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Cross-Primming as a Predominant Mechanism for Inducing CD8$^+$ T Cell Responses in Gene Gun DNA Immunization

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DNA immunization induces CD8$^+$ CTL responses by bone marrow-derived APCs, which are directly transfected with a plasmid DNA and/or acquire Ags from DNA-transfected non-APCs. To investigate the relative contribution of DNA-transfected APCs vs non-APCs to the initiation of CD8$^+$ T cell responses, we used tissue-specific promoter-directed gene expression and adoptive transfer systems in gene gun DNA immunization. In this study, we demonstrated that non-APC-specific gene expressions induced significant CD8$^+$ CTL and IFN-γ-producing cells and Ab responses, whereas APC-specific gene expressions led to moderate CTL and IFN-γ-producers, but no Ab responses. Interestingly, mice immunized with a non-APC-specific plasmid induced more rapid, vigorous, and prolonged proliferation of adaptively transferred Ag-specific CD8$^+$ T cells than APC-specific plasmid-immunized mice. In addition, the in vivo proliferative responses elicited by a non-APC-specific plasmid administration were dependent on TAP, but were independent of CD4$^+$ T cell help. Collectively, our results suggest that cross-priming, in which Ags expressed in non-APCs are taken up, processed, and presented by APCs, plays an important role in the initiation, magnitude, and maintenance of CD8$^+$ T cell responses in gene gun DNA immunization.

insight into the relative contribution of direct priming vs cross-priming to the initiation, magnitude, and maintenance of CD8+ T cell responses in gene gun DNA immunization.

Materials and Methods

Mice
Female BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). MHCI- and TAP-deficient C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Transgenic C57BL/6 mice for a TCR recognizing the OVA257–264 epitope of the hen egg OVA in association with H-2Kb molecules were generously provided by Dr. W. R. Heath (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) (18). The mice were bred and maintained in specific pathogen-free conditions. All mice used here were 6–8 wk old.

Plasmid construction
To generate the pCMV-Luc, firefly luciferase cDNA from pGL2-control vector (Promega, Madison, WI) was inserted into the pCMV vector containing the CMV promoter and the SV40 polyadenylation signal (poly(A)). The CMV promoter of the pCMV-Luc was then replaced by the 2.2-kb human katatin 14 (K14) promoter (a kind gift from Dr. E. Fuchs, University of Chicago, Chicago, IL; see Ref. 19), the 1.7-kb human CD11b promoter (a kind gift from Dr. D. G. Tenen, Harvard Medical School, Boston, MA; see Ref. 20), and the 2.1-kb murine MHCI gene promoter (a kind gift from Dr. C. Benoist and Dr. D. Mathis, Institut de Genetique et de Biologie Moléculaire et Cellulaire, Illkirch, Strasbourg, France; see Ref. 21) to produce the pK14- , pCD11b-, and pMHCI-Luc, respectively. For investigating Ag-specific immune responses, 1.5-kb nucleoprotein (NP) cDNA of the A/P/R/8/34 influenza virus or 1.2-kb OVA cDNA was used to replace the luciferase gene of the pCMV-, pK14-, pCD11b-, and pMHCI-Luc to generate pCMV-, pK14-, pCD11b-, and pMHCI-NP (or -OVA), respectively. We also constructed pTV-GM-CSF and pTV-IL-4 encoding GM-CSF and IL-4, respectively, as previously described (22). These plasmids were propagated in Escherichia coli and were purified using an Endofree plasmid purification kit (Qiagen, Chatsworth, CA).

Cell culture
HaCaT (human keratinocyte cell line), RAW264.7 (mouse macrophage cell line), and COS-7 (monkey kidney cell line) cells were maintained in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml 2-ME, and 2 mM glutamine (all purchased from Life Technologies, Rockville, MD).

Cell transfection and luciferase assay
Transfection was performed by electroporation using the Gene Pulser (Bio-Rad, Hercules, CA) set at 220–280 V and 960 µF capacitance. Cells (5 × 10⁶) were resuspended in 0.4 ml of supplemented DMEM and were transfected with 45 µg of each luciferase reporter plasmid together with 5 µg of the plasmid pCH110 (Amersham Pharmacia Biotech, Piscataway, NJ), which expresses β-galactosidase (β-gal), as a control for differences in transfection efficiency between and within various cell lines, in 0.4-cm electrode gap cuvettes (Bio-Rad). The luciferase and β-gal activities were measured 48 h after transfection using Promega assay systems as previously described (23).

