Prime-Boost with Alternating DNA Vaccines Designed to Engage Different Antigen Presentation Pathways Generates High Frequencies of Peptide-Specific CD8⁺ T Cells

Joanna N. Radcliffe, Joanne S. Roddick, Peter S. Friedmann, Freda K. Stevenson and Stephen M. Thirdborough

*J Immunol* 2006; 177:6626-6633; doi: 10.4049/jimmunol.177.10.6626

http://www.jimmunol.org/content/177/10/6626

---

**Why The JI?**

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**References**

This article cites 42 articles, 27 of which you can access for free at:

http://www.jimmunol.org/content/177/10/6626.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Prime-Boost with Alternating DNA Vaccines Designed to Engage Different Antigen Presentation Pathways Generates High Frequencies of Peptide-Specific CD8⁺ T Cells

Joanna N. Radcliffe, Joanne S. Roddick, Peter S. Friedmann, Freda K. Stevenson, and Stephen M. Thirdborough

The route for presentation of Ag to CD8⁺ or CD4⁺ T cells following DNA vaccination is critical for determining outcome, but the pathways involved are unclear. In this study, we compare two different DNA vaccine designs aimed to elicit CD8⁺ T cell responses against a specific peptide-epitope either by direct- or cross-presentation. Each carries sequences from tetanus toxin (TT) to provide essential CD4⁺ T cell help. In the first already proven design, the peptide-epitope is fused to the N-terminal domain of fragment C from TT. This appears to act mainly by cross-presentation. In the second design, the peptide-epitope is encoded by a minigene, with induction of Th responses mediated by coexpression of a hybrid invariant chain molecule, incorporating a single determinant from TT (p30) in exchange for class II-associated invariant chain peptide. This design appears to act mainly via direct presentation from transfected APCs. Both vaccines mediated Th-dependent priming of CD8⁺ T cells in mice, but the kinetics and level of the responses differed markedly, consistent with engagement of distinct pathways of Ag presentation. Importantly, the vaccines could be combined in an alternating prime-boost regime, in either order, generating substantially expanded memory CD8⁺ T cells, with potent effector function. Taken together, these results demonstrate that vaccination protocols involving different modes of Ag presentation at prime and boost can significantly improve the effectiveness of immunization. The Journal of Immunology, 2006, 177: 6626–6633.

CD8⁺ T cells play a critical role in immunity to tumors and intracellular pathogens. Consequently, there is great interest in developing vaccines that generate potent CD8⁺ T cell memory responses. Numerous approaches have been developed in recent years, many of which are based on the use of recombinant viruses or plasmid DNA. These “genetic vaccines” aim to deliver Ags to the MHC class I processing and presentation machinery in professional APC, particularly dendritic cells (DCs). DCs have the apparently unique capacity to acquire Ag in the periphery and migrate to secondary lymphoid organs, where they up-regulate costimulatory molecules and activate naïve (i.e., Ag-inexperienced) T cells. This activation event triggers CD8⁺ T cell expansion, functional differentiation, and dissemination into peripheral tissues. The requirement for “help” during the priming of CD8⁺ T cell responses to non-inflammatory immunogens has been recognized for some time, and is thought to involve the activation of DCs through cognate interactions with CD4⁺ T cells. For responses to plasmid DNA vaccines, immunostimulatory CpG motifs present in bacterial DNA may bypass the requirement for CD4⁺ T cell help by directly activating DCs (3, 4). Nevertheless, although primary CD8⁺ T cell responses to DNA vaccines may be helper-independent (5, 6), the generation of stable and functional CD8⁺ T cell memory is critically dependent on CD4⁺ T cell help at priming (7–10). Therefore, vaccines designed to generate potent memory CD8⁺ T cells also need to code deliver CD4⁺ T cell determinants. In cancer, and possibly chronic infection, persistence of Ag may tolerate the available CD4⁺ T cell repertoire (11). The strategy we have explored has been to mobilize alternative CD4⁺ T cell help by fusing minimal class I peptide-epitopes directly to the N-terminal domain of tetanus toxin fragment C (N.FrC). This pathogen-derived sequence contains the universal Th determinant (THd) p30, known to bind various MHC class II allomorphs with high affinity (12). Such fusion vaccines induce memory CD8⁺ T cells able to provide significant protection in preclinical models (13, 14), and are currently in phase I/II trials for prostate cancer.

