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J Immunol 2007; 179:6725-6733; doi: 10.4049/jimmunol.179.10.6725
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The duration of Ag expression in vivo has been reported to have a minimal impact on both the magnitude and kinetics of contraction of a pathogen-induced CD8+ T cell response. In this study, we controlled the duration of Ag expression by excising the ear pinnae following intradermal ear pinnae DNA immunization. This resulted in decreased magnitude, accelerated contraction and differentiation, and surprisingly greater secondary CD8+ T cell responses. Furthermore, we found delayed and prolonged Ag presentation in the immunized mice; however, this presentation was considerably decreased when the depot Ag was eliminated. These findings suggest that the magnitude and the contraction phase of the CD8+ T cell response following intradermal DNA immunization is regulated by the duration rather than the initial exposure to Ag.

The kinetics of Ag-specific CD8+ T cell expansion, differentiation, and contraction have been carefully delineated. Ag-specific CD8+ T cells undergo a phase of massive expansion following infection or vaccination, during which time they acquire effector functions and disseminate throughout the body. This large population of effector cells decreases in number after Ag clearance and differentiates into long-lived memory CD8+ T cells (1). Following re-exposure to cognate Ag, the memory CD8+ T cells expand rapidly, regaining effector function and differentiating into effector-memory cells (2).

The contribution of Ag persistence to the kinetics of a CD8+ T cell response has been questioned. A brief exposure to Ag can drive the clonal expansion of CD8+ T cells and their subsequent differentiation into the memory compartment. In vitro studies have shown that as little as 24 h of Ag stimulation is sufficient to induce the proliferation and differentiation of these cells (3, 4). Consistent with these findings, shortening the duration of active Listeria monocytogenes infection in vivo to <48 h by antibiotic treatment does not affect the magnitude of the elicited CD8+ T cell population, the kinetics of contraction of those cells, or their ability to generate a secondary immune response (5–7). However, in these Listeria studies, the pathogen was administrated systemically and caused inflammation, which can enhance Ag presentation and obscure the need for Ag persistence (8). In fact, Prlic et al. (9) found that elimination of peptide-pulsed dendritic cells (DC)3 after less than a 48-h exposure decreased the magnitude of the induced CD8+ T cell. In addition, it has been shown that protracted Ag-presenting activity following acute infection with vesicular stomatitis virus and influenza virus can alter antiviral immunity (10–12). These observations suggest that the duration of Ag presentation may shape the expansion of effector CD8+ T cells.

Because CD4+ T cells can be important for the maintenance of memory CD8+ T cells following an infection (13), the duration of Ag expression in vivo may affect the Ag-specific CD8+ T cells through an effect on CD4+ T cells. CD4+ T cells have been reported to require durable Ag stimulation during the period of their expansion (14, 15) and the absence of help from CD4+ T cells may impact the secondary expansion of CD8+ T cells (16). A recent study has even suggested that CD4+ T cells can contribute to prolonged Ag-presenting activity by protecting APCs from CTL-mediated killing (17). Therefore, the duration of Ag expression in vivo may be affecting CD8+ T cell responses through an indirect mechanism.

To evaluate the impact of Ag persistence on the generation and differentiation of CD8+ T cells in vivo, we immunized mice in the ear pinnae with plasmid DNA and removed the ear pinnae at various times postimmunization. Using this model to control depot Ag expression, we demonstrate that the duration of Ag expression following intradermal DNA immunization has a substantial impact on the elicited CD8+ T cell response.

Materials and Methods

Abs and reagents

The Abs used in this study were directly coupled to FITC, PE, allophycocyanin, allophycocyanin-Cy7, PerCP-Cy5.5, Alexa Fluor 700, or PE-Cy7. The following mAbs were used: anti-CD62 ligand (CD62L, MEL-14; BD Biosciences), anti-IL-2 (JHS6-5H4; BD Biosciences), anti-CD8α (53-6.7; BD Biosciences), anti-IFN-γ (XMG1.2; BD Biosciences), anti-IL-2 (JHS6-5H4; BD Biosciences).
anti-CD127 (ATR34; eBioscience), anti-CD27 (LG.7F9; eBioscience), anti-CD4 (GK1.5; BD Biosciences), and anti-Thy.1.2 (53-2.1; BD Biosciences). The SIINFEKL–specific MHC class I tetramers were purchased from Beckman Coulter.