Gene gun delivery of plasmid DNA
Sodium pentobarbital-anesthetized mice received two or four nonoverlapping abdominal deliveries of 0.5 mg of gold beads (1 µm) coated with various plasmid DNA using the Helios gene gun system (Bio-Rad) at a voltage of 280 V and 960 Ω (1). The mice were electroporated in carbonate buffer, pH 9.5, and incubated at room temperature by several washes with distilled water. After drying, spots were collected and then analyzed using CellQuest software (BD Biosciences).

ELISA for anti-NP IgG and CTL assay
For determination of NP-specific Ab responses, microtiter plates were coated with NP at 2 µg/ml (100 µl) in carbonate buffer, pH 9.5, and individually diluted in PBS. These plates were then incubated with anti-β-gal mAb or by ELISA as previously described (24). For determination of NP-specific CTL responses, pooled splenocytes were cultured for 6 days at 2 × 10⁶ cells/ml in the presence of 10 µM H-2Kb-restricted NP147–155 peptide (TYQRTRALV; Alberta Peptide Institute, Alberta, Canada) and 10 U/ml murine rIL-2 (BD Pharmingen, San Diego, CA). Cell-mediated cytotoxicity was determined by a standard 51Cr release assay using P815 (H-2d) mastocytoma cells as previously described (22). Nonspecific lysis was evaluated using P815 cells pulsed with an irrelevant H-2Ld-restricted β-gal peptide (TPHPARIGL; Alberta Peptide Institute). In our condition for CTL assay, lysis of P815 cells pulsed with irrelevant β-gal peptide was <5% in all groups of mice (data not shown).

LDA for CTL
Limiting dilution analysis (LDA) for cytotoxicity was performed as previously reported (26). Pooled splenocytes were 3-fold serially diluted in 96-well U-bottom plates (24 replicates/dilution). The 10 µM NP147–155 peptide and 10 U/ml murine rIL-2 were added to each well. On day 6, 100 µl of cultured cells from each well was transferred to 96-well plates containing 10 µM NP147–155 peptide-pulsed 51Cr-labeled P815 cells for a standard 51Cr release assay. Wells were scored as positive for CTL recognition if the specific lysis exceeded 3 SD above the mean control release from the target cells pulsed with irrelevant β-gal peptide. The CTL precursor (C11p) frequency was estimated at which 37% of the wells were negative from the slope of a regression plot of the log percentage of negative vs input cell numbers.

ELISPOT assay
An ELISPOT assay was performed as previously described (27). Nitrocellulose plates (96-well; Millipore, Bedford, MA) were coated with the anti-mouse IFN-γ Ab (5 µg/ml; BD PharMingen). Pooled splenocytes were 3-fold serially diluted and incubated for 24 h with 10 µM NP147–155 peptide or irrelevant β-gal peptide in the presence of 10 U/ml murine rIL-2. Each dilution was seeded in quadruplicate. The plates were washed six times with PBS containing 0.05% Tween 20. To detect IFN-γ-specific spots, 2.5 µg/ml biotinylated anti-mouse IFN-γ mAb (BD Pharmingen) was added and incubated at room temperature for 2 h, followed by alkaline phosphatase-coupled streptavidin (BD Pharmingen) for 1 h. Spots of IFN-γ-secreting cells were visualized by adding 5-bromo-4-chloro-3-indolyl phosphate/tetranitroblue tetrazolium (TNBT) substrate solution (Calbiochem, San Diego, CA). The reaction was stopped after 15–20 min at room temperature by several washes with distilled water. After drying, spots were counted under a dissecting microscope. The frequency of peptide-specific T cells is expressed as the number of IFN-γ-secreting cells per 10⁵ splenocytes.

