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A Matter of Timing: Unsynchronized Antigen Expression and Antigen Presentation Diminish Secondary T Cell Responses

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Despite the low and short expression of secondary Ag, prime-boost immunizations using homologous or heterologous vectors are capable of amplifying memory CD8⁺ T cells. This is mainly attributed to the rapid presentation of Ag by APCs and the high proliferative capacity of memory CD8⁺ T cells. Nevertheless, certain viruses and vectors often require prolonged Ag presentation for optimal T cell priming, and the influence of such a prolonged presentation during secondary immune induction is not clear. To address this issue, we primed and boosted mice intradermally (i.d.) with plasmid DNA that was recently reported to require prolonged Ag presentation for maximal CD8⁺ T cell priming. Although functional memory CD8⁺ T cells were present in the mice after i.d. priming, the secondary CD8⁺ T cell response elicited was limited and reached a similar level of that observed during priming. The initial levels of secondary Ag expressed in the boosted mice were sufficient to prime CD8⁺ T cell response in naive hosts, suggesting that lower Ag load alone does not explain the limited secondary immune responses observed. Removal of the injection site 5 or 10 days after i.d. boosting immunization resulted in diminished Ag presentation and no expansion of memory CD8⁺ T cells. In fact, Ag-presenting activity following boost occurred mainly two weeks postimmunization, a time when the Ag was no longer expressed in situ. These findings suggest that when the boosting vector triggers prolonged Ag presentation, the lack of synchronicity between Ag accessibility and Ag presentation limits secondary immune responses. *The Journal of Immunology, 2009, 183: 1013–1021.

Cell vaccination usually involves prime-boost immunization strategies to generate robust, pathogen-specific CD8⁺ T cell responses. These boosted CD8⁺ T cells mainly develop into effector-memory cells and persist in the host for a long time (1, 2). It has been shown that the nature of the boosting immunogen shapes the magnitude, differentiation, and function of the responding T lymphocytes (2, 3). Numerous studies have also demonstrated that secondary CD8⁺ T cells are highly protective against various intracellular pathogens (4, 5). However, boosting CD8⁺ T cells is not a trivial task, and relatively few combinations of heterologous vectors are capable of eliciting vigorous expansion of primed CD8⁺ T cells. There is, therefore, great interest in studying the immune mechanisms that regulate the induction of secondary CD8⁺ T cell responses.

The generation of strong secondary T cell responses depends largely on the establishment of memory CD8⁺ T cells following priming. These cells require the help of CD4⁺ T cells to maintain an effective memory pool (6, 7). However, a recent study suggested that memory CD8⁺ T cells can also restrict their own secondary expansion by limiting Ag presentation following the boosting immunization (8). A second factor that limits secondary immune responses is the presence of anti-vector immunity following homologous prime-boost immunization with viral- or bacterial-based vectors. This vector-specific immunity decreases antigenic stimulation during boosting immunization, resulting in a weaker expansion of the boosted T cells. Plasmid DNA does not engender vector-specific immunity and thus can be administered repeatedly to elevate the frequency of Ag-specific T cells. Indeed, in many human and nonhuman primate vaccine trials, plasmid DNA is given multiple times to prime the immune response before exposing the host to heterologous immunogens (9–12). Because of this feature, DNA immunization is an attractive system to study the interplay between Ag expression and presentation during secondary immune responses.

We recently reported that following intradermal (i.d.) plasmid DNA immunization, durable expression of the Ag is required for priming optimal CD8⁺ T cell response (13). This was due to prolonged Ag-presenting activity that occurs in the local draining lymph nodes (LNs) following immunization. Protracted Ag presentation was also reported following infection or immunization with many viruses, such as vesicular stomatitis virus, influenza virus, herpes simplex virus, Sendai virus, and lentivirus (14–19). These durable presentations of the viral Ags were found to influence the virus-specific T cells generated in the host. Because the magnitude and duration of primary Ag expression are larger than expression following secondary exposure to the Ag, it is not clear how the relatively brief exposure to the Ag affects presentation following secondary immunization. This study was initiated to address this question, using an immunization strategy where prolonged Ag presenting activity is known to play a critical role during priming.

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.