**Vectors**

The codon-optimized HIV-1 HXB2 env or the firefly luciferase genes were cloned into the VRC vector (DNA-gp120 and DNA-luc, respectively). The empty VRC vector was provided by Dr. G. Nabel (Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health). The recombinant replication-defective adenovirus human serotype 5 containing the HIV-1 HXB2 env (rAd-gp140) was provided by Dr. G. Nabel. The pACB-OVA plasmid was a gift from Dr. M. Corr (University of California, San Diego, CA). All plasmids used in this study were prepared using the EndoFree plasmid giga kit (Qiagen) and the levels of endotoxin in the DNA preparations were always <0.1 endotoxin unit/μg DNA.

**Mice and immunizations**

Six- to 8-wk-old female BALB/c, B6, OT-1 Thy1.2+ and B6 Thy1.1+ mice were purchased from The Jackson Laboratory and maintained under specific pathogen-free conditions. Research on mice was approved by the Beth Israel Deaconess Institutional Animal Care and Use Committee. Mice were anesthetized with ketamine/xylazine mix and then injected in the ear pinnae by using a 31-gauge needle with DNA-gp120, DNA-luc, or pACB-OVA (50 μg of DNA in 80 μl of total injection volume, 40 μl was delivered into each ear). For i.m. injection, the mice received the DNA-gp120 and DNA-luc (50 μg of DNA in 100 μl of total injection volume, 50 μl was delivered into each quadriceps muscle). In some experiments, mice were boosted i.m. 10 wk after the first immunization with rAd-gp140 (10^6 particles). Removal of the ear pinnae was performed after the mice were anesthetized with ketamine/xylazine mix and by using sterile scissors.

**Phenotypic T lymphocyte analyses**

Tetrameric H-2Dd complexes folded with the gp120/p18 epitope peptide (RGPGRAFVTI) (18) were prepared as previously described (19). Blood was collected from individual mice in RPMI 1640 medium containing 400 μl of heparin/ml and PBMCs were isolated using Lympholyte-M (Cedarlane Laboratories). Cells were washed with PBS containing 2% FBS and stained for 15 min at room temperature with PE-conjugated H-2Dd/p18 tetramer. The cells were then stained with anti-CD8α, anti-CD62L, anti-CD127, and anti-CD27 for an additional 15 min at room temperature, washed once, and fixed with PBS containing 2% paraformaldehyde. In certain experiments, single-cell suspensions were prepared from spleens of individual animals in PBS plus 2% FBS and the staining was performed as described above. Samples were collected on an LSR II instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Splenocyte stimulation and intracellular cytokine staining**

Splenocytes were harvested from individual mice and RBC were lysed by anesthetized with ketamine/xylazine mix and then injected in the ear pinnae of mice at various times and treated with collagenase type II (1 mg/ml; Worthington Biochemicals) and DNase I (1 mg/ml; Roche) solution in RPMI 1640 plus 10% FBS for 20 min at 37°C in a shaker bath. After washing, the splenocytes were resuspended in 1× Perm/Wash buffer (BD Biosciences) and loaded with anti-cytokine mAb. After an additional washing step with 1× Perm/Wash buffer, the cells were fixed in 2% formaldehyde–PBS. Samples were collected on an LSR II instrument and analyzed using FlowJo software.

**Bioimaging of luciferase protein expression**

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

**T cell stimulation assay**

The draining lymph nodes (LN) were collected from immunized mice at various times and treated with collagenase type II (1 mg/ml; Worthington Biochemicals) and DNase I (1 mg/ml; Roche) solution in RPMI 1640 plus 10% FBS. Lymphocytes were isolated and cultured for 18 h in 96-well plates at 10^4 cells/well. The RF33.70 hybridoma (a gift from Dr. K. Rock, University of Massachusetts, Worcester, MA), which produces IL-2 upon specific recognition of SIINFEKL–specific MHC class I tetramers, was included in all cytokine assays as a control. The RF33.70 hybridoma (a gift from Dr. K. Rock, University of Massachusetts, Worcester, MA), which produces IL-2 upon specific recognition of SIINFEKL–specific MHC class I tetramers, was included in all cytokine assays as a control. The RF33.70 hybridoma (a gift from Dr. K. Rock, University of Massachusetts, Worcester, MA), which produces IL-2 upon specific recognition of SIINFEKL–specific MHC class I tetramers, was included in all cytokine assays as a control. The RF33.70 hybridoma (a gift from Dr. K. Rock, University of Massachusetts, Worcester, MA), which produces IL-2 upon specific recognition of SIINFEKL–specific MHC class I tetramers, was included in all cytokine assays as a control.