Preparation of OT-I cells for adoptive transfer
Transgenic OVA-specific MHCI class I-restricted CD8+ T (OT-I) cells were purified from the spleen and LNs of OT-I mice as previously described (28). Cell suspensions were treated with J11d (anti-HSA), RI/72 (anti-CD4), and M5/14.15.2 (anti-MHCI) for 30 min on ice, and then depleted by treatment with rabbit complement (Calbiochem) for 30 min at 37 °C. The purified OT-I cells were resuspended at 5 × 10⁶ cells/ml in PBS containing 0.1% BSA and then incubated with 10 µM CFSE (Molecular Probes, Eugene, OR) for 10 min at 37 °C. Cells were washed twice with cold RPMI 1640 containing 10% FBS followed by two washes in PBS. CD8+Vα1Vβ5–1 CFSE+ OT-I cells (2 × 10⁶) in 200 µl of PBS were injected into the tail vein of mice.

FACS analysis
Inguinal LN cells were isolated and stained with PE-conjugated anti-CD8 (53-6.7) mAb, PE- or biotin-conjugated anti-Vα2 TCR (B20.1), and anti-Vβ5.1/2 TCR (MR9-4) mAb (BD Pharmingen). Biotin-labeled mAbs were detected with streptavidin-conjugated PerCP (BD Biosciences), for 1 h. Spots of IFN-γ-secreting cells were visualized by adding 5-bromo-4-chloro-3-indolyl phosphate/tetranitroblue tetrazolium (TNBT) substrate solution (Calbiochem, San Diego, CA). The reaction was stopped after 15–20 min at room temperature by several washes with distilled water. After drying, spots were counted under a dissecting microscope. The frequency of peptide-specific T cells is expressed as the number of IFN-γ-secreting cells per 10⁵ splenocytes.

Results
Plasmid vectors with the K14, CD11b, or MHCI gene promoter can drive the cell type-specific expression of a reporter gene in various cell lines
To separately investigate the in vivo function of DNA-transfected APCs and non-APCs in gene gun DNA immunization, we used a
specialized plasmid DNA with well-characterized tissue- and cell-type-specific promoters that selectively produce DNA-encoded Ags in either APCs or non-APCs in vivo. Gene gun administration of a plasmid DNA is known to primarily transfect non-APCs such as keratinocytes, which play a role as an Ag reservoir in the epidermis of the bombarded skin (29–32). In contrast, the gene gun immunization has been shown that a small number of APCs such as DCs were transsected (7, 8, 31). Although Langerhans cells and dermal DCs are known to be potent dendritic APCs in the bombarded skin, macrophages are also likely candidates for APC function. Thus, we used two APC-specific promoters, the CD11b and the MHCII gene promoter, which are specific for all skin APCs, including macrophages, Langerhans cells, and dermal DCs (20, 21, 33–35). The K14 promoter was also selected for specific gene expression in squamous epithelial cells such as keratinocytes (19, 36, 37).

The tissue and cell-type specificities of these promoters have been well known in various transgenic mice systems (19–21, 33, 34, 36). To further confirm the strength and tissue specificities of these promoters in cultured cell lines, we constructed various plasmid DNA encoding the luciferase gene under the control of the K14, the CD11b, the MHCII, or the CMV promoter as a positive control (Fig. 1A). These plasmids were transiently transfected into various cell lines and their relative luciferase activities were analyzed (Fig. 1B). Significant luciferase activity from pK14-Luc was observed only in the keratinocyte-derived cell line (HaCaT), but pCD11b- and pMHCII-Luc showed luciferase activity exclusively in the macrophase-derived cell line (RAW264.7). In contrast, there was no luciferase activity in the nonspecific COS-7 cell line. We also could not detect any significant luciferase activity in other cell lines including the muscle, kidney, melanoma, ovary, colon, cervix, liver, and fibroblast cell lines (data not shown). Similar results were also obtained when the green fluorescent protein (GFP) was used as the reporter gene (data not shown). It was notable that the relative luciferase activity driven by the K14 promoter in HaCaT cells was similar to that driven by the CD11b or the MHCII promoter in RAW264.7 cells, although their activities were approximately two to three times lower than those driven by the CMV promoter. These results indicate that a plasmid DNA containing the CD11b, the MHCII, or the K14 promoter would allow us to separately investigate the in vivo function of DNA-transfected APCs and non-APCs after gene gun DNA immunization.