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The following mAbs were used: anti-CD26 (MEL-14; eBioscience), anti-CD8ε (53–6.7; BD Biosciences), anti-IFN-γ (XMG1.2; BD Biosciences), anti-IL-2 (JHS6–5H4; BD Biosciences) and anti-CD4 (GK1.5; BD Biosciences). Tetrameric H-2D\(^{b}\) complexes folded with the gp120 p18 epitope peptide (RGPGRAVFTL) (20) were prepared as previously described (21), and SIINFEKL H-2K\(^{b}\) tetramers were purchased from Beckman Coulter. CFSE was purchased from Molecular Probes.

**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. Gary Nabel (National Institute of Allergy and Infectious Diseases, Bethesda, MD). The pACB-OVA plasmid was a gift from Dr. Maripat Corr (University of California San Diego, La Jolla, CA). The recombinant replication-defective adenovirus human serotype 5 containing the HIV-1 HXB2 env (rAd-gp140) was provided by Dr. Norman Letvin (Harvard University, Boston, MA). All plasmids used in this study were prepared using the Endo-Free plasmid Giga kit (Qiagen) and the levels of endotoxin in the DNA preparations were always lower than 0.1 EU/μg DNA.

**Mice and immunizations**

Six- to eight-week-old female BALB/c, B6, B6 Thy1.1\(^{+}\), and OT-I Thy1.2\(^{+}\) mice were purchased from The Jackson Laboratory and maintained under specific pathogenic free conditions. Research on mice was approved by the Hebrew University Institutional Animal Care and Ethics Committee. Mice were anesthetized with ketamine/xylazine mix and then i.d. injected with VRC-gp120, VRC-luc, or pACB-OVA in the ear pinna (50 μg DNA in 40 μl total injection volume, 40 μl was delivered into each ear) with a 31-gauge needle. For i.m. injection, the mice received the noted plasmids and 50 μg of DNA in 100 μl total injection volume. Fifty microliters were delivered into each quadriceps muscle. Ten weeks after the first immunization, mice were homologously boosted via the same route and quantity as described for the priming immunization. In some experiments, mice were boosted i.m. with Ad-gp140 (10\(^{4}\) particles) 10 wk after the first immunization. Removal of the ear pinna was performed after the mice were anesthetized with ketamine/xylazine mix and by using sterile scissors.

**Tetramer and phenotypic analysis of the T cells**

Blood was collected from individual mice in RPMI 1640 medium containing 40 U/ml heparin and PBMC were isolated using Lympholyte-M (Cedarlane Laboratories). Cells were washed with PBS containing 2% FBS and stained for 15 min at room temperature with H-2D\(^{a}\)/I-A\(^{d}\) Abs for an additional 15 min at room temperature, washed 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 a LSR II instrument (BD Biosciences) and analyzed using the FlowJo software.

**Splenocyte stimulation and intracellular cytokine staining**

Splenocytes were harvested from individual mice and RBC were lysed by using ACK buffer. The cells were then washed with PBS plus 2% FBS, counted, and stained with PE-conjugated H-2D\(^{a}\) and anti-CD8ε Abs and then i.d. injected with VRC-gp120, VRC-luc, or pACB-OVA in the ear pinna (50 μg DNA in 40 μl total injection volume, 40 μl was delivered into each ear) with a 31-gauge needle. For i.m. injection, the mice received the noted plasmids and 50 μg of DNA in 100 μl total injection volume. Fifty microliters were delivered into each quadriceps muscle. Ten weeks after the first immunization, mice were homologously boosted via the same route and quantity as described for the priming immunization. In some experiments, mice were boosted i.m. with Ad-gp140 (10\(^{4}\) particles) 10 wk after the first immunization. Removal of the ear pinna was performed after the mice were anesthetized with ketamine/xylazine mix and by using sterile scissors.

2% formaldehyde-PBS. Samples were collected on a LSR II instrument and analyzed using FlowJo software.

**Bioimaging of luciferase protein expression**

Bioimaging of vector expressing firefly luciferase was done using the In Vivo Imaging System 110, IVIS-110 (Xenogen). Mice were anesthetized with ketamine/xylazine mix and injected i.p. with 100 μl of an isotonic salt solution containing 30 mg/ml D-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 (Xenogen) to identify and measure regions of interest.