A major hurdle to developing effective T cell vaccines is immunodominance. Contributing to this complex phenomenon is competition between CD8⁺ T cells specific for vector-derived determinants and those of desired specificity (15). Plasmid DNA vectors by their nature provide a minimum of extraneous Ag. However, the necessity of incorporating foreign helper sequences to promote CD8⁺ T cell responses to weak determinants may unwittingly focus secondary responses on irrelevant immunodominant peptides. Therefore, we sought to develop a DNA vaccine that delivered nominal CD8⁺ and CD4⁺ T cell determinants without additional “antigenic baggage.” The strategy we have now explored is based on using only the p30 THd, rather than the large (256 aas) N.FrC sequence, to induce the helper response. To ensure direct delivery into the MHC class II Ag-presentation pathway, the p30 sequence has been inserted into the 31-kDa isoform of mouse invariant chain (Ii), in exchange for the class II-associated Ii peptide (CLIP) sequence. This strategy was based on several reports demonstrating that optimal loading of MHC class II
with endogenously synthesized THd can be achieved through genetic exchange of the core CLIP sequence with that of the selected epitope (16, 17). In addition to the recombinant Ii-hybrid protein, the plasmid vector also expresses a minimal class I peptide-epitope under the control of a separate promoter. This is therefore code-livered, and a leader sequence directs the minigene product into the endoplasmic reticulum (ER). This vaccine design should favor presentation of CD4+ and CD8+ T cell determinants by direct transfection of APCs. In contrast, pDOM-peptide vaccines are more likely to induce Th-dependent CD8+ T cell responses by cross-priming, offering an opportunity to compare and combine the two pathways. In this study, we explore the modes of Ag presentation of the two vaccine designs and investigate prime-boost strategies using alternating injections. The outcome is induction of high levels of IFN-γ-producing and cytolytic CD8+ T cells.

Materials and Methods

Abs, reagents, and cells

The H-2Kb/SIINFEKL-specific Ab 25-D1.16 (18) and the CD4-depleting Ab YTS191.1 were provided by Cancer Research U.K. Directly conjugated Abs were purchased from BD Biosciences. PE-labeled H-2Kb/SIINFEKL tetramers were obtained from Proimmune. Peptides were custom synthesized by Peptide Protein Research. RMA, RMA-S, and P815 cells were transfected with pSL8. An open reading frame for E3/19K-SIINFEKL was cloned into pCI as a NotI-KpnI fragment in frame with the signal peptide.

pRES-DOM-SL8 and pRES-SL8. An open reading frame for E3/19K (Promega) was digested with NotI and EcoRI restriction sites was amplified by PCR using pRES-SL8 as a template and subcloned into pRES2-EGFP (Clontech Laboratories). To construct pRES-(M)SL8, two complementary oligonucleotides encoding the Kozak consensus sequence (CCACCATG) and SIINFEKL were annealed and ligated into pRES2-EGFP as a NotI-EcoRI fragment.

Plasmid DNA was purified for vaccination using a QIAfilter Giga kit (Qiagen). All constructs were sequenced and checked for expression in vitro using the TNT T7 Coupled Reticulocyte Lysate System (Promega).

In vitro transfection

RMA, RMA-S, and P815 cells were transfected with pDUO and pDOM-SL8 by electroporation (340 V, 800 μF in 400 μl of serum-free RPMI 1640) using a Gene Pulser II (Bio-Rad). DCs were electroporated with 20 μg in vitro-transcribed mRNA as described previously (21).

Mice and in vivo experiments

C57BL/6 mice, bred in-house, were vaccinated at 8–10 wk of age with a total of 50 μg of plasmid DNA in normal saline injected into two sites in the quadriceps. CD4 T cell depletion (>95% efficiency) was conducted by i.p. injection of 150 μg of anti-CD4 Ab (YTS191.1) at intervals of 2 days starting 3 days before vaccination. For adoptive transfer experiments, splenocytes from TCR transgenic OT-1 mice were resuspended at 10⁷ to 10⁸ cells/ml in RPMI 1640, and CFSE (Molecular Probes) was added to a final concentration of 5 μM. After 10 min at 37°C, labeling was quenched with serum. Labeled cells (1 × 10⁶) were transfected by i.v. injection into sex-matched C57BL/6 mice. After 2 days, recipients were injected s.c. into the flanks with Ag-positive donor cells. Animal experiments were conducted according to the U.K. Home Office license guidelines and approved by the University of Southampton’s ethical committee.