**Immunization with pK14-NP, but not with pCD11b- or pMHCII-NP, induces significant NP-specific Ab responses**

Using these promoters described above, we investigated whether DNA-transfected APCs or non-APCs are capable of producing a sufficient level of Ags for inducing Ab responses after gene gun DNA immunization. Female BALB/c mice were immunized at 0 and 3 wk by two nonoverlapping gene gun deliveries with gold beads coated with pCMV–, pK14–, pCD11b–, or pMHCII-NP plasmid encoding influenza virus NP or with a mock plasmid DNA. At 5 wk after the first DNA immunization, pooled sera from the immunized mice were analyzed for NP-specific Ab responses.

As expected, mice immunized with pK14-NP that directs NP expression in keratinocytes produced significant levels of anti-NP IgG responses, suggesting that keratinocytes are the major cells that take up the injected plasmid DNA and produce enough Ags to generate Ab responses (Fig. 2A). These data support a previous study in which nonmigratory cells such as keratinocytes influenced the magnitude of Ab responses after gene gun DNA immunization (7). It is of interest that there were no detectable anti-NP IgG responses in mice immunized with pCD11b– or pMHCII-NP that directs NP expression in APCs. However, we could detect a few GFP-positive DCs in draining LNs of pCD11b– or pMHCII-GFP-immunized mice (data not shown). Given that the relative strength of the CD11b or the MHCII promoter was similar to that of the K14 promoter (Fig. 1B), the lack of Ab responses in pCD11b– or pMHCII-NP-immunized mice is not likely to result from the weak promoter activities or no in vivo transfection of APCs. Alternatively, this may be caused by the insufficient amount of Ags produced by a small number of DNA-transfected APCs in vivo. As a positive control, pCMV-NP-immunized mice induced slightly higher anti-NP IgG responses than pK14-NP-immunized mice (Fig. 2A), presumably due to an increased amount of Ags resulting from a stronger and broader specificity into various cell types of the CMV promoter in comparison with the K14 promoter (Fig. 1B). In contrast, there were no anti-NP IgG responses in mock plasmid DNA-immunized mice as a negative control (Fig. 2A).

To study whether the tissue-specific Ag expression affects the isotypes of anti-NP IgG responses, the relative levels of anti-NP IgG1 and IgG2a responses were determined from individual sera of the immunized mice described above. It was previously reported...
that gene gun DNA immunization using a CMV promoter-containing plasmid favors the development of IgG1 dominant responses compared with i.m. DNA immunization (38). Similarly, a preferential IgG1 response appeared to be induced in pK14-NP-immunized mice because the relative ratio of IgG1 to IgG2a is comparable with that of pCMV-NP-immunized mice (1.24 vs 1.29; Fig. 2).

**Immunization with pK14-NP induces higher NP-specific CTL and IFN-γ-producing T cells than pCD11b- or pMHCII-NP immunization**

Although directly transfected APCs are generally accepted to be essential for CTL priming in gene gun DNA immunization (6–8), the role of DNA-transfected non-APCs has been unclear. Thus, we wanted to determine the relative contribution of DNA-transfected APCs and non-APCs to the induction of CTL responses in gene gun DNA immunization.

At 3 wk after the first DNA immunization, we determined NP-specific CTL responses in pooled splenocytes from the immunized mice described in Fig. 2. As shown in Fig. 3A, mice immunized with pCMV-NP induced robust levels of NP-specific CTL responses, whereas mock vector-immunized mice as a negative control did not. Interestingly, pK14-NP-immunized mice elicited higher levels of NP-specific CTL responses than pCD11b- or pMHCII-NP-immunized mice, but induced slightly lower responses than pCMV-NP-immunized mice. We also observed the similar pattern of NP-specific CTL responses at 2 and 5 wk after the booster DNA immunization (data not shown).