**Adoptive transfer**

B6 Thy1.1+ mice were immunized in the ear pinnae with pACB-OVA plasmid 10, 5, or 3 days before the adoptive transfer. LN cells were obtained from OT-1 Thy1.2+ mice, washed with PBS, and -6 × 10^6 CD8+ T cells were transferred into the preimmunized B6 Thy1.1+ mice by i.v. tail injection. On various days after transfer, the mice were bled and percentages of the transferred cells in the PBMCs were determined by flow cytometry using anti-Thy.1.2 Ab and SIINFEKL–specific tetramers.

**Statistical analysis**

Data were expressed as means ± SEM. Statistical tests were performed using one-way ANOVA or Student’s t test and p < 0.05 was considered significant.

**Results**

Transgene expression and CD8+ T cell responses following intradermal immunization with plasmid DNA

We were interested in exploring the impact of the duration of Ag expression on vaccine-elicted cellular immune responses. To create a system in which this could be studied, we developed a model in which mice were immunized intradermally in the ear pinnae and vaccine Ag was eliminated at various times following vaccination by surgical removal of the ear pinnae. Because most studies of plasmid DNA immunization have been done by delivering DNA–vectorized immunogen via the i.m. route, we first characterized the kinetics of transgene expression and the development of CD8+ T cell responses following intradermal plasmid DNA immunization. Mice were immunized intradermally in the ear pinnae or, for purposes of comparison, i.m. with a plasmid DNA–luciferase construct, and the expression of the luciferase protein was monitored in vivo using an IVIS-110. Following both the intradermal and i.m. injections, the expression of luciferase was localized at the sites of injection; however, the subsequent kinetics of transgene expression varied with the route of plasmid DNA administration (Fig. 1, A and B). Following an intradermal immunization, a rapid expression of luciferase protein was observed at the site of injection and was maximal 1 day after injection. This expression decreased precipitously over the ensuing days, but low levels of expression were still detectable 10 wk postimmunization. Administration of plasmid DNA into the muscle resulted in a higher peak level of transgene expression, and this expression reached its maximal level more slowly, with peak luciferase protein expression observed 1–2 wk postimmunization. The luciferase expression decreased very slowly thereafter and was detectable for >100 days.
To evaluate the generation of Ag-specific CD8$^+$ T cells by these two routes of inoculation, we immunized mice with a plasmid DNA-gp120 construct and assessed the magnitude and phenotype of the vaccine-elicited dominant epitope-specific CD8$^+$ T cell populations. A higher percentage of p18 epitope-specific CD8$^+$ T cells were found in PBMCs of intradermally injected mice than in PBMCs of mice immunized i.m. (Fig. 1C). However, this larger CD8$^+$ T cell population contracted rapidly, and the magnitudes of the p18-specific CD8$^+$ T cell populations were comparable in both groups of vaccinated mice 50 days postimmunization. The kinetics of the expression of the memory-associated molecules CD62L, CD127 but not CD27 on p18-specific CD8$^+$ T cells was comparable in mice vaccinated intradermally or i.m. (Fig. 1D). These findings therefore indicate that intradermally and i.m. administered plasmid DNA have distinct kinetics of transgene expression and elicit CD8$^+$ T cell responses with distinct kinetics of expansion, contraction, and differentiation.

**Shortening the duration of Ag expression modifies both the magnitude and kinetics of contraction of the elicited CD8$^+$ T cell population**