**Ag presentation assays**

The draining LNs were collected from immunized mice at various times after priming with collagen-coated 1 ml/well Wellman-24 well Bio-Scoot insert (StemCell Technologies). The purified OT-1 splenocytes (5\(^{10}\) cells/well) were incubated with CD11c\(^{+}\) cells from OT-I CD8\(^{+}\) T cells were incubated with negative selection by EasySep mouse CD8\(^{+}\) T cell enrichment kit according to the manufacturer’s instruction.

To compare the level of Ag presenting activity in primed vs boosted mice, CD11c\(^{+}\) cells were obtained from the collected LNs by positive isolation using MACS Microbeads according to the manufacturer’s instruction. The purified OT-1 CD8\(^{+}\) T cells were incubated with same volume of 5 μg CFSE in HBSS, for a final concentration of 2.5 μM, for 10 min at 37°C. Labeling was quenched by adding excess of ice-cold RPMI 1640 complete medium and cells were washed twice with culture medium. CFSE-labeled OT-I CD8\(^{+}\) T cells (5 \(\times\) 10\(^{5}\) cells/well) were incubated with CD11c\(^{+}\) cells (2 \(\times\) 10\(^{5}\)well) in 96-well U-Plate (Nunc). The cells were then incubated for 48 or 60 h and the dilution of CFSE fluorescence was analyzed by the LSR II instrument.

**Adoptive transfer**

Lymphocytes were obtained from OT-I Thy1.2\(^{+}\) mice, washed with PBS, and 5 \(\times\) 10\(^{6}\) CD8\(^{+}\) T cells were transferred into B6 Thy1.1\(^{+}\) mice by i.v. tail injection. The mice were immunized in the ear pinna with pACB-OVA plasmid 24 h later, as described earlier, and boosted 10 wk later via the same route and quantity of this plasmid. The mice were bled at various times and percentages of the transferred cells in the PBMC were determined by flow cytometry using anti-Thy1.2 and anti-CD8 Abs.

**Statistical analysis**

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

**Results**

**Limited secondary T cell responses after i.d. plasmid DNA immunization**

Cohorts of mice were primed i.d. or i.m. with plasmid DNA encoding the HIV-1 gp120 protein. Ten weeks later, the mice were boosted with the noted plasmid via the same route and quantity. Induction of primary and secondary CD8\(^{+}\) T cell responses was monitored by using H-2D\(^{d}\) Mhc class I tetramers specific to the p18 immunodominant epitope of the gp120 protein. Following priming, i.d.-immunized mice generated considerably higher percentages of p18-specific CD8\(^{+}\) T cells than i.m.-immunized mice (Fig. 1A). This, however, changed over time, as before the boosting immunization, similar percentages of tetramer positive cells were found in both i.d.- and i.m.-immunized mice. Homologous i.m.-boosting of i.m.-primed mice resulted in a large expansion of the p18-specific CD8\(^{+}\) T cells, as would be expected by a typical
secondary immune response. Surprisingly, i.d.-primed p18-specific CD8\(^+\) T cells did not expand robustly following i.d. homologous boost immunization. In fact, the percentage of tetramer-specific CD8\(^+\) T cells we observed during secondary responses in i.d. prime-boost mice were similar, or lower than, those detected after the priming immunization (Fig. 1A). Similar percentages of Ag-specific CD8\(^+\) T cells were found in the spleen and liver, whereas in the gut mucosa the frequencies of these cells were very low (data not shown). To quantify the relative numbers of Ag-specific CD8\(^+\) T cells generated by each immunization regimen, we measured the total number of tetramer-specific CD8\(^+\) T cells in the spleen. The size, weight, and overall percentages of T cell subsets in the spleen were indistinguishable across the different groups of mice (data not shown). However, the numbers of Ag-specific cells varied greatly between the immunized groups; priming i.d. with plasmid DNA elicited more than three times p18-specific CD8\(^+\) T cells than those observed in i.m.-primed mice (Fig. 1B). Consistent with the percentages in the blood, by 10 wks the relative number of tetramer-positive CD8\(^+\) T cells in both i.d.-priming failed to expand robustly following secondary i.d. homologous immunization.