We next measured the number of NP-specific IFN-γ-producing CD8⁺ T cells by an ELISPOT assay (Fig. 3B). As expected, pK14-NP-immunized mice induced a 3.5 and 4.2 times higher number of NP-specific IFN-γ-producing CD8⁺ T cells than pCD11b- and pMHCII-NP-immunized mice, respectively. In addition, pCMV-NP-immunized mice induced the highest number of NP-specific IFN-γ-producing CD8⁺ T cells, whereas mock vector-immunized mice failed to induce significant IFN-γ-producers.

We further confirmed the above results by performing LDA of NP-specific CTLp frequency (Table 1). Mice immunized with pK14-NP induced one CTLp in every 87,500–77,000 splenocytes, whereas mock vector-immunized mice showed less than one CTLp in every 1,200,000 splenocytes.

Because keratinocytes are incapable of directly priming T cell responses because of their lack of costimulatory molecules (13), the ability of keratinocytes to efficiently induce CD8⁺ T cell responses appeared to depend on cross-priming in which Ags produced by DNA-transfected keratinocytes must be transferred to bone marrow-derived APCs. Thus, our results showed that cross-priming alone sufficiently contributes to CD8⁺ T cell priming in gene gun DNA immunization using various promoter-driven NP-expressing plasmids. Groups of BALB/c mice were immunized as described in Fig. 2. At 3 wk after the first DNA immunization, pooled splenocytes from three mice per group were assayed for determining the MHC class I-restricted NP147–155 peptide-specific CTL responses (A) and IFN-γ-secreting CD8⁺ T cells (B) by performing CTL and ELISPOT assay, respectively. The data were represented as the mean ± SD of three independent experiments.
To investigate the efficacy and kinetics of the induction of CD8⁺ T cell responses directly in vivo after gene gun DNA immunization, naive OT-I cells were labeled with CFSE, which allows us to monitor cell division in vivo, and they were then adoptively transferred into female C57BL/6 mice. One day after adoptive transfer, the mice were immunized once by two nonoverlapping gene gun deliveries with gold particles coated with pCMV-, pK14-, pCD11b-, or pMHCI-OVA plasmid encoding OVA or with a mock plasmid DNA. At various time points after DNA immunization, cells isolated from draining LNs of the immunized mice were analyzed for detecting in vivo proliferation of CFSE-labeled OT-I cells by flow cytometry.

The OT-I cells in pCMV-OVA-immunized mice began to proliferate on day 3 (data not shown), some of them dividing more than five times on day 4, and continued to proliferate for up to 21 days (Fig. 4A). As a negative control, there was no detectable proliferation of OT-I cells in mock vector-immunized mice (Fig. 4E). These results indicate that OT-I cells can be activated and subsequently proliferate in draining LNs in response to OVA-derived epitope presented in association with MHC class I molecules on direct- or cross-presented APCs after gene gun DNA immunization. Interestingly, the OT-I cells in pK14-OVA-immunized mice began to proliferate 2–3 days earlier than those in pCD11b- or pMHCI-OVA-immunized mice (4 vs 7 days; Fig. 4, B–D). These results indicate that cross-priming is not only involved in priming CD8⁺ T cell responses, but can also initiate more rapid responses than direct priming in vivo. In addition to earlier OT-I T cell responses, more vigorous OT-I cell proliferation was detected at 7 and 21 days, and an obvious proliferation signal, albeit weak, was also detected at 35 days after pK14-OVA immunization (Fig. 6). In contrast, there was no detectable OT-I cell proliferation at 49 days after pK14-OVA immunization, which is enough to prime adoptively transferred OT-I cells in vivo.

In vivo proliferation of OT-I cells after pK14-OVA immunization was dependent on TAP, but was independent of CD4⁺ T cell help

It has been previously reported that in vivo cross-priming occurs in either a TAP-dependent (44, 45) or a TAP-independent manner

<table>
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<th>Plasmids</th>
<th>CTLp Frequency *10^6</th>
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<tr>
<td>Mock DNA</td>
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<td>&gt; 1.20 ± 10^6</td>
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</table>

*1 Groups of BALB/c mice were immunized as described in Fig. 2. At 3 wk after the first DNA immunization, pooled splenocytes from three mice per group were incubated in vitro with 10 μM NP147–155 peptide for 6 days, and the NP-specific CTLp frequency in the spleen of the immunized mice was estimated by LDA as described in Materials and Methods.