Flow cytometry

For tetramer analysis, lymph node/spleen cells were stained with PE-labeled H-2Kb/SIINFEKL tetramers and allophycocyanin-labeled anti-CD8-α. Data were collected using a FACScan (BD Biosciences) cytometer and analyzed using WinMDI 2.8 software.

IFN-γ ELISPOT assay

ELISPOT analysis was performed using the BD ELISPOT Set (BD Biosciences) for murine IFN-γ. Spots were developed using 5-bromo-4-chloro-3-indolyl phosphate (Zymed Laboratories) and counted with a Trans regulation 1300 ELISPOT reader (AID Diagnostika).

<table>
<thead>
<tr>
<th>Designation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDOM-peptide</td>
<td>Fusion protein with nominal SIINFEKL peptide in frame with n.FrC</td>
</tr>
<tr>
<td>pSL8</td>
<td>SIINFEKL expressed as an ER-targeted minigene product (SL8)</td>
</tr>
<tr>
<td>pDUO</td>
<td>SL8 co-expressed with an Ii hybrid with CLIP region replaced with a promiscuous THd from n.FrC (p30)</td>
</tr>
</tbody>
</table>

FIGURE 1. Representative structures of plasmids used for vaccination.
C57BL/6 mice were vaccinated by i.m. injection with naked DNA inducing SIINFEKL-specific T cell responses in vivo. Naive less vector levels comparable to those obtained by vaccination with the "help-sion reduced the magnitude of the SIINFEKL-specific response to was to deliver nominal CD8 antigenic baggage provided by the N.FrC sequence. The second (Fig. 1). The first aim of this novel DNA vaccine was to minimize a core peptide sequence from p30, a universal THd from N.FrC the 31-kDa isoform of mouse Ii with the CLIP region replaced by cassette was incorporated within the plasmid backbone encoding help for the SIINFEKL minigene-product, a separate expression (257–264) as an ER-targeted minimal peptide (pSL8) yielding comparable levels of transgene expression and peptide pro-
cessing (data not shown).

Vaccination with pDUO and pDOM-peptide elicit Th-dependent CD8 T cell responses with distinct kinetics

We initially compared the efficacy of pDUO and pDOM-SL8 for inducing SIINFEKL-specific T cell responses in vivo. Naive C57BL/6 mice were vaccinated by i.m. injection with naked DNA plasmid, and on day 12, the number of peptide-specific T cells induced was measured directly ex vivo by IFN-γ ELISPOT. Both vaccines primed specific CD8+ and CD4+ T cells (Fig. 2A); however, the magnitude of the responses were consistently different, with pDUO eliciting ~2- to 3-fold higher numbers of IFN-γ-pro-
ducing cells than pDOM-SL8. The requirement for CD4+ T cell help for both vaccines was established by the effect of depletion of CD4+ cells before vaccination. For pDUO or pDOM-SL8, deple-
tion reduced the magnitude of the SIINFEKL-specific response to levels comparable to those obtained by vaccination with the “helpless” vector pSL8 (Fig. 2A). Supporting the efficiency of CD4+ T cell depletion, responses to the p30 peptide were completely abrogated (Fig. 2A). Thus, both vaccines prime Th-dependent CD8+ T cell responses.

To ensure that our findings of the enhanced immunogenicity of pDUO were not attributable simply to differences in kinetics of T cell expansion, we performed IFN-γ ELISPOT analysis between days 6 and 40 postvaccination (Fig. 2, B and C). By day 8, small but detectable numbers of SIINFEKL-reactive T cells were present in the spleens of mice vaccinated with pDUO (Fig. 2B). These numbers were comparable to those induced by the helpless vector pSL8. However, after day 10, the number of IFN-γ-producing cells fell abruptly with pSL8 immunization. In contrast, CD8+ T cell expansion to pDUO increased dramati-
cally over this period, peaking on day 14, again illustrating the importance of CD4+ T cell help. The kinetics of the response induced by pDOM-SL8 differed from that of pDUO, with the CD8+ T cell response being detectable earlier, such that the number of IFN-γ-producing cells was substantially higher by day 8 (Fig. 2B). However, these effector cells were near to their

