immunization (GG) on the shaved abdomen. Two days after immunization, inguinal and axillary LN were pooled from each mouse and analyzed for CD11c$^+$MHC-II$^+$ cells, along with groups of naive controls. Data are means ± SD of $n = 4$ per group. *B*, Representative density plots of mice analyzed in *A*. *C*, Transcription of βGal DNA vaccine in draining LN of gene gun-immunized Lang-DTR mice. Mice were left untreated (−) or injected with 1 μg of DT (+) 2 days before abdominal immunization. At the indicated points of time postimmunization, inguinal and axillary LN were pooled from each mouse and analyzed by RT-PCR. Two immunized mice per condition and one naive control each are shown. Arp, housekeeping gene; nt, no template controls.

The kinetics of CD11c$^+$ MHC-II$^+$ cell immigration and vaccine gene expression in draining LN is not affected by LC ablation

The finding that epidermal gene gun immunization could induce humoral and cellular immune reactions in the absence of skin LC provoked the question as to whether other CD11c$^+$ cells in the skin might be involved in Ag presentation. For example, dermal DC could capture Ag from transfected skin cells or express Ag after direct transfection. Therefore, we investigated the effects of gene gun immunization on CD11c$^+$ cells in the draining LN. When isolated 2 days after the gene gun shot, the frequency of CD11c$^+$ MHC-II$^+$ cells was increased relative to naive mice. The observed increase in frequency among total LN cells may even be an underestimate of actual numbers of immigrating DC due to considerable LN swelling caused by concomitant lymphocyte immigration and/or proliferation. Importantly, however, there was no difference between DT-treated and untreated groups (Fig. 7, *A* and *B*). Thus, our results are consistent with the hypothesis that, upon gene gun immunization, migratory dermal DC could be activated and participate in Ag presentation in draining LN. Moreover, RT-PCR analyses revealed that DNA vaccine-specific transcripts appeared in draining LN 2 days after a gene gun shot, whereas no such transcripts were detected 24 h after the shot (Fig. 7*C*). Again, the same kinetics was observed in untreated control mice and in mice that had been injected with DT 2 days before immunization. This observation supports the idea that directly transfected DC could be involved in Ag presentation even in the absence of LC.

Discussion

In the present study, we have investigated the dependence of gene gun-induced humoral and cell-mediated immune reactions on the presence of epidermal LC. We have used a recently developed Lang-DTR knockin mouse strain that allows for conditional ablation of Langerin-positive cells. After a single injection of 1 μg of DT, LC completely disappeared from the epidermis for at least 8 days, but gradually recovered ∼2–3 wk later (33). Furthermore, examination of gene gun-immunized skin at short time intervals revealed that inflammatory reactions originating from the gene gun shot did not induce accelerated de novo recruitment of LC to the epidermis within 1 wk after DT injection. We also did not detect CD207$^+$ cells in dermal sheets of DT-treated mice (data not shown). Therefore, with repetitive injections of DT at weekly intervals, we were able to maintain LC ablation for a sufficient period to conduct long-term experiments.

Gene gun immunization of LC-depleted mice induced proliferation of CD4$^+$ and CD8$^+$ T cells of TCR transgenic OT-II or OT-I donor mice, respectively, indicating the activation of both MHC-I as well as MHC-II pathways of Ag presentation. This effect was confined to draining LN, suggesting that proliferation had probably been stimulated by skin-derived APC, rather than systemically distributed Ag. Gene gun immunization under continuous ablation of LC elicited Abs of the same isotype spectrum and comparable serum titers as in untreated Lang-DTR or wild-type mice. CD8$^+$ cytotoxic effector T cells, as defined by IFN-γ secretion in response to CTL peptide restimulation, were induced, and CTL peptide-pulsed target cells were cleared in situ with comparable efficiency in the presence or absence of LC. Moreover, gene gun immunization established protective antitumor immunity without the contribution of LC.

The critical role of skin DC for the initiation of gene gun-induced immune reactions has early been recognized and corroborated by experimental evidence. Gene gun immunization induced skin DC maturation and migration of transfected DC to draining LN (11, 14). Functionally, a role for migratory skin cells was demonstrated by transfer of gene gun-treated skin biopsies to recipient mice. Thus, grafting within a short time span after treatment induced immunity in naive recipient mice, but was gradually less effective at later time points (34). Moreover, vaccine constructs with promoter elements of fascin, CD11c, or dectin-2 induced T cell responses that were in some cases comparable to those achieved with the ubiquitously active CMV promoter (15, 18, 35). Larregina et al. (13) observed that, after gene gun treatment, CD1a$^+$ LC emigrated from epidermal sheets of human skin explants and were able to induce IFN-γ production by an Ag-specific T cell clone. Similarly, Garg et al. (12) demonstrated, in a most elegant approach, that directly transfected CD11c$^+$CD205$^+$ cells emigrated from the site of gene gun treatment to draining LN, and these cells were able to induce proliferation and IFN-γ production ex vivo. This cell preparation was also found positive for Langerin. Unfortunately, however, Langerin expression was assessed by RT-PCR on the whole population so that the possibility remains that a proportion of the transfected cells contained in this preparation were Langerin-negative DC, presumably dermal DC.

