CD4+ T lymphocytes mediate in vivo clearance of plasmid DNA vaccine antigen expression and potentiate CD8+ T-cell immune responses

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There is evidence that the limited immunogenicity of plasmid DNA vaccines is the result, at least in part, of the rapid clearance of vaccine antigen expression by antigen-specific immune responses. However, the cell types responsible for the clearance of plasmid DNA vaccine antigens are not known. Here we demonstrate that macrophages, NK cells, and CD8+ T cells did not significantly contribute to the DNA antigen clearance but CD4+ T cells played the crucial role in attenuating plasmid DNA vaccine antigen expression. Adoptive transfer experiments demonstrate that CD4+ T cells facilitated DNA vaccine antigen clearance in a Fas/FasL-dependent manner. Furthermore, we show that depletion of CD4+ T cells prevented the clearance of vaccine antigen and the appearance of a CD8+ T-cell immune response. Inoculation of major histocompatibility complex class II KO mice with the plasmid DNA led to persistent antigen expression and ablation of a CD8+ T-cell immune response. Importantly, the prolongation of antigen expression by disrupting the CD4+ T-cell Fas/FasL myocytes signaling led to a 3- to 5-fold increase of antigen-specific CD8+ T-cell responses. These data demonstrate a dominant role of CD4+ T cell-mediated cytotoxicity in plasmid DNA vaccine antigen clearance. (Blood. 2008;112:4585-4590)

Introduction

Plasmid DNA vaccines are a promising modality for immunization against a variety of infectious agents because they are safe, readily scalable, and easy distributed. Plasmid DNA vaccine vectors can elicit CD8+ cytotoxic T lymphocytes (CTLs), CD4+ T helper cell immune responses, as well as humoral immune responses. Nonetheless, the utility of DNA immunogens has been limited by their failure to elicit sufficiently potent immune responses. One potential explanation for the limited immunogenicity of plasmid DNA is that vaccine antigen expression is generated at only transient and at low levels.1

Immune-mediated destruction of antigen-producing muscle fibers appears to play a significant role in limiting vaccine antigen expression. Clearance of antigen-expressing myocytes has been shown to be dependent both on the immunogenicity of the antigen and the presence of a functional immune system.2,3 However, the cell types responsible for this destruction remain to be determined. We have shown that damping of plasmid DNA vaccine antigen expression in vivo occurs coincident with the emergence of major histocompatibility complex (MHC) class I-restricted T-cell responses. In addition, we observed that vaccine antigen expression persists in Fas receptor knockout mice, suggesting a role in this process for T cell–mediated apoptosis via the Fas/FasL pathway.4 Based on these data, we hypothesized that CD8+ T cells mediated vaccine antigen clearance through Fas-dependent apoptosis. Alternatively, other studies have suggested that the limited antigen expression in this setting may be a result of antibody-dependent cell-mediated cytotoxicity or complement-mediated lysis.4 In addition to adaptive immune responses, innate immune responses, such as those mediated by macrophages and NK cells, have also been implicated as potential contributors to the destruction of antigen-producing myocytes.5,6

In the present study, we investigated the cell types responsible for antigen clearance in plasmid DNA vaccinated mice. We used an In Vivo Imaging System (IVIS), which enabled us to measure antigen expression in vivo precisely, without serial killing of the animals. Using knockout (KO) mice and antibody-depletion experiments, we investigated the relative contribution of NK cells, macrophages, CD8+ T cells, and CD4+ T cells to the damping of antigen expression in vaccinated animals. Surprisingly, we observed that CD4+ T cells were both necessary and sufficient to mediate plasmid DNA vaccine antigen clearance. These findings demonstrate a central role for CD4+ T cells in vaccine antigen clearance.

Methods

Animals and immunizations

Six- to 8-week-old wild-type C57BL/6, C57BL/6.82 M KO, C57BL/6.MHC II KO, Rag1 KO, and NK-function-deficient beige mice (C57BL/6-Ly5+/−6) were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed and maintained in accordance with the Guide for the Care and Use of Laboratory Animals and all studies and procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Harvard University.

For immunizations, 50 μg plasmid DNA in 100 μL sterile saline was divided between quadriceps muscles.
Vectors
The plasmid DNA-luciferase (DNA-Luc) with the AL11-tag was constructed as described previously. This vector contains the GLA.10 luciferase gene (Promega, Madison, WI) and the immunodominant H-2D\(^{b}\)-restricted SIV Gag AL11 epitope (AVYKNWMTQTL) flanked by triple-alanine spacers. Plasmid DNA was prepared using an Endotoxin-free QIAGEN Giga-prep kit (Valencia, CA).