Phenotype and functional analysis of the secondary CD8\(^+\) T cells

We next examined the characteristics of the tetramer "CD8\(^+\)" T cells elicited following i.d. prime-boost immunization. The secondary p18-specific CD8\(^+\) T cells express low levels of the memory-associated molecule CD62L on their surface throughout the experiment (Fig. 2A). The total number of CD62L\(^-\) Ag-specific CD8\(^+\) T cells was also low in the spleen of these mice (Fig. 2B). The level of CD62L expression and the low number of p18-specific CD62L-positive was similar to what is observed in mice primed and boosted by the i.m. route. Such pattern of expression is typical to secondary CD8\(^+\) T cells that differentiate mainly into effector-memory cells but not into memory cells (1, 2). In contrast, the primary p18-specific CD8\(^+\) T cells evolved rapidly into memory cells as indicated by the higher expression of CD62L (Fig. 2A).

We next asked whether the CD8\(^+\) T cells elicited by the secondary i.d.-immunization expanded from memory cells generated during priming or were differentiating de novo from naive T cell precursors. B6 Thy1.1\(^+\) mice were adoptively transferred with CD8\(^+\) T cells purified from naive OT-I Thy1.2\(^+\) mice, primed i.d. with the pACB-OVA plasmid and 10 wk later boosted i.d. with the same plasmid. As shown in Fig. 2C, the increase in the tetramer-specific CD8\(^+\) T cells observed following secondary i.d. immunization was due to expansion of the memory SIINFEKL-specific OT-I CD8\(^+\) T cells presented in the mice rather than de novo expansion of naive precursors. Similar results were obtained when the mice were primed and boosted with various combinations of i.d. and i.m. immunizations (supplemental Fig. S1). We next measured the capability of the DNA vaccine-elicited secondary CD8\(^+\) T cells to produce IFN-\(\gamma\) and IL-2 upon peptide stimulation. Splenocytes from DNA-gp120 immunized mice were collected and incubated with the gp120 CD8\(^+\) T cell immunodominant p18 peptide. Considerable production of IFN-\(\gamma\) and IL-2 was measured by p18-specific CD8\(^+\) T cells obtained from mice primed and boosted i.d. (Fig. 2D). The frequencies of p18-specific cells producing IFN-\(\gamma\) or IL-2 were similar to that measured by CD8\(^+\) T cells from i.m.-immunized mice. Finally, we measured the contribution of the cross-presentation mechanism to the induction of secondary CD8\(^+\) T cells. Primed mice were i.d.-boosted with pACB-OVA plasmid or with plasmid encoding the OVA Ag under the control of the keratinocyte-specific promotor K14 (a gift from Dr. Joshy Jacobs, Emory University, Atlanta, GA). The magnitude of the Ag-specific CD8\(^+\) T cells generated in both groups was comparable, thus indicating the importance of cross-presentation in our system (supplemental Fig. S2). Our data thus suggests that despite generating reduced secondary immune responses, the phenotype and function of i.d.-elicited CD8\(^+\) T cells are typical to secondary T cell immune response.

Intact progression of CD8\(^+\) T cells into memory cells following i.d. priming

The development of memory T cells after priming is important for the efficient expansion of secondary CD8\(^+\) T cells following the boosting immunization (22). Analysis of CD62L expression on i.d.-elicited primary p18-specific CD8\(^+\) T cells showed normal

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4 The online version of this article contains supplemental material.
mice, and represent the mean of five mice per group ± SE. B, Splenocytes were collected from DNA-gp120 i.d.-primed/boosted mice or i.m.-primed/boosted mice, and the total number of Ag-specific memory CD8+ T cells was calculated. Data are presented as the number of CD62L+ p18-tetramer+ CD8+ T cells in the spleen of the immunized mice, and represent the mean of five mice per group ± SE. C, Lymphocytes from OT-1 Thy1.2+ mice (~5 x 10^6 CD8+ T cells per mouse) were transferred into B6 Thy1.1+ mice by i.v. tail injection. The mice were primed i.d. with pACB-OVA plasmid 24 h after the transfer and 10 wk later boosted via the same route and quantity of this plasmid. The percentages of the transferred cells in the peripheral blood of the mice were measured before the secondary immunization and 2 wk later. Representative flow plots with the percentages of CD8+ Thy1.2+ lymphocytes (transferred cells) are shown. D, Splenocytes from BALB/c mice immunized with the DNA-gp120 plasmid were harvested at the time of the peak secondary immune responses, and exposed for 6 h to medium alone (Med.) or p18 peptide (2 μg/ml) to test their capability to produce cytokines upon stimulation. Data are presented as the percentages of tetramer+ CD8+ T cells staining positively for IFN-γ and IL-2, and represent the means of five mice per group ± SE.