Results

DNA vaccine designs

We generated a series of plasmid DNA constructs that expressed the H-2Kb-binding peptide SIINFEKL from chicken OVA (aas 257–264) as an ER-targeted minimal peptide (pSL8) or as a chimeric fusion protein. In this latter construct, designated pDOM-SL8, the SIINFEKL peptide is fused in frame with N.FrC and is cotranslationally directed into the ER (Fig. 1). This is a proven vaccine design (13, 14), thought to induce CD8+ T cell immunity by the concomitant activation of CD4+ T cell help. To provide help for the SIINFEKL minigene-product, a separate expression cassette was incorporated within the plasmid backbone encoding the 31-kDa isoform of mouse Ii with the CLIP region replaced by a core peptide sequence from p30, a universal THd from N.FrC (Fig. 1). The first aim of this novel DNA vaccine was to minimize antigenic baggage provided by the N.FrC sequence. The second was to deliver nominal CD8+ and CD4+ T cell determinants by direct presentation. The new design was given the generic name pDUO. Transfection of RMA cells with pDUO or pDOM-SL8 led to similar levels of surface Kb/SIINFEKL expression, as detected by the Kb/SIINFEKL complex-specific mAb 25-D1.16 (18), indicating comparable levels of transgene expression and peptide pres-
cessing (data not shown).

FIGURE 2. Vaccination with pDUO and pDOM-peptide elicit Th-dep-
dendent CD8+ T cell responses with distinct kinetics. A, C57BL/6 mice (4/group) were treated with a CD4-depleting Ab (clone YTS191.1) prior to vaccination with the constructs indicated. The number of peptide-specific T cells induced was determined 12 days later by IFN-γ ELISPOT. Both vaccines primed specific CD8+ and CD4+ T cells (Fig. 2A); however, the magnitude of the responses were consistently different, with pDUO eliciting ~2- to 3-fold higher numbers of IFN-γ-pro-
ducing cells than pDOM-SL8. The requirement for CD4+ T cell help for both vaccines was established by the effect of depletion of CD4+ cells before vaccination. For pDUO or pDOM-SL8, deple-
tion reduced the magnitude of the SIINFEKL-specific response to levels comparable to those obtained by vaccination with the “helpless” vector pSL8 (Fig. 2A). Supporting the efficiency of CD4+ T cell depletion, responses to the p30 peptide were completely abrogated (Fig. 2A). Thus, both vaccines prime Th-dependent CD8+ T cell responses.

To ensure that our findings of the enhanced immunogenicity of pDUO were not attributable simply to differences in kinetics of T cell expansion, we performed IFN-γ ELISPOT analysis between days 6 and 40 postvaccination (Fig. 2, B and C). By day 8, small but detectable numbers of SIINFEKL-reactive T cells were present in the spleens of mice vaccinated with pDUO (Fig. 2B). These numbers were comparable to those induced by the helpless vector pSL8. However, after day 10, the number of IFN-γ-producing cells fell abruptly with pSL8 immunization. In contrast, CD8+ T cell expansion to pDUO increased dramati-
cally over this period, peaking on day 14, again illustrating the importance of CD4+ T cell help. The kinetics of the response induced by pDOM-SL8 differed from that of pDUO, with the CD8+ T cell response being detectable earlier, such that the number of IFN-γ-producing cells was substantially higher by day 8 (Fig. 2B). However, these effector cells were near to their
peak magnitude at this time point; the absolute number was 3-fold lower than at the peak of the response to pDUO. Furthermore, the size of this effector population contracted rapidly after day 10, falling to \(\approx 10\%\) of the original peak response by day 20 (Fig. 2B). In contrast, the contraction phase of the CD8\(^+\) T cell response to pDUO was more protracted, declining to \(\approx 45\%\) of the peak response by day 20.