Since shortening the duration of a recombinant *L. monocytogenes* infection did not alter the magnitude or kinetics of primary or secondary CD8$^+$ T cell responses, it has been suggested that the duration of Ag expression has little impact on vaccine-elicited cellular immune responses (6, 7). However, unlike bacterial vectors, expression of a transgene following plasmid DNA immunization occurs via host cellular machinery and the transgene is expressed mainly by cells at the injection site. We considered the possibility that differences in the way Ag is produced and distributed in vivo can alter the characteristics of an elicited CD8$^+$ T cell response. To explore this possibility, we excised the ear pinnae of the immunized mice at various times postimmunization and assessed the ramifications of this procedure on the elicited CD8$^+$ T cell response. Removal of the ear pinnae 5, 24, or 48 h postimmunization abrogated the induction of p18-specific CD8$^+$ T cells; plasmid DNA-induced CD8$^+$ T cells were detectable only after 72 h of persistent plasmid DNA Ag expression (data not shown). Moreover, elimination of the vaccine Ag 5, 10, or 19 days postimmunization resulted in lower and earlier peak p18-specific CD8$^+$ T cell responses. (Fig. 2). To determine whether the kinetics of the contraction of the CD8$^+$ T cell response is also modified by the duration of the persistence of vaccine Ag, we excised the ear pinnae 4 days after the peak immune response, when the CD8$^+$ T cells response was beginning to decline. We observed that the p18-specific CD8$^+$ T cell population decreased in magnitude more rapidly when the Ag was eliminated (*p* < 0.05) while the memory set points in both groups were comparable over time (Fig. 2B). Therefore, these data indicate that early elimination of intradermally administered plasmid DNA causes a reduction in the magnitude of the vaccine-elicited CD8$^+$ T cell population, an acceleration of the onset of the contraction of these cells, and an alteration in the kinetics of the contraction phase of the immune response.

**Evidence of prolonged Ag-presenting activity after intradermal plasmid DNA immunization**

The kinetics of the expansion and contraction of this Ag-specific CD8$^+$ T lymphocyte population suggested that Ag presentation by APCs in local draining LN may be occurring continuously over a period of days to weeks following the intradermal inoculation of plasmid DNA. However, previous studies have suggested that Ag presentation to CD8$^+$ T cells by APCs occurs 24–48 h after exposure to Ag, and the elimination of depot Ag 2 or 3 days after immunization has no effect on the kinetics of the induced CD8$^+$ T cell response. To resolve this apparent inconsistency, we examined the kinetics of Ag presentation following intradermal immunization with plasmid DNA. We immunized mice intradermally in the ear pinnae with plasmid DNA expressing the OVA Ag and collected cells from the local draining LN at various times postimmunization. These collected LN cells were then cocultured with a T cell hybridoma specific for the SIINFEKL epitope. Because this
T cell hybridoma secretes IL-2 upon activation as a response to Ag presented by APCs, the hybridoma can be used as an indicator of APC activity by monitoring its production of IL-2. APCs capable of presenting processed OVA Ag were present in the draining LN of the immunized mice in largest numbers on days 10 through 15 and could still be detected on day 21 postimmunization (Fig. 3A). Moreover, elimination of depot Ag by removing the ear pinnae as late as day 15 postimmunization significantly decreased presentation of processed Ag in the draining LN. These data also indicate that Ag is presented by APCs in the LN even after elimination of depot Ag by removal of the ear pinnae.

To determine whether this vaccine-associated Ag-presenting activity might be capable of triggering an expansion of Ag-specific T cells, we immunized Thy1.1+/H11001 mice with an OVA plasmid DNA vector and adoptively transferred into them CD8+/H11001 T cells from OT-1 Thy1.2+/H11001 naive mice, transgenic mice whose T lymphocytes

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**FIGURE 2.** The impact of the duration of Ag expression on the kinetics of vaccine-elicited CD8⁺ T cells. Mice were immunized in the ear pinnae with DNA-gp120 (50 μg) and 5, 10, or 19 days following immunization the pinnae of the mice were removed. A. Representative flow plots display percentages of CD8⁺ tetramer⁺ T cells in the PBMCs of the immunized mice. B. Kinetics of p18-specific CD8⁺ T cells in the PBMCs of the immunized mice (upper panel) and kinetics of the contraction of the p18-specific CD8⁺ T cells when the pinnae was excised 4 days following the time of the peak immune response (lower panel). Data are shown as the percentage of CD8⁺ tetramer⁺ T cells and represent the means of five mice per group ± SE.