In view of such evidence, together with the ideal position of LC in the epidermis, it is not surprising that, with time, the assumption that LC are the principal APC involved in gene gun-mediated immune reactions has become paradigmatic (36–38). Nevertheless, a direct dependence of gene gun-induced immunity on LC functions has never been demonstrated. Such analysis has only become possible by the availability of transgenic mice that allow for the conditional ablation of LC without affecting other DC types. Most surprisingly, however, in all of our experiments there was neither a difference in quality nor in magnitude of gene gun-induced immune reactions between LC-depleted and intact mice. Our data provide, for the first time, unequivocal evidence that the induction of humoral as well as cell-mediated immunity by gene gun vaccination does not depend on LC. These findings may, therefore,

have important implications on our current understanding of gene gun immunization and, possibly, other immune reactions originating from skin. With respect to this, it may be of note that knockin mice for constitutive (39) or conditional (23, 24) LC ablation have recently been introduced by three independent groups. All of these mice were still able to mount contact hypersensitivity reactions to skin-painted haptens, suggesting that LC were also dispensable for this type of immune response. Under constitutive LC deficiency, contact hypersensitivity reactions were even more pronounced than in wild-type mice, pointing to a regulatory role of LC under certain conditions (39). Likewise, LC did not contribute to Ag presentation after herpes simplex infection confined to mucosal or epidermal epithelia (40, 41), and LC were not required for Ag presentation after infection with *Leishmania major* (42).

The apparent independence on LC raises the question of which type of APC might then initiate gene gun immune reactions. Histological examination of gene gun-treated skin revealed that, under the conditions chosen in our experiments, the majority of gold particles was found in the epidermis. However, a minor fraction of particles had also penetrated into the dermal layers. Dermal DC, which do not express Langerin and are therefore resistant to DT-mediated cytotoxicity in Lang-DTR mice (24), are reasonable candidates. In situ, a majority of these cells may resemble macrophages, as defined by their expression of MGL/CD301 (43) that may transform into CD11c$^+$ DC upon activation, similar to what has been described for i.d. injected macrophages (44). In fact, we found elevated numbers of CD11c$^+$ MHC-II$^+$ cells in the draining LN of gene gun-immunized mice, and these changes were also observed in DT-pretreated individuals. Also, DNA vaccine transcripts appeared 2 days after gene gun immunization in the draining LN, irrespectively of DT treatment. Yet, it is still questionable, although possible, whether a minority of transfected dermal DC would initiate immune reactions at the same magnitude as seen in wild types in which an intact population of LC could contribute to Ag presentation. As an alternative to direct transfection of APC, classical and cross-presentation of exogenous Ag acquired from transfected nonimmune cells could offer another explanation for the activation of CD4$^+$ and CD8$^+$ T cells. In this case, the complete independence on LC of gene gun-induced immunity would suggest that dermal DC are the primary APC for cross-priming in skin tissue. In contrast, cross-presentation properties of dermal DC have not been studied hitherto. Therefore, a comparison with LC, in which cross-presenting capacity has been described recently (20), is not possible at this point. Interestingly, in human skin, Angel et al. (45) have recently identified two subsets of dermal APC that are distinct of LC. One of these, a CD1a^{dim}CD14$^-$CD207$^-$ population of HLA-DR^{high} cells, also expressed the chemokine receptor CCR7. These cells were able to migrate toward CCL19 or CCL21 gradients and strongly promoted the proliferation of allogenic CD4$^+$ T cells. Moreover, inflammation caused by the gene gun shot could induce de novo recruitment of APC to the site of immunization. It seems conceivable that such cells would participate in the uptake and presentation of Ag expressed in immunized skin tissue. Finally, B cells have also been shown to contribute to cross-presentation after DNA vaccination (18). However, the significance and relative contribution of B cell-mediated cross-priming in the initial phase of an immune response remain to be clarified. Moreover, the paucity of B cells in the healthy skin would render this possibility unlikely, and we have observed unimpaired proliferation of CFSE-labeled TCR transgenic T cells in gene gun-immunized B cell-deficient μMT mice (data not shown).

Our results show that LC are not required for gene gun-mediated T and B cell responses in the mouse. They do not exclude the possibility that LC are principally capable of being activated by gene gun and, in turn, migrate to lymphoid organs to activate T cells. In fact, gene gun-induced transfection and maturation of epidermal LC and their emigration to LN have been shown experimentally, and the ability of such cells to activate T cells has also been demonstrated, at least, ex vivo (12, 13). Thus, it remains to be explained why the complete ablation of such important APC that are located at the most favorable anatomical site for gene gun immunization has no detectable consequence on any of the parameters of humoral or cell-mediated immunity that have been examined in this study.

In conclusion, our data show that LC are dispensable for the induction of both humoral and cell-mediated immune reactions by gene gun immunization through murine skin. Therefore, the identification of the primary type of APC involved in gene gun-induced immunity warrants further investigation and may aid in a more complete understanding of underlying mechanisms and the development of efficient vaccines.

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Disclosures

The authors have no financial conflict of interest.

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