Antibodies
Fluorescein isothiocyanate, peridinin-chlorophyll-protein complex, allophycocyanin, and phycoerythrin-labeled antibodies were used for the flow cytometric analysis. The dye-coupled antibodies anti–CD14-fluorescein isothiocyanate (mC5-3), anti–CD8\(^{b}\)-peridinin-chlorophyll-protein complex-Cy5.5 (53-6.7), anti–CD4-allophycocyanin-Cy7 (L3T4), and anti–IL-2-phycoerythrin (JES6-5H4) were purchased from BD Biosciences (San Jose, CA).

Immunologic assays
H-2D\(^{b}\)/AL11 tetramers were prepared and used to stain epitope-specific CD8\(^{b}\) T cells as previously described. Peripheral blood was collected and lysed with BD Pharmlyse buffer (BD Biosciences). The samples were then analyzed on a FACS Array (BD Biosciences), and CD8\(^{b}\) T lymphocytes were examined by staining with the H-2D\(^{b}\)/AL11 tetramer. CD8\(^{b}\) T lymphocytes from control mice immunized with untagged plasmid DNA-Luc exhibited less than 0.1% tetramer staining. Intracellular cytokine staining was performed as previously described. For T-cell stimulation, cells were incubated with a luciferase peptide pool at 2 \(\mu\)g/mL for each peptide. The luciferase peptide pool of 67 18-mers, overlapping by 10, was synthesized by Quality Control Biochemicals (Hopkinton, MA).

In vivo bioluminescence measurement
Animals were injected intraperitoneally with 100 \(\mu\)L of a 30-mg/mL solution of firefly luciferin (Xenogen, Alameda, CA) in phosphate-buffered saline, as well as 100 \(\mu\)L of a 20-mg/mL ketamine and 1.72-\(\mu\)g/mL xylazine mixture. After 20 minutes, imaging was performed using the IVIS Series 100 (Xenogen) with an integration time of one minute. Overlay images and luminescence measurements were made using Living Image software (version 2.50.1; Xenogen).

Measurement of antigen expression
To convert the in vivo bioluminescence relative light units (RLU) of the different vectors into quantity of antigen expressed, we prepared a calibration curve with values of emitted light per minute for different amounts of recombinant luciferase protein. There was a linear correlation between amount of protein injected (10 ng to 50 \(\mu\)g) and light emitted, enabling us to calculate antigen expression from the different vectors according to the following formula:

\[
\text{Concentration of antigen in ng} = \text{antilog} \left( \frac{\log \text{RLU} - 4.2}{0.76} \right)
\]

Cell depletion
To deplete macrophages, clodronate-loaded liposomes and control liposomes were prepared as previously described. Phosphatidylcholine was obtained from Lipoid (Ludwigshafen, Germany). Cholesterol was purchased from Sigma-Aldrich (St Louis, MO). Liposomes were obtained from Lipoid (Ludwigshafen, Germany). Cholesterol was purified on a FACS Array (BD Biosciences), and CD8\(^{b}\) lymphocytes were analyzed with BD Pharmlyse buffer (BD Biosciences). The samples were then analyzed on a FACS Array (BD Biosciences), and CD8\(^{b}\) T lymphocytes were examined by staining with the H-2D\(^{b}\)/AL11 tetramer. CD8\(^{b}\) T lymphocytes from control mice immunized with untagged plasmid DNA-Luc exhibited less than 0.1% tetramer staining. Intracellular cytokine staining was performed as previously described.

Adoptive transfer
Wild-type mice were immunized with the plasmid DNA-Luc vaccine construct. Fourteen days later, spleens were placed in RPMI medium (Sigma-Aldrich) and gently teased apart between frosted microscope slides. To reduce red blood cell contamination, the splenocyte suspensions were treated with M-Lyse buffer (R&D Systems, Minneapolis, MN). Leukocyte suspensions were incubated with a mixture of monoclonal antibodies and then loaded onto the Mouse T Cell Subset Column Kit (catalog #MCD4-1000, R&D Systems) according to the manufacturer’s instructions. B cells, nonselected T cells, and monocytes were then bound to glass beads coated with anti-immunoglobulin via both F(ab) and Fc interactions, and purified CD4\(^{b}\) T cells were eluted. A total of 10 million purified CD4\(^{b}\) T cells were injected intraperitoneally into RAG-deficient mice. Purity was tested by anti-CD4 and anti-CD8\(\alpha\) antibody staining and flow cytometric analysis. Cells were greater than 95% CD4\(^{b}\) T cell positive.