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**FIGURE 3.** Prolonged Ag-presenting activity following intradermal plasmid DNA immunization. A. Mice were immunized with pACB-OVA and 3, 5, 10, or 15 days after immunization the pinnae of the immunized mice were excised. At various times thereafter, the local draining LN cells were collected, irradiated, and cocultured with the SIINFEKL-specific T cell hybridoma for 24 h, and the IL-2 concentration in the supernatants was quantitated by ELISA. The presented data are the IL-2 concentrations in the supernatants and represent the means of four mice per group ± SE. B. Schematic diagram of the experimental system. Different groups of Thy1.1⁺ mice were immunized with pACB-OVA 10, 5, or 3 days before the adoptive transfer of 10⁶ LN cells from naive OT-1 Thy1.2⁺ mice. Six, 11, or 16 days after the transfer the mice were bled and the expansion of CD8⁺ T cells was assessed (C). Representative flow plots, gating on CD8⁺ lymphocytes with the percentages of SIINFEKL tetramer-binding/Thy1.2⁺ and SIINFEKL tetramer-binding/Thy1.2⁺ cells are shown.
express a TCR that recognizes the SIINFEKL epitope. The resulting expansion of the transferred Thy1.2\(^+\) CD8\(^+\) T cells was then quantitated using cell staining and flow cytometry (Fig. 3B). The naive Thy1.2\(^+\) OT-1 cells transferred into immunized mice on days 3, 5, and 10 following intradermal inoculation of plasmid DNA expanded in the recipient mice. Moreover, the naive Thy1.2\(^+\) OT-1 CD8\(^+\) T cells that were transferred into mice on day 10 after immunization expanded more rapidly than naive Thy1.2\(^+\) OT-1 CD8\(^+\) T cells that were transferred 3 or 5 days following immunization (Fig. 3C). We also observed that the largest expansion of CD8\(^+\) Thy1.2\(^+\) cells transferred on day 10 postimmunization was found 11 days after transfer, whereas the largest expansion of these cells transferred 3 or 5 days postimmunization was seen 16 days posttransfer.

These findings suggest that robust Ag presentation occurs between days 10 and 21 postimmunization. However, these results also indicate that low levels of Ag presentation can be demonstrated before day 10 postimmunization. This earlier Ag presentation might contribute to optimal recruitment of CD8\(^+\) T cells to the immune response, because transferring the Thy1.2\(^+\) OT-1 CD8\(^+\) T cells early after the immunization (day 3 or 5 vs day 10) resulted in a particularly dramatic expansion of these cells 3 wk postimmunization. These data therefore demonstrate that during the first 2 wk after immunization, after the peak immune response did not result in a decrement in IL-2 production by peptide-stimulated CD4\(^+\) T cells. Interestingly, we did not detect significant production of IFN-\(\gamma\) by the peptide-exposed CD4\(^+\) T cells of the immunized mice. These data therefore suggest that CD8\(^+\) T cell function is not altered by the duration of Ag persistence. In contrast, the function of CD4\(^+\) T cells influences by the duration of the persistence of depot Ag during the first 2 wk after immunization.

### Rapid elimination of Ag accelerates the acquisition of memory-associated cell surface molecules

Previous studies have suggested that the persistence of Ag influences the differentiation of Ag-specific CD8\(^+\) T cells to memory cells. To examine whether the rapid elimination of plasmid DNA Ag affects the maturation of p18-specific CD8\(^+\) T cells, we monitored the expression of the memory-associated molecules CD62L, CD27, and CD127 on these same vaccine-elicited cells. The p18-specific CD8\(^+\) T cells expressed the highest percentages of CD62L and CD27 in the mice in which the ear pinnae was excised early following immunization (Fig. 5A). However, expression of CD127 on the p18-specific CD8\(^+\) T cells was not affected by the early removal of the depot vaccine Ag.

We also determined the absolute numbers of tetramer\(^+\) CD8\(^+\) T cells expressing these memory-associated molecules in the spleens of the plasmid DNA-immunized mice. Ten weeks after immunization, larger numbers of p18-specific CD8\(^+\) T cells were found in the spleens of mice in which depot Ag was present for longer periods of time (Fig. 5B). Larger numbers of CD27\(^+\) p18-specific CD8\(^+\) T cells were also observed in the spleens of the mice having depot Ag for a longer period of time. In contrast, however, the numbers of splenic CD62L\(^+\) p18-specific CD8\(^+\) T cells were comparable in the different groups of mice. These findings suggest that...
the duration of Ag persistence modifies the kinetics of differentiation of memory CD8\(^+\) T cells. However, the eventual absolute number of memory CD8\(^+\) T cells as determined by CD62L expression was not affected by the early removal of the depot Ag.