The CD4\(^+\) T cell response to pDUO, like the CD8 response, also peaked on day 14 (Fig. 2C). However, p30-specific cells were discernable earlier, with high numbers of IFN-\(\gamma\)-producing cells present by day 8. For pDOM-SL8, the p30-specific response was also evident by day 8, but did not increase substantially over the next 4 days, and then fell sharply (Fig. 2C). These differences in the kinetics of T cell expansion and contraction were observed over three independent experiments, suggesting that pDUO and pDOM-SL8 were priming T cells by distinct pathways.

pDUO and pDOM-peptide differ in their mode of \(K^\alpha/\text{SIINFEKL}\) presentation

We next investigated the pathways by which pDUO and pDOM-SL8 were presenting CD8\(^+\) T cell determinants. To establish a role for direct- and/or cross-presentation, we injected mice with MHC-matched or mismatched Ag-positive donor cells. As Ag donors, we used syngeneic RMA (H-2\(^b\)) or allogeneic P815 (H-2\(^d\)) cells transiently transfected with pDUO or pDOM-SL8. To detect priming, we transferred naive CFSE-labeled TCR transgenic OT-1 T cells specific for \(K^\alpha/\text{SIINFEKL}\) into C57BL/6 mice. Two days after T cell transfer, the recipient mice were injected s.c. with the donor cells. After a further 5 days, OT-1 proliferation was examined in draining lymph nodes. As indicated by the decrease in CFSE fluorescence, OT-1 cells had proliferated strongly upon injection of both RMA and P815 cells transfected with pDOM-SL8 (Fig. 3A). In sharp contrast, OT-1 division was only observed in mice that

![Figure 3](http://www.jimmunol.org/Downloadedfrom/6629)

**FIGURE 3.** A minimal CD8\(^+\) T cell determinant can be cross-presented when fused to NFrC. A, C57BL/6 mice (3/group) that had received CFSE-labeled \(K^\alpha/\text{SIINFEKL}\)-specific TCR transgenic T-cells (OT-1) 2 days previously were injected s.c. with RMA or P815 cells transiently transfected with pDOM-SL8 or pDUO. Five days later, draining lymph node cells were pooled and OT-1 proliferation assessed by flow cytometry. B, Schematic representation of bicistronic expression constructs pIRES-DOM-SL8 and pIRES-(M)SL8. C, RMA and the TAP2-deficient derivative RMA-S were transfected with equimolar amounts of pIRES-DOM-SL8 or pIRES-(M)SL8 and stained 18 h later with the mAb 25-D1.16, which is specific for \(K^\alpha/\text{SIINFEKL}\) (18). D, C57BL/6 mice (3/group) were injected s.c. with RMA or RMA-S transiently transfected with pIRES-DOM-SL8 and pIRES-(M)SL8. Five days later, draining lymph node cells were pooled and the frequency of peptide-specific CD8\(^+\) T cells measured by \(K^\alpha/\text{SIINFEKL}\) tetramer staining. These experiments were repeated twice with similar findings.
received MHC-matched and not mismatched cells transfected with pDUO (Fig. 3A). These data suggest that the SIINFEKL peptide-epitope encoded by pDUO is presented only by direct transfection, whereas pDOM-SL8 will also cross-prime.

To confirm that the nominal SIINFEKL peptide can be cross-presented when fused to N.FrC in pDOM-SL8, complementary experiments were performed using Ag-positive, TAP2-deficient RMA-S cells (22). Because of a failure to load nascent MHC class I molecules with the antigenic peptides, these cells are unable to prime CD8\(^+\) T cell responses directly (23). RMA or RMA-S were transfected with a plasmid vector that coexpressed N.FrC-SL8 with GFP from a single bicistronic transcript (pIRES-DOM-SL8; Fig. 3B). We initially established that the RMA-S transfecants were unable to load K\(^\*\) with SIINFEKL by staining with the mAb 25-D1.16. Thus, while RMA-S cells transfected with pIRES-DOM-SL8 expressed GFP, they were 25-D1.16 negative (Fig. 3C). In contrast, RMA transfecants showed efficient peptide loading (Fig. 3C).