**FIGURE 2.** Phenotypic and functional analysis of the secondary CD8+ T cells. Mice were primed and boosted i.d. (ID) or i.m. (IM) with DNA-gp120 as described in Materials and Methods. A, Expression of the memory-associated molecule CD62L on primary and secondary p18-specific CD8+ T cells in the peripheral blood. Data are presented as the percentage of tetramer positive CD8+ T cells that were CD62Lhigh and represent the mean of five mice per group ± SE. B, Splenocytes were collected from DNA-gp120 i.d.-primed/boosted mice or i.m.-primed/boosted mice, and the total number of Ag-specific memory CD8+ T cells was calculated. Data are presented as the number of CD62L+ p18-tetramer+ CD8+ T cells in the spleen of the immunized mice, and represent the mean of five mice per group ± SE. C, Lymphocytes from OT-1 Thy1.2+ mice (~5 x 10^6 CD8+ T cells per mouse) were transferred into B6 Thy1.1+ mice by i.v. tail injection. The mice were primed i.d. with pACB-OVA plasmid 24 h after the transfer and 10 wk later boosted via the same route and quantity of this plasmid. The percentages of the transferred cells in the peripheral blood of the mice were measured before the secondary immunization and 2 wk later. Representative flow plots with the percentages of CD8+ Thy1.2+ lymphocytes (transferred cells) are shown. D, Splenocytes from BALB/c mice immunized with the DNA-gp120 plasmid were harvested at the time of the peak secondary immune responses, and exposed for 6 h to medium alone (Med.) or p18 peptide (2 μg/ml) to test their capability to produce cytokines upon stimulation. Data are presented as the percentages of tetramer+ CD8+ T cells staining positively for IFN-γ and IL-2, and represent the means of five mice per group ± SE.

progression into the memory compartment (Fig. 2A). In addition, these CD8+ T cells generated by i.d. or i.m. immunization showed no differences in their progression into effector memory (CD127+CD62L−) or central memory (CD127−CD62L+) cells (supplemental Fig. S3). The total number of CD62L+expressing p18-specific CD8+ T cells in the spleen was also similar following i.d.- or i.m.-priming immunization (Fig. 3A). To examine whether the limited secondary response found in i.d.-immunized mice is due to a defect in the primary T cells, we analyzed the function of these cells. We first measured the capability of the T cells to produce cytokines upon p18 peptide stimulation. Our analysis indicate that p18-specific CD8+ T cells from i.d.-primed mice produce IFN-γ or IL-2, and the percentages of the cells producing these cytokines is similar to those generated in i.m.-primed mice (Fig. 3B). Because the help provided by CD4+ T cells during priming was thought to be central for secondary CD8+ T cell expansion, we also examined the CD4+ T cell response. We found production of IL-2 by peptide-exposed CD4+ T cells from i.d.-primed mice. Interestingly, the percentages of IL-2 producing cells were significantly higher than those generated by i.m.-primed mice (Fig. 3C). To test the capability of the i.d.- and i.m.-primed CD8+ T cells to expand following identical boosting immunization, we exposed the mice to suboptimal doses (10^7 particles) of recombinant adenovirus expressing the HIV-1 gp140 protein and evaluated the secondary CD8+ T cell responses. This suboptimal dose of rAd-gp140 was chosen to facilitate the discrimination of quantitative differences between the priming immunizations in the different groups of mice. As shown in Fig. 3D, mice that were primed with plasmid DNA, either i.d. or i.m., generated comparable rAd-induced secondary CD8+ T cell responses that were greater in magnitude than those seen in unprimed mice. To further show that the primary T cell responses were intact, we primed and boosted mice with various combinations of i.m. and i.d. immunizations. As demonstrated by Fig. 3E, secondary i.m. immunization resulted in robust secondary CD8+ T cell expansion both in i.d.- and i.m.-primed mice. In contrast, boosting the mice i.d. failed to generate higher frequency of CD8+ T cells than that observed by i.d. priming. These findings demonstrate that the limited secondary CD8+ T cell response observed following i.d. plasmid DNA immunization is not a result of impaired primary T cell responses.