Persistence of depot Ag interferes with the induction of secondary CD8\(^+\) T cell responses

Having demonstrated that early elimination of depot Ag in the immunized mice did not alter the absolute numbers or function of vaccine-elicited memory CD62L\(^+\)CD8\(^+\) T cells, and that both CD8\(^+\) and CD4\(^+\) T cells responses were optimal when the source of Ag was removed near the time of the peak cellular immune response, we asked whether the duration of Ag expression following the priming immunization had an impact on a vaccine-elicited secondary CD8\(^+\) T cell response. To address this question, we boosted these different groups of plasmid DNA-gp120-immunized mice with suboptimal doses (10\(^6\) particles) of recombinant adenovirus expressing the gp140 protein (rAd-gp140) and evaluated the secondary CD8\(^+\) T cell responses. This suboptimal dose of rAd-gp140 was chosen to facilitate the discrimination of quantitative and qualitative differences between the priming immunizations in the different groups of mice. As shown in Fig. 6A, all mice that were primed with DNA-gp120-generated rAd-induced secondary CD8\(^+\) T cell responses that were greater in magnitude than those seen in unprimed mice (PBS control). The magnitudes of the secondary CD8\(^+\) T cell responses, however, were substantially affected by the duration of Ag persistence following the priming immunization. Elimination of the ear pinnae 5 days after the priming immunization was associated with a secondary response that was similar in magnitude to that generated by mice in which the depot Ag was not eliminated. However, elimination of the ear pinnae 10 or 19 days after vaccine administration was associated with a secondary CD8\(^+\) T cell response that was considerably greater in magnitude than that generated in the mice whose depot priming Ag was not eliminated. The fold increase in number of secondary CD8\(^+\) T cells shown in Fig. 6B demonstrates that the absence of Ag enhances the ability of the CD8\(^+\) T cells to expand following re-exposure to the Ag. Despite the differences in the magnitudes of the secondary CD8\(^+\) T cell responses in the different experimental groups of mice, the proportion of secondary

FIGURE 5. Rapid differentiation of CD8\(^+\) T cells into memory cells after early elimination of Ag. Mice were immunized with DNA-gp120 and 5, 10, or 19 days after the immunization the pinnas of the immunized mice were excised. A, Expression of CD62L, CD27, and CD127 on CD8\(^+\) tetramer\(^+\) T cells in the PBMCs of the immunized mice. Data are presented as the percentage of CD8\(^+\) tetramer\(^+\) T cells that were CD62L\(^+\), CD127\(^+\), or CD27\(^+\) and represent the means of five mice per group \pm SE. B, Total numbers of memory p18-specific CD8\(^+\) T cell subsets in the spleens of the immunized mice. The data are the means of five mice per group \pm SE.

FIGURE 6. The magnitude and function of the secondary CD8\(^+\) T cells elicited in mice previously primed with plasmid DNA. Mice were immunized intradermally with DNA-gp120 (50 \(\mu\)g) and the ear pinnae were removed at the indicated times. Ten weeks later, the mice were immunized i.m. with 10\(^6\) particles of rAd-gp140. A, p18-specific CD8\(^+\) T cells generated following the secondary immunization. B, Fold increase in the level of secondary CD8\(^+\) T cell responses. C, Two weeks after the secondary immunization, splenocytes were exposed for 6 h to medium alone (Med.) or p18 peptide (2 \(\mu\)g/ml). Data are the percentages of p18-specific CD8\(^+\) T cells staining positively for IFN-\(\gamma\) or IL-2 and represent the means of five mice per group \pm SE.
p18-specific CD8+ T cells producing IFN-γ and IL-2 was not affected by the time of elimination of the priming Ag (Fig. 6C). These data suggest that long-term Ag persistence following primary immunization can interfere with the generation of a subsequent secondary CD8+ T cell response. It also indicates that the magnitude of a secondary CD8+ T cell response can be increased by removing the Ag near the time of the peak primary cellular immune response.

Discussion

In the present study, we found that expression of Ag by a plasmid DNA for more than 2 wk was required for the optimal expansion of a CD8+ T cell population. Consistent with this finding, we observed prolonged and delayed Ag-presenting activity in the LN of the immunized mice during this period of time. The Ag-presenting activity increased slowly over time following vaccination, was maximal 2 wk after vaccine administration, and elimination of depot Ag diminished this activity. These data indicate that expansion of CD8+ T cell populations is tightly controlled by the level of ongoing Ag presentation.