To assess the ability of pDOM-SL8 to prime by cross-presentation, naive C57BL/6 mice were injected s.c. with the transfecants, and 6 days later, the frequency of peptide-specific CD8\(^+\) T cells induced was measured directly ex vivo with K\(^\*\)/SIINFEKL tetramers. Tetramer-positive CD8\(^+\) T cells were detected in the draining lymph nodes of mice upon injection of both RMA and RMA-S cells expressing N.FrC-SL8 (Fig. 3D), supporting the premise that the fusion protein is a suitable substrate for cross-presentation. However, there remained the possibility that RMA-S cells could have a residual capacity to directly present the peptide-epitope in vivo even though they were surface negative for K\(^\*\)/SIINFEKL complexes by 25-D1.16 staining. To ensure that the CD8\(^+\) T cell response induced by pIRES-DOM-SL8 transfecants was in fact the result of cross-presentation, we performed the following control experiment. RMA or RMA-S cells were transfected with plasmid vectors that coexpressed GFP with a leader-minus MSIINFEKL sequence (where M designates an initiating methionine; Fig. 3B). Similar to pIRES-DOM-SL8 transfecants, RMA-S cells transfected with pIRES-(M)SL8 were 25-D1.16 negative, whereas RMA cells expressed surface K\(^\*\)/SIINFEKL complexes (Fig. 3C). Importantly, however, only the TAP-sufficient cells induced a CD8\(^+\) T cell response (Fig. 3D).

Collectively, these data demonstrate that fusion of a minimal CD8\(^+\) T cell determinant to the C terminus of N.FrC facilitates its cross-presentation. However, the analysis does not eliminate the possibility that direct presentation might also occur from pDOM-peptide. The question is whether this route is operative in vivo.

pDOM-SL8 induces Th-dependent CD8\(^+\) T cell responses by cross-priming

Although pDOM-SL8 can generate SIINFEKL peptide for direct presentation in vitro, we hypothesized that vaccination with pDOM-SL8 would prime SIINFEKL-specific CD8\(^+\) T cells predominately by cross-presentation. The rationale for this was that CD8\(^+\) T cells are required for optimal CD8\(^+\) T cell induction by pDOM-peptide (Fig. 2A), and that unless specifically targeted to endolysosomal compartments, endogenous proteins are preferentially channeled into the MHC class I-processing pathway with little opportunity to access a class II-loading compartment (24). We therefore reasoned for pDOM-SL8 to provide cognate help it would be as a result of uptake of N.FrC from an exogenous source; in which case, SIINFEKL would be cross-presented. However, we could not exclude the possibility that professional APC would “regurgitate” secreted N.FrC-SL8 protein to present both class I and class II epitopes at their cell surface. To address this possibility, bone marrow-derived DCs were electroporated with GFP, N.FrC, or II-p30 mRNA, and used as APCs. Production of IFN-γ by p30-specific T cells was used as an indicator of the ability of the DCs to process and present class II determinants from endogenously synthesized N.FrC. No significant T cell activation was observed by DC electroporated with N.FrC mRNA, as compared with the robust stimulation observed using DC pulsed with exogenous N.FrC protein or electroporated with II-p30 mRNA (Fig. 4). These data demonstrate that DC are unable to present THd from endogenously synthesized N.FrC protein, and support the premise that pDOM-peptide would induce Th-dependent CD8\(^+\) T cell responses by cross-priming.

Alternating prime-boost with pDUO and pDOM-peptide potentially induces IFN-γ-producing and cytolytic CD8\(^+\) T cells

Given the fact that pDUO and pDOM-peptide were apparently operating by distinct pathways of T cell priming, we next asked whether the vaccines could be used sequentially in a prime-boost setting. Mice primed with pDUO were revaccinated on day 50 with either pDUO or pDOM-SL8, and ex vivo H-2K\(^\*\)/SIINFEKL tetramer analysis was performed 10 days later. This time point represented the highest T cell response in both groups (data not shown). By day 60 after a single vaccination with pDUO, 0.8–1% of the CD8\(^+\) T cell population was tetramer positive (Fig. 5A, i). Boosting with pDUO expanded that memory population ~5-fold (Fig. 5A, ii). In contrast, using pDOM-SL8 for the boost substantially increased the size of the tetramer-positive CD8\(^+\) T cell population, ~25-fold (Fig. 5A, iii).

When functional IFN-γ ELISPOT analysis was used to measure the number of SIINFEKL-reactive T cells in the spleen, an even greater differential efficacy of heterologous boosting was observed (Fig. 5B). Following priming with pDUO, a second injection of the same vaccine at day 50 raised the level of functional CD8\(^+\) T cells only slightly above the unboosted levels (Fig. 5B). In contrast, injection of heterologous pDOM-SL8 expanded the memory CD8\(^+\) T cell response ~16-fold. This increased secondary expansion also occurred with the alternative combination, i.e., pDOM-SL8 prime followed by pDUO boost (Fig. 5B).