In vivo proliferation of adoptively transferred CFSE-labeled OT-I cells after gene gun immunization with various promoter-driven OVA-expressing plasmids. C57BL/6 mice were i.v. injected with 2 × 10⁶ CFSE-labeled OT-I cells, and 1 day later were immunized once by two nonoverlapping gene gun deliveries of gold particles coated with 1 μg of pCMV-OVA (A), pK14-OVA (B), pCD11b-OVA (C), pMHCI-OVA (D), or mock plasmid DNA (E). At the indicated time points after DNA immunization represented on the right of this figure, pooled draining inguinal LNs from two mice per group were analyzed by flow cytometry. Profiles were gated on CD8⁺ cells. Data are represented on a contour plot showing CFSE (x axis) vs CD8 fluorescence (y axis). The data are representative of two independent experiments with similar results.
were gated on CD8^+ LNs from two mice per group were analyzed by immunization. On day 3 after adoptive transfer, pooled draining inguinal DNA immunization, pooled draining inguinal LNs from two mice per each of pCD11b-OVA, pTV-GM-CSF, and pTV-IL-4 (D). At 4 days after DNA immunization, cells from draining LNs of the immunized mice were analyzed by flow cytometry. Profiles were gated on CD8^+ cells. Data are represented on a contour plot showing CFSE (x axis) vs CD8 fluorescence (y axis). The data are representative of two independent experiments with similar results.

To investigate whether TAP is required for OT-I cell proliferation after pK14-OVA immunization, TAP-deficient mice (TAP^{−/−}) or wild-type (TAP^{+/+}) mice adoptively transferred with CFSE-labeled OT-I cells were immunized once with pK14-OVA or mock DNA-coated gold particles. At 9 days after DNA immunization, cells from draining LNs of the immunized mice were analyzed by flow cytometry. Significant OT-I cell proliferation was detected in TAP^{+/+} mice, whereas there was no detectable OT-I cell proliferation in TAP^{−/−} mice (Fig. 7, A and B). In addition, there was no detectable OT-I cell proliferation in both TAP^{+/+} and TAP^{−/−} mice after mock DNA immunization (data not shown). These results further support the absolute requirement of TAP for cross-priming to occur in vivo.

In DNA immunization, the question of whether the induction of CD8^+ T cell responses is dependent on CD4^+ T cell help has remained controversial (49–53). To investigate whether CD4^+ T cell help is required for OT-I cell proliferation, MHCII-deficient mice (MHCII^{−/−}) or wild-type (MHCII^{+/+}) mice adoptively transferred with CFSE-labeled OT-I cells were immunized once with pK14-OVA- or mock DNA-coated gold particles. At 9 days after DNA immunization, cells from draining LNs from the immunized mice were analyzed by flow cytometry. Interestingly, both MHCII^{−/−} and MHCII^{+/+} mice showed significant OT-I cell proliferation after pK14-OVA but not mock DNA (data not shown) immunization (Fig. 7, C and D), suggesting CD4^+ Th-independent cross-priming. It is worth noting that OT-I cell proliferation in MHCII^{+/+} mice appeared to be a little higher than that in MHCII^{−/−} mice. These results agree well with the previous observation that the induction of CD8^+ T cell responses is independent of CD4^+ T cell help after DNA immunization (51). Similarly, it has been reported that significant CD8^+ T cell responses can be induced after injection with a plasmid DNA encoding a CTL epitope only (52, 53). Thus, our results further reinforce the notion that CD4^+ T cell help is not essential for the induction of CD8^+ T cell responses in DNA immunization.