**Analysis of Ag expression following secondary i.d. immunization**

To better understand the immune mechanisms of i.d. boosting immunization, we measured the kinetics of Ag expression in vivo. Mice were primed or boosted either i.d. or i.m. with plasmid DNA encoding the luciferase gene, and the level of luciferase expression was determined using IVIS. Administration of the plasmid i.m. resulted in higher levels of luciferase expression both after priming and boosting than those seen in i.d.-immunized mice (Fig. 4, A and B). In addition, following boost, the duration of Ag expression was considerably shorter than that detected during priming regardless of the immunization route. The level of Ag expression differed as well, and was much lower in the boosted mouse. Nevertheless, the decrease in the expression level of luciferase following secondary immunization was more pronounced in i.m.-boosted mice (Fig. 4C). For example, on the first day after secondary i.d. immunization, the expression level of luciferase was three times lower than at the same day in the primed mice. In contrast, in i.m.-boosted mice, luciferase expression level was 10 times lower than the priming immunization. Still, it is possible that the diminished level of
Ag expression after i.d.-boosting immunization is not sufficient to provide optimal triggering for CD8+ T cells. To examine this possibility, we immunized naive mice with decreasing amounts of plasmid DNA-gp120 (50, 25, 10, 5, and 0.5 μg) to find the lowest Ag level capable of generating optimal immunogenicity. We found that immunization of mice with 10-fold lower dose of DNA-gp120 plasmid (5 μg instead of 50 μg) did not affect the magnitude of the Ag-specific CD8+ T cell responses elicited in vivo (Fig. 5A). When similar concentrations of plasmids were used to examine the kinetics of luciferase expression via IVIS, we observed lower levels of luciferase in mice that received 5 μg of DNA, a level that was comparable to that observed in i.d. boosted mice until day 5 (Fig. 5B). However, in contrast to i.d.-boosted mice, considerable levels of luciferase were still maintained for more than 15 days after the i.d. administration of 5-μg VRC-luc. It is important to mention that a 0.5-μg dose of plasmid DNA (100 times lower than our original dose) was still capable of priming considerable, though not optimal, CD8+ T cell responses, whereas the level of Ag expression was much lower than that seen in the boosted mice (data not shown). Taken together, our data suggest that during i.d. boosting immunization, it is the duration of secondary Ag expression rather than the magnitude of initial Ag expression that influences the secondary expansion of CD8+ T cells.

Prolonged Ag expression is required for maximal secondary T cell responses

We previously reported that during i.d. priming with plasmid DNA, durable Ag expression is essential for eliciting optimal CD8+ T cells response (13). Thus, we also asked whether similar...
phenomenon occurs after the boosting immunization. Mice were primed and boosted i.d. with DNA-gp120 and the ear pinna was removed at different times postimmunization. It is important to mention that amputation of the ear pinna removes Ag expressed only at the injection site and not at other sites, such as the LNs where limited expression of the Ag occurred shortly after the immunization (data not shown). Excision of the ear pinna 5 or 10 days postimmunization resulted in no secondary CD8\(^+\) T cell response (Fig. 6A). Premature removal of the Ag also affected the induction of secondary gp120-specific CD4\(^+\) T cells response (Fig. 6B). In contrast, excision of the ear pinna 15 days after the boosting immunization did not alter the magnitude of the secondary T cell responses in the immunized mice. Thus, as with primary i.d. immunization, durable expression of the DNA-encoded Ag is necessary to generate secondary CD8\(^+\) and CD4\(^+\) T cell responses. It also suggests that expression of the Ag at the ear pinna and not at other sites controls the development of the T cell response.