Finally, we sought to demonstrate the killing potential of the SIINFEKL-specific populations in vivo. Differentially, CFSE-labeled splenocytes from naive mice were pulsed with (CFSE\(^{high}\)) or without SIINFEKL (CFSE\(^{low}\)) and i.v. injected into immunized animals. After 5 h, cytolytic responses were quantified by flow cytometry. Consistent with the frequency data, mice primed with pDUO and boosted with pDOM-SL8 exhibited significantly stronger killing than those animals boosted with pDUO or empty vector.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** DCs fail to present THd from endogenously synthesized N.FrC protein. Responder cells from p30-peptide-vaccinated animals were restimulated in vitro with bone marrow-derived DCs electroporated with GFP, N.FrC, or II-p30 mRNA. To confirm that the DCs were capable of processing exogenous Ag, cells were also pulsed with recombinant N.FrC protein (100 μg/ml). IFN-γ production was detected 40 h later by ELISPOT. These studies were repeated twice with similar findings.
Thus, alternating vaccination with pDUO and pDOM-peptide provides a very efficient strategy for expanding high frequencies of effector CD8+ T cells.

Discussion

Therapeutic vaccination aimed at promoting CD8+ T cell immunity requires three key components: activation of the innate system, optimal presentation of MHC class I-binding peptides, and provision of CD4+ T cell help (25). DNA vaccines have the potential to supply all of these, but rational design is hampered by lack of knowledge of their mechanism of action. Although it is well established that bone marrow-derived APCs are required for priming T cell responses to DNA vaccines (26–28), it is not clear whether the encoded Ags are presented directly, indirectly (via cross-presentation), or both (28–34). A second restriction is the low ability of DNA vaccines to boost a primed response, necessitating the use of a second delivery system, usually a recombinant virus. Although this appears to improve performance, it raises additional problems including preexisting or induced antivector immunity. In this study, by manipulating antigenic sequences to favor direct- or cross-presentation, we demonstrate that plasmid DNA vaccines can be designed to activate Th-dependent CD8+ T cell responses by either route. By combining these in a prime-boost setting, effective boosting of IFN-γ-producing and cytolytic CD8+ T cells was achieved.

Previously, we have shown that vaccination with plasmid DNA encoding a minimal class I-determinant fused to N.FrC elicits potent effector CD8+ T cells (13, 14). The efficacy of the pDOM-peptide design was thought to reflect its ability to activate cognate CD4+ T cell help for the CD8+ T cell response, and this was confirmed in the current study. Despite the efficacy of this vaccine for priming, immunodomination by CD8+ T cells specific for determinants from the relatively large N.FrC domain (256 aas) may limit responses to weak target peptides following boosting. Therefore, we attempted to minimize the antigenic-baggage encoded by the vaccine. If the fused sequence was reduced to encode only an individual THd from N.FrC (p30), performance fell dramatically (13). Although it was difficult to draw firm conclusions, this might have been due to the increased sensitivity of the smaller oligopeptide to proteolytic degradation and epitope destruction. In this study, we sought to deliver nominal MHC class I- and class II-restricted T cell determinants by direct presentation. The approach we explored again used a sequence derived from N.FrC to amplify the CD8+ T cell response. However, rather than generating a DNA fusion vaccine, we chose to coexpress a hybrid II molecule, incorporating p30 in exchange for CLIP, with an ER-targeted minimal CD8+ T cell determinant. This dual epitope expression vector was given the generic name pDUO.

Both pDUO and pDOM-peptide induced Th-dependent CD8+ T cell responses when delivered as naked DNA; however, the kinetics of the expansion and contraction phases were strikingly different. Vaccination with pDOM-peptide elicited a CD8+ T cell response, faster in onset compared with pDUO, but less sustained resulting in a net lower peak burst size. Such dynamic differences likely reflect the frequency and quality of peptide-bearing DCs in vivo. This in turn may be due to the vaccines operating by distinct

(FIG. 5C). Thus, alternating vaccination with pDUO and pDOM-peptide provides a very efficient strategy for expanding high frequencies of effector CD8+ T cells.