Discussion

In the present study, we have shown that cross-priming contributes predominantly to the initiation, magnitude, and maintenance of CD8^+ T cell responses in gene gun DNA immunization. Our observations are partially inconsistent with previous results reported by Porgador et al. (8). The discrepancies could be caused by differences in the experimental methods for measuring the ability of APCs to prime CD8^+ T cells. In particular, the previous experiments were performed in the in vitro condition in which APCs isolated from draining LNs within 24 h after gene gun DNA immunization were incubated with a CD8^+ T cell clone to measure IFN-γ secretion (8). It has been previously reported that CD8^+ T

FIGURE 5. The effect of delivery number and cytokine genes on in vivo OT-I cell proliferation. C57BL/6 mice were i.v. injected with 2 × 10^6 CFSE-labeled OT-I cells. One day later, mice were immunized once by two (A) and four (B–D) nonoverlapping gene gun deliveries of gold particles coated with 1 µg of pCD11b-OVA (B), or cocoated with 0.5 µg of each of pCD11b-OVA and pTV-GM-CSF (C), or cocoated with 0.5 µg of each of pCD11b-OVA, pTV-GM-CSF, and pTV-IL-4 (D). At 4 days after DNA immunization, pooled draining inguinal LNs from two mice per group were analyzed by flow cytometry. Profiles were gated on CD8^+ cells. Data are represented on a contour plot showing CFSE (x axis) vs CD8 fluorescence (y axis). The data are representative of two independent experiments with similar results.

FIGURE 6. The period of Ag persistence required for in vivo OT-I cell proliferation after pK14-OVA immunization. C57BL/6 mice were immunized once by two nonoverlapping gene gun deliveries of gold particles coated with 1 µg of pK14-OVA and i.v. injected with 2 × 10^6 CFSE-labeled OT-I cells at days 7 (A), 21 (B), 35 (C), and 49 (D) after DNA immunization. On day 3 after adoptive transfer, pooled draining inguinal LNs from two mice per group were analyzed by flow cytometry. Profiles were gated on CD8^+ cells. Data are represented on a contour plot showing CFSE (x axis) vs CD8 fluorescence (y axis). The data are representative of two independent experiments with similar results.

FIGURE 7. The dependency of TAP or CD4^+ T cell help on in vivo OT-I cell proliferation after pK14-OVA immunization. TAP^{+/+} (A) and TAP^{−/−} (B) mice, as well as MHCII^{+/+} (C) and MHCII^{−/−} (D) mice were i.v. injected with 2 × 10^6 CFSE-labeled OT-I cells, and 1 day later were immunized once by two nonoverlapping gene gun deliveries of gold particles coated with 1 µg of pK14-OVA. At 9 days after DNA immunization, pooled draining inguinal LNs from two mice per group were analyzed by flow cytometry. Profiles were gated on CD8^+ cells. Data are represented on a contour plot showing CFSE (x axis) vs CD8 fluorescence (y axis). The data are representative of two independent experiments with similar results.
cell clones have a lower activation threshold in proliferation after Ag stimulation (54–56), presumably allowing the CD8\(^+\) T cell clones to be activated at earlier time points and at lower doses of Ag stimulation than would be required in naive CD8\(^+\) T cells in vivo. These could explain why in vivo proliferation of OT-I cells in our studies was not detected until 3 days after the pCMV-OVA immunization (data not shown). Thus, DNA-transfected APCs in draining LNs within 24 h after gene gun DNA immunization appear to be sufficient to stimulate a CD8\(^+\) T cell clone in vitro (8), but not naive CD8\(^+\) T cells in vivo. In support of this hypothesis, it was previously reported that excision of the epidermal site 24 h after cutaneous DNA immunization abrogated the induction of CTL responses (57) despite DNA-transfected DCs being detected in draining LNs less than 24 h after gene gun DNA immunization (6, 8). This indicates that the Ag presentation by a very small percentage of DNA-transfected DCs in draining LNs within 24 h after gene gun DNA immunization is insufficient to induce detectable CD8\(^+\) T cell responses.