Although other investigators have recently reported that the duration of Ag expression can modulate an elicited cellular immune response, they have described a considerably shorter time frame from immunization to peak Ag-presenting activity than we observed in the present study. Although Prlic et al. (9) demonstrated that shortening the period of TCR signaling to <54 h resulted in smaller populations of responding CD8+ T cells, they found that long-term presentation of Ag was not necessary to elicit maximal responses. This different requirement for Ag persistence may be attributed to the fact that Prlic et al. (9) inoculated mice systemically with peptide-pulsed DC and OT-1 T cells, both of which might minimize the period of time needed for maximal recruitment of naïve CD8+ T cells. In another study, a prolonged period of Ag-presenting activity was reported in the setting of HSV-1 skin infection (20). In contrast to what was seen following plasmid DNA vaccination, however, presentation of the HSV-1 Ag was maximal 2 days after infection and gradually declined thereafter.

Because Ag-presenting activity following HSV-1 infection did not require trafficking of virus to the LN (17), it is possible that local inflammation occurring as a consequence of HSV-1 infection accelerated Ag-presenting activity. Indeed, several studies (21, 22) have shown the rapid migration of DC to LN following administration of proinflammatory mediators. Interestingly, the plasmids used for immunization in the present study contain CpG motifs that may provide costimulatory signals and induce migration of DC to the LN (21, 23). It is not clear however how these immunostimulatory motifs impact the immune response generated following intradermal DNA immunization, since migration of DC from the ear pinnae seems to be relatively slow and similar to that found during steady state (24).

The present study suggests that the duration of expression of depot Ag can determine the onset of contraction of the elicited CD8+ T cell population, as elimination of the Ag occurs coincident with the onset of this contraction. However, it is not clear what triggered the onset of the contraction of the CD8+ T cells in the control mice, since Ag was expressed in these mice for a long period of time after the onset of contraction. It is possible that when Ag is not limiting, factors such as the availability of survival cytokines may determine the onset of the contraction of the CD8+ T cells (25, 26). Interestingly, we observed in the present study that expression of Ag after the time of peak Ag presentation resulted in a slight delay in the kinetics of CD8+ T cell contraction. Although the mechanism mediating this effect was not defined, this observation is consistent with the delayed contraction of CD8+ T cell populations seen in association with prolonged interactions between CD27 and its ligand CD70 (27). Because CD27-CD70 signaling is tightly controlled by the presence of the Ag, Ag persistence may alter the kinetics of the contraction of CD8+ T cell populations via this receptor-ligand interaction.

Because effector CD8+ T cells differentiate in a linear fashion into memory cells, similar factors may drive the generation and survival of CD8+ T cells (28). Although elimination of the ear pinnae 5–19 days following immunization reduced the magnitude of the expansion of vaccine-elicited CD8+ T cells, the percentage of CD8+ T cells that expressed CD62L increased over time. Consequently, the absolute number of CD62L+CD8+ T cells in the spleens of the immunized mice after immunization remained relatively constant. In contrast to the finding of Williams et al. (6), these results suggest that the size of an effector CD8+ T cell population generated during the expansion phase of the immune response does not necessarily dictate the size of the induced memory CD8+ T cell population. Thus, effector and memory CD8+ T cells may receive different signals early after immunization that determine the fate of their differentiation.

Despite the comparable numbers of memory CD62L+CD8+ T cells found in the spleens of all immunized mice, a more robust secondary CD8+ T cell response was generated in mice in which Ag was eliminated 10 or 19 days after the priming immunization. These results suggest that Ag persistence shapes the magnitude of secondary CD8+ T cell responses by a yet undefined mechanism. Quantifying memory CD8+ T cells simply by measuring the expression of CD62L may not be sufficient to accurately determine the maturation status of the CD8+ T cells in immunized mice. However, other memory-associated characteristics of the Ag-specific CD8+ T cells such as expression of CD127 and the ability to produce IL-2 and IFN-γ were comparable in all groups of immunized mice. This observation supports our contention that similar numbers of memory cells were generated in these groups of mice. It is also possible that durable expression of Ag induced high numbers of effector memory CD8+ T cells in the control mice. Because effector memory CD8+ T cells seem to have better effector function than central memory CD8+ T cells, they may reduce the magnitude of the secondary CD8+ T cell response by decreasing the level of antigenic stimulation after the boosting immunization by eliminating APCs (29, 30). It is also possible that Ag-specific CD8+ T cells in the mice in which the ears were removed received more stimulation following the secondary immunization. In those groups, lower frequencies of p18-specific CD8+ T cells were seen before the boosting immunization, and therefore these cells might have less competition for stimulatory signals than those in the unmanipulated mice. However, in some experiments, removal of the ear pinnae after the peak immune response resulted in a frequency of Ag-specific CD8+ T cells that was similar to that seen in unmanipulated mice (data not shown). Boosting these mice with rAd-gp140 also resulted in a robust secondary response in mice in which the ear pinnae was removed. This finding suggests that competition for stimulatory signals may not be the only cause for the higher secondary response observed in the treated mice.