Data analysis
The statistical significance between 2 experimental groups was determined using the Mann-Whitney test. A \(P\) value less than .05 was considered to be significant. Statistical calculations were performed using software for personal use only. Please see the online article for more details.

Results
We previously observed that vaccine antigen expression was more durable in Fas receptor KO mice than in wild-type mice, suggesting a role for T cell–mediated apoptosis via the Fas/FasL pathway in vaccine antigen clearance. To exclude the possibility that this observed persistence of vaccine antigen expression was a consequence of an idiosyncrasy of the Fas receptor KO mouse, we monitored plasmid DNA vaccine antigen expression and immune responses in Fas ligand knockout mice (FasL KO). We immunized mice intramuscularly with the plasmid DNA-Luc construct encoding both the luciferase gene and the H-2D\(^{b}\)-restricted CTL epitope of SIV gag, AL11. Interestingly, the AL11-specific CD8\(^{b}\) T-cell response was 3- to 5-fold greater in the FasL knockout than in the wild-type mice starting at day 14 after inoculation (\(P = .03\); Figure 2A). We observed a damping of transgene expression in wild-type mice by day 21 after plasmid DNA inoculation that was not observed in the FasL KO mice (\(P = .03\); Figure 1B,C). These findings are consistent with the notion that T cells mediate the damping of plasmid DNA vaccine antigen expression via a Fas/FasL interaction.

Our demonstration that CD8\(^{b}\) T-cell responses to the AL11 epitope lagged behind the damping of luciferase expression by 7 days suggested that this vaccine antigen clearance might be CD8\(^{b}\) T cell mediated. To evaluate this possibility, we inoculated beta-2-microglobulin KO (\(\beta^{2m}\) KO) and wild-type mice with a plasmid DNA-Luc construct and monitored luciferase expression over time. Because MHC class I presentation of peptide antigen to CD8\(^{b}\) T cells requires \(\beta^{2m}\), the \(\beta^{2m}\) KO mice cannot generate antigen-specific CD8\(^{b}\) T-cell responses. Surprisingly, we did not observe a difference in the kinetics of expression of luciferase between the \(\beta^{2m}\) KO and wild-type mice (Figure 2A). We next depleted CD8\(^{b}\) T cells from wild-type mice by treating them with an anti-CD8\(\alpha\) antibody before immunization with plasmid DNA-Luc (Figure 2B). This treatment resulted in a greater than 95% depletion of the CD8\(^{b}\) T cells as confirmed by monoclonal antibody staining and flow cytometric analysis (data not shown). Consistent with the results of the study in the \(\beta^{2m}\) KO mice, the kinetics of luciferase expression were not altered in the CD8\(^{b}\)
These animals are reported to have nonfunctional NK cells.\textsuperscript{7-9} We responses were measured by D\textsuperscript{P}AL11 tetramer staining of CD8\textsuperscript{T} cells. Epitope-specific CD8\textsuperscript{T} cell responses in vaccinated wild-type and FasL KO mice. Epitope-specific CD8\textsuperscript{T} cell responses were measured by D\textsuperscript{P}AL11 tetramer staining of CD8\textsuperscript{T} cells at the indicated times after plasmid DNA vaccine inoculation. (B) Photo images overlaid with visualized infrared signal readouts of wild-type and FasL knockout (FasL KO) mice. Wild-type and FasL knockout mice were inoculated with plasmid DNA-Luc (n = 4), and luciferase expression in vivo was measured by IVIS imaging. (B) Luciferase expression in vaccinated wild-type and CD8\textsuperscript{T} cell-depleted mice. CD8\textsuperscript{T} T cells were depleted by administration of anti-CD8 antibody 3 days before and every third day after plasmid DNA-Luc inoculation for a 28-day period, and in vivo luciferase expression was measured. The line graphs represent mean values; error bars represent SEM.

Figure 1. Damping of luciferase expression from a plasmid DNA vaccine construct is FasL-mediated. (A) SIV-gag AL11 epitope-specific CD8\textsuperscript{T} T-cell responses in vaccinated wild-type and FasL KO mice. Epitope-specific CD8\textsuperscript{T} T-cell responses were measured by D\textsuperscript{P}AL11 tetramer staining of CD8\textsuperscript{T} T cells at the indicated times after plasmid DNA vaccine inoculation. (B) Photo images overlaid with visualized infrared signal readouts of wild-type and FasL knockout (FasL KO) mice. Wild-type and FasL knockout mice were inoculated with plasmid DNA-Luc (n = 4), and the in vivo expression of luciferase was measured by IVIS imaging. Comparison of luciferase antigen expression between wild-type and FasL KO mice. (C) Representative infrared signal images of luciferase activity are shown as relative light units (RLU) at 0.2, 2, 7, 21, and 28 days after plasmid DNA inoculation. The line graphs represent mean values; error bars represent SEM. Statistically significant differences at specific times were determined by the Mann-Whitney test. *Significant difference ($P < .05$).