Interestingly, our results demonstrated that DNA-transfected APCs were shown to initiate CD8\(^+\) T cell responses slower than APCs that take up and present Ags produced by DNA-transfected non-APCs (Fig. 4, B–D), which might be explained by the following reasons. First, the number of DNA-transfected APCs appears to be a critical parameter in determining earlier CD8\(^+\) T cell responses. It is possible that the number of DNA-transfected APCs might not be enough to induce an earlier proliferation of OT-I cells after pCD11b- or pMHCII-OVA immunization. It was previously reported that the number of DNA-transfected APCs was proportionally increased by the delivery number of DNA-coated gold particles (8). However, the kinetics of initial OT-I cell proliferation was not changed even by four nonoverlapping gene gun deliveries with pCD11b-OVA (Fig. 5B), although the magnitude of proliferative responses was slightly increased at later time points (days 7 and 9; data not shown). It is likely that the very small number of DNA-transfected APCs in draining LNs appears to be an intrinsic problem in gene gun DNA immunization, because a 2-fold increase of their number was still insufficient to induce an earlier proliferation of OT-I cells after pCD11b- or pMHCII-OVA immunization. Second, it is possible that DNA-transfected APCs might transfer their antigenic materials into other APCs in vivo because short-lived immature peripheral DCs were reported to transfer their Ags to resident DCs in draining LNs (58). It was previously reported that the dose of Ags could affect the kinetics of proliferation of CD8\(^+\) T cells in vitro (59) and that cross-priming occurred only in situations in which the dose of Ags exogenously produced was beyond a certain threshold level (28). These previous reports allow us to postulate that a very small number of DNA-transfected APCs after gene gun DNA immunization (8) produced only the low amount of Ags that cannot mediate efficient cross-priming in vivo, thereby leading to the delayed OT-I cell proliferation in pCD11b- or pMHCII-OVA-immunized mice (Fig. 4, C and D).

It was previously reported that naive transgenic CD4\(^+\) T cells were not activated when adoptively transferred into mice that had been s.c. immunized with a plasmid DNA 20 days before (13). However, Doe et al. (3) showed that significant CD8\(^+\) CTL responses were induced when immunocompetent spleen and bone marrow cells were transferred into histoincompatible SCID mice at 21 days after i.m. DNA immunization. These discrepancies may be due to the route of DNA administration (s.c. vs i.m.), the nature of encoded Ags, the difference of activation threshold between CD4\(^+\) and CD8\(^+\) T cells, and the methods for evaluating the induced T cell responses. In the present study, we demonstrated that adaptively transferred naive OT-I cells can proliferate when mice immunized with pK14-OVA at 35 days before adoptive transfer (Fig. 6). Considering the short half-life of both DCs (60, 61) and extracellular plasmid DNA in vivo (62), it is likely that DNA-transfected keratinocytes persistently produce Ags up to at least 5 wk after pK14-OVA immunization, thereby allowing APCs to take up and present them to naive OT-I cells in vivo. Our results agree well with the previous results that DNA-transfected keratinocytes could produce Ags for long periods after gene gun DNA immunization (32).

It has been reported that cross-priming plays an important role in inducing CD8\(^+\) T cell responses to peripheral self, viral, tumor, and bacterial Ags (28, 45, 46, 63). This indicates that cross-priming is a general mechanism for the induction of CD8\(^+\) T cell immunity and/or tolerance. However, it remains to be determined how Ags produced by DNA-transfected keratinocytes are transferred into APCs. One possibility is that heat shock protein carrying antigenic peptides might be released from transfected keratinocytes and be transferred into APCs. Recent studies have suggested that the transfer of heat shock protein 70-linked peptides into APCs may induce CD8\(^+\) T cell responses after DNA immunization (64). Alternatively, the transfected keratinocytes might somehow undergo either necrosis or apoptosis by which proteins could be taken up and processed by APCs to induce CD8\(^+\) T cell responses (65–67). It was previously reported that apoptotic but not intact keratinocytes transfected with a plasmid DNA resulted in the activation of an Ag-specific T cell line via cross-priming in vitro (13). In addition, it was recently shown that a cell-associated Ag is more efficient for inducing CD8\(^+\) T cell responses in vivo than a soluble Ag (68).

To our knowledge, these studies are the first to show the kinetics of in vivo proliferation of Ag-specific CD8\(^+\) T cells after gene gun DNA immunization, providing clear in vivo evidence that cross-priming plays an important role in the initiation, magnitude, and maintenance of CD8\(^+\) T cell responses in gene gun DNA immunization. Our findings further suggest that appropriate methods that facilitate Ag transfer from DNA-transfected non-APCs to APCs will be one of the critical factors for designing optimal DNA vaccines to induce faster and stronger CD8\(^+\) T cell responses in gene gun DNA immunization.

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