Whether the higher secondary immune responses seen in the manipulated mice might be associated with better protection against a viral infection is not clear. Secondary CD8+ T cells differentiate preferentially into effector-memory cells, a lymphocyte population that is more protective than central memory cells (2). It is therefore most likely that removal of the ear pinnae after the priming immunization will lead to a better protective immunity. Moreover, large numbers of circulating effector-memory CD8+ T cells should be associated with large numbers of pathogen-specific
CD8+ T cells at the portal of pathogen entry, which should contribute to protection. In contrast, the quality of CD8+ T cells is also important for generating protective cell-mediated immunity. Recent studies have demonstrated a correlation between the ability of pathogen-specific T cells to manifest multifunctional effector function and their ability to provide protection (31). It will therefore be important to determine whether the early removal of ear pinnae during a primary immune response has an impact on the multifunctionality of the secondary T cells.

In contrast to the expression of CD62L and CD27, expression of CD127 on Ag-specific CD8+ T cells was not influenced by the persistence of depot Ag. Instead, consistent with observations in previous studies (32), a small population of the vaccine-induced CD8+ T cells expressed the CD127 molecule a few days after the immunization, and the percentage of these CD8+ T cells expressing CD127 increased over time. CD127 expressed on lymphocytes delivers survival signals to these cells, in part by regulating apoptosis via bcl-2 family members (33). The findings in the present study suggest that early events determine the expression level of CD127 on CD8+ T cells, and persistence of Ag or contraction of the CD8+ T cells population does not affect the expression of this cell surface molecule. However, the disparity between CD62L and CD127 expression by Ag-specific CD8+ T cells in this study provides further evidence that CD127 expression may not identify memory CD8+ T cells (34, 35).

The impact of the duration of Ag expression on CD4+ T cell responses has been unclear. Several studies have suggested that Ag persistence is not necessary for the generation of CD4+ T cell responses (36, 37). However, a recent study by Obst et al. (14) demonstrated that CD4+ T cells are critically dependent on the continuous presentation of the Ag in vivo. In the present study, maximal levels of Ag-specific CD4+ T cells were found only when Ag was expressed for 19 days postimmunization. We were, however, able to detect CD4+ T cell responses even when Ag was eliminated 5 days after immunization, although in smaller numbers than in the control mice. This small CD4+ T cell population appeared to be fully functional, because the CD4 help-dependent evolution of memory CD8+ T cells and secondary immune responses were intact in these mice (16, 38). Additionally, the duration of Ag expression seems to have no effect on the differentiation process of the CD4+ T cells, because 10 wk after the immunization these cells differentiated into memory cells according to their ability to secrete IL-2 but not IFN-γ (39, 40). These findings in the present study are consistent with the possibility that the duration of Ag expression following plasmid DNA immunization shapes the magnitude of the Ag-specific CD4+ T cell population but not their differentiation into memory cells.

Other studies have investigated the role of Ag expression and presentation following intradermal plasmid DNA immunization (41–43). However, in the majority of these studies, the plasmid DNA was delivered into the skin by gene gun and, as a result, expression of the Ag may be required only for a very short period of time. Moreover, these studies assessed the level of Ag-specific CD8+ T cell responses using CTL or ELISPOT assays. These assays may be less sensitive than the tetrameric MHC class I complexes used in the present study, since tetramer detects all Ag-specific CD8+ T cells rather than a subset of these cells with a specific function. The data in the present study therefore demonstrate the important role of Ag persistence on the elicitation of primary and secondary CD8+ T cell responses following intradermal DNA immunization.

Acknowledgments
We are grateful to Itaï Roni Eyal for their help during this study. The HIV-1 IIIB gp120 overlapping peptides were provided by the European Union Program European Vaccine Against AIDS/Medical Research Council Centralized Facility for AIDS Reagents, National Institute for Biological Standards and Control, United Kingdom.

Disclosures
The authors have no financial conflict of interest.

References