T cell–depleted mice. These findings suggested that damping of the in vivo expression of DNA vaccine antigen may not be mediated by CD8\textsuperscript{+} T cells.

We then investigated the role of cells of the innate immune system in facilitating the T cell–mediated killing of antigen-expressing myocytes. To examine the role of NK cells, we immunized beige mice with the plasmid DNA-Luc construct. These animals are reported to have nonfunctional NK cells.\textsuperscript{7-9} We observed no difference in the kinetics of antigen expression in beige mice compared with wild-type mice (Figure 3A). We also examined the role of macrophages in plasmid DNA vaccine antigen clearance. We depleted macrophages by injecting mice with clodronate-loaded liposomes. Depletion was greater than 95% as confirmed by flow cytometric analysis with fluorescence-labeled anti-CD14 antibody (data not shown). Interestingly, we did not observe a difference in the clearance of vaccine antigen expression after macrophage depletion, suggesting that these cells also did not play an important role in this clearance (Figure 3B).

In light of evidence that CD8\textsuperscript{+} T cells did not appear to mediate vaccine antigen clearance, we examined whether T cells play a significant role in this phenomenon. We inoculated TCR-\beta/TCR-\delta KO mice with the plasmid DNA-Luc vaccine construct and quantified antigen expression over time (Figure 3C). Consistent with our previous results, we observed persistent antigen expression in T cell–deficient TCR-\beta/TCR-\delta KO mice with significant differences between wild-type and KO mice starting at day 14 ($P = .03$). Similarly, we observed persistent antigen expression in MHC class I/MHC class II knockout mice, with significant differences at day 14 after inoculation ($P < .01$) in the damping of antigen expression between wild-type and KO mice (Figure 3D). These findings argue strongly for a role for T cells in plasmid DNA vaccine antigen clearance.

The observation that T cells, but not CD8\textsuperscript{+} T cells, mediate vaccine antigen clearance suggested that CD4\textsuperscript{+} T cells may play a central role in this clearance. To evaluate the contribution of CD4\textsuperscript{+} T cells directly, we inoculated MHC class II KO mice with plasmid DNA-Luc and confirmed that the animals were indeed CD4\textsuperscript{+} T cell–deficient by anti-CD4 antibody staining of peripheral blood mononuclear cells and flow cytometric analysis (data not shown). In contrast to the MHC class I KO mice, we observed persistent vaccine antigen expression in the MHC class II KO mice (Figure 4A). Significant differences in the clearance of vaccine antigen expression between wild-type and KO mice started at day 14 after vaccination ($P = .04$). To confirm these findings in wild-type mice, we infused mice with an anti-CD4 antibody before plasmid DNA-Luc inoculation to deplete CD4\textsuperscript{+} T cells. The depletion of CD4\textsuperscript{+} T cells was confirmed with an anti-CD4 antibody staining of...
Peripheral blood mononuclear cells and by flow cytometric analysis (data not shown). Persistent vaccine antigen expression was observed in the absence of CD4\(^+\) T cells with a difference between control and antibody-treated groups starting at day 21 after vaccination (\(P < .03\); Figure 4B). These data suggested that vaccine antigen clearance requires CD4\(^+\) T cells. To investigate whether CD4\(^+\) T cells can also influence the CD8\(^+\) T-cell immune response, we measured the AL11-specific CD8\(^+\) T-cell immune response as part of the luciferase antigen construct in the MHC class II KO and the CD4\(^+\) T-cell antibody–depleted mice. We found that depletion of CD4\(^+\) T-cell immune response abolished any CD8\(^+\) T-cell immune response (Figure 4C,D).

The observation that CD4\(^+\) T cells are involved in plasmid DNA vaccine antigen clearance suggested that plasmid DNA vaccine antigen expression elicits an MHC class II-restricted antigen-specific immune response. To evaluate this possibility, we performed intercellular cytokine staining of splenic CD4\(^+\) T cells of plasmid DNA-Luc immunized mice after stimulation with pooled 18-mer peptides spanning the luciferase gene. We observed potent antigen-