FIGURE 5. Alternating injections of pDUO and pDOM-SL8 potently induces high frequencies of peptide-specific CD8+ T cells. A, C57BL/6 mice (4/group) primed with pDUO were revaccinated on day 50 with the constructs listed, and ex vivo H-2Kb/SIINFEKL tetramer analysis was performed 10 days later on pooled splenocytes. B, IFN-γ ELISPOT analysis of pooled splenocytes from mice (4/group) primed with pDOM-SL8 or pDUO and boosted on day 50 with the constructs listed. C, Mice primed with pDUO were revaccinated on day 50 with the constructs listed, and in vivo killing of SIINFEKL-pulsed targets was measured 10 days later, as described in Materials and Methods. Each data point represents the percentage killing from an individual mouse. These studies were repeated twice with similar findings.
modes of Ag acquisition and processing. For example, Cho et al. (34) have demonstrated that DNA-transfected APC initiate CD8+ T cell responses slower than APC that have cross-presented Ags produced by somatic cells.

Recent studies have suggested that cross-priming is dependent on transfer of whole or partially degraded proteins, rather than mature peptides (35–38). Therefore, we hypothesized that the minigene-expressing vector pDUO would induce specific CD8+ T cells predominantly by direct priming. In support of this hypothesis, MHC mismatched Ag-donor cells, transfected with pDUO, failed to cross-prime. In sharp contrast, cross-priming was efficient with pDOM-peptide transfectants, and although priming could also occur by a direct route, the requirement for CD4+ T cell made it less likely.

Compatible with pDUO and pDOM-peptide-inducing CD8+ T cells by distinct pathways was the finding that both vaccines could be combined in a prime-boost regimen to generate substantial T cell memory. Thus, boosting with pDOM-peptide elicited a significantly greater recall response from pDUO-primecd CD8+ T cells than restimulating with pDUO. Neither vaccine was effective in a “homologous prime-boost” setting for expanding memory T cells. Why the heterologous strategy should be so effective is not entirely clear, but one possible explanation is differential requirements for Ag density at prime and boost. Pamer and colleagues (39) have demonstrated that effector responses limit secondary memory T cell expansion by rapidly clearing Ag-bearing APCs upon restimulation. This could be overcome by using a recombinant virus that expressed more Ag at boost. Similarly, we have shown that electroporation at boost leads to increased Ag expression and greater recall responses to naked DNA vaccines (40). If pDOM-peptide vaccination results in a larger number of Ag-bearing professional APCs than pDUO, as suggested by our kinetic data, one might predict that sequential immunization with pDUO and boosting with pDOM-peptide should elicit a greater recall response. Surprisingly, however, the increased secondary expansion occurred irrespective of the order of vaccine delivery. Recent evidence has suggested that CTL-mediated elimination of APCs occurs in the tissues and not lymph nodes (41). Perhaps, pDUO and pDOM-peptide differentially influence the site of priming and subsequent dissemination of effector T cells, such that boosting with the reciprocal vector overcomes effector T cell responses that otherwise restrict central memory T cell expansion (39). To strengthen this hypothesis, we clearly need to identify the professional APCs active in this process, their phenotype, activation state and peptide-MHC ligand display. We also plan to investigate potentially synergistic combinations of the two vaccines injected together or in separate sites. Incorporation of the two designs into a single vector, which would be attractive for clinical translation, will also be assessed.

In summary, we have described two DNA vaccine designs able to induce high frequencies of CD8+ T cells against defined minimal class I-determinants by different routes. The disadvantage of both is that the MHC class I-binding peptide must be known, but candidate peptides are emerging as our understanding of Ag processing advances (24). Single-epitope vaccines may also allow escape by epitope loss or modulation, but combination with vectors encoding additional determinants, ideally injected into separate sites, should overcome this. The success of the li-p30 hybrid-containing vaccine pDUO depends on the promiscuity of p30 for human MHC class II haplotypes, and this requires further analysis. Alternative universal THd can be tested, as well as peptides aimed to induce effector CD4+ T cells against tumor or pathogen-derived Ags. Overall, this study highlights the power of DNA vaccines and, in particular, the ease with which antigenic sequences can be manipulated to promote the induction of high levels of effector and memory T cells, an undoubted requirement for providing immunity to cancer and virulent pathogens (42).

Acknowledgments

We thank Dr. R. N. Germain for providing permission to use the 25-D1.16 Ab, and members of the biomedical facility for their technical assistance.

Disclosures

The authors have no financial conflict of interest.

References