**Ag presentation following secondary i.d. immunization is reduced**

We next examined whether the requirement for prolonged expression of the Ag we observed is due to slower kinetics of Ag-presenting activity in the draining LNs. To measure the level of Ag presentation, we primed and boosted B6 mice i.d. or i.m. with plasmid DNA encoding the OVA gene. At various times after the boosting immunization, LNs were collected and LN cells were cocultured with a T cell hybridoma specific for the OVA-immunodominant 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. Presentation of the SIINFEKL epitope by APCs was detected in a low level during the first 10 days post-boosting immunization (Fig. 7A). However, a considerable higher level of Ag-presenting activity was found 15 days after the secondary immunization. Termination of depot Ag expression on day 5 or 10 postimmunization, by excising the ear pinna, dramatically inhibited Ag-presenting activity to nearly background levels. Analysis of SIINFEKL-presentation following i.m. boosting immunization demonstrated entirely different kinetics. An elevated level of Ag presentation was observed 5 days after the boosting immunization, and this presentation decreased thereafter (Fig. 7B). These findings suggest that secondary Ag-presenting activity occurs far more rapidly following i.m. than i.d. boosting immunization. Our results indicate that the kinetics of Ag-presenting activity exerts critical influence on the capability to generate secondary T cell expansion, as the Ag is available for a short period of time (less than 10 days) during the boosting immunization. To examine this hypothesis, we collected LN cells after priming or boosting i.d. or i.m. immunizations (15 or 5 days after the immunization, respectively) and incubated them with CFSE-labeled CD8\(^+\) T cells from OT-I mice. Two to three days later, the cultures were analyzed to measure the reduction in CFSE staining that represents the proliferative capacity of the CD8\(^+\) T cells. Fig. 7C demonstrates that incubation of CFSE-labeled OT-I CD8\(^+\) T cells with CD11c\(^+\) cells purified from i.d.-boosted mice resulted in less dilution in the CFSE levels than those induced by

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**FIGURE 5.** Determining the lowest plasmid DNA dose necessary for optimal immune induction. BALB/c mice were primed i.d. with DNA-gp120, DNA-luc, or sham plasmid (50 or 5 \(\mu\)g of plasmid DNA). A, Kinetics of p18-specific CD8\(^+\) T cells in the peripheral blood of DNA-gp120-immunized mice that represent the percentages of tetramer\(^{+}\)CD8\(^+\) T cells and are the means of four mice \(\pm\) SE. B, The mean relative light unit (RLU) values expressed by groups of four mice (50 or 5 \(\mu\)g) following priming with DNA-gp120 and the ear pinna was immunized with DNA-luc.

**FIGURE 6.** The impact of the duration of Ag expression on the kinetics of secondary CD8\(^+\) T cells. BALB/c mice were immunized in the ear pinna with DNA-gp120, and 5, 10, or 15 days following the immunization the pinna of the mice were removed. A, Kinetics of the secondary p18-specific CD8\(^+\) T cells in the PBMC of the immunized mice, as indicated by tetramer staining and represent the mean of five mice \(\pm\) SE. B, The influence of the excision of the pinna on the CD4\(^+\) T cells. Splenocytes were collected from the mice 3 wk after the secondary i.d. immunization and were exposed to gp120 peptides. Data are presented as the percentages of IL-2\(^+\) CD4\(^+\) T cells, and are the mean of five mice per group \(\pm\) SE. Ctrl, Control.
CD11c^+ cells from i.d.-primed mice. In contrast, no differences in the dilution levels of CFSE was observed following incubation of the labeled CD8^+ T cells with CD11c^+ cells derived from i.m.-primed or i.m.-boosted mice. These data suggest that Ag presentation is reduced during secondary i.d. immunization. Taken together, we propose that due to the delayed kinetics of Ag presentation is reduced during secondary i.d. immunization. We demonstrated that, following homologous prime-boost immunization with vaccinia or adenovirus vectors (3). Nevertheless, such immunization strategies have been shown to increase the frequencies of Ag-specific CD8^+ T cells three to eight times over the amount elicited by primary alone (3). In the present study, considerably more durable expression of the secondary Ag was observed in both i.m.- and i.d.-immunized mice. This suggests that the reduced level of Ag expression observed following i.d. boosting immunization is not the only reason for the weak secondary CD8^+ T cell response. In a similar manner, it has been shown that Ag clearance is not the critical factor determining the reduction of recall immune responses during lymphocytic choriomeningitis virus and Listeria monocytogenes infections (25–27). Our finding that the initial levels of Ag expression found in i.d.-boosted mice were capable of priming optimal CD8^+ T cell response in naive mice provide strong support for this notion (Fig. 5). These data suggest that it is the duration, rather than the initial level of Ag expression, that serves as the limiting factor for secondary T cell expansion following i.d. boosting immunization. It was expected that, despite the lower level of Ag expression, the presence of higher frequencies of memory CD8^+ T cells at the time of the i.d. boosting immunization would result in enhanced secondary expansion, as seen in i.m.-boosted mice. Memory cells are known to enhance proliferative capacity more than naive CD8^+ T cell precursors (28, 29), and the lack of enhanced expansion suggests that the Ag was not efficiently presented following i.d. boosting immunization. Indeed, throughout the secondary Ag expression period (< day 10), Ag presenting activity was meager in i.d.-boosted mice, whereas in i.m.-boosted mice the majority of Ag presentation had already been completed by that point (Fig. 6).
Because i.d.- and i.m.-immunized mice were injected with the same quality, quantity, and order of plasmid DNA, the differences in their immunogenicity can be attributed to the injection site. In contrast to i.m., immunization, by which CD8+ T cell priming occurs rapidly, priming of CD8+ T cells via i.d. injection takes longer and requires durable expression of the transgene (13, 30). It is not yet clear what the basis for these differences is, and we are currently investigating this question. A recent study demonstrated that Ag presentation following skin infection requires transfer of skin-derived Ag by cutaneous-dendritic cells (DCs) to LNs-resident DCs for efficient CD8+ T cell priming (31). The time this process takes following injection of plasmid DNA to the skin suggests that Ag expression is required for a longer time. Regardless of the specific mechanism involved, our study demonstrates that when prolonged Ag presentation takes place during secondary exposure to a cognate Ag, it might diminish immunogenicity because of a lack of synchronization between Ag viability and kinetics of its presentation by APCs. This might explain why cutaneous re-exposure to herpes simplex virus, a virus that triggers prolonged Ag presentation during primary infection, resulted in diminished secondary CTL responses (17, 32).

Belz et al. recently reported that, whereas prolonged Ag presentation was observed during primary influenza infection, secondary presentation of the Ag was very short, ending 4 days after reinfection. This was due to a perforin-mediated killing of Ag-bearing APCs by memory CD8+ T cells, a phenomenon that does not occur during the primary infection (24). We think that it is unlikely that the limited secondary immune response observed in i.d.-immunized mice is attributable to the rapid elimination of APCs by memory CD8+ T cells. Firstly, Ag-presenting activity was mainly detected 15 days postinmunization. Secondly, excision of the ear pinna 10 days after the boosting immunization reduced the immune responses, indicating that migrating APCs still continued living at this time.

Expansion of secondary CD8+ T cells can be facilitated by increasing the amount of Ag during the boosting immunization (23). A more recent study also indicates that better targeting of the Ag to DCs enhances Ag presentation and consequently CD8+ T cells expansion (8). However, increasing the concentration of plasmid DNA may not be applicable in our system, as increasing the amount of vector injected into the skin 2-fold during priming (100 μg instead of 50 μg) resulted in considerably lower magnitude of CD8+ T cells (data not shown). To overcome such a problem, Radcliffe et al. suggest priming and boosting with plasmid DNA using different Ag-presentation pathways (33). This enhances the frequencies of secondary CD8+ T cells without increasing the amount of vector at the boosting immunization. It has been also suggested that the immunogenicity of plasmid DNA can be enhanced by extending the immunization intervals (34). However, we found no significant improvement in the frequencies of secondary CD8+ T cells even when the boosting immunization was given 24 wk after priming (data not shown).

In conclusion, our study suggests an additional way by which secondary T cell immune responses can be regulated when prolonged Ag presentation plays a critical role in T cell induction. Despite the presence of sufficient Ag load to elicit T cell expansion, the protracted kinetics of the uptake and processing by APCs decrease its chance to efficiently present to T cells. This information is important in vaccine design, as secondary immunogens should be selected also on the basis of their capability to initiate rapid and effective Ag presentation.

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Disclosures
The authors have no financial conflict of interest.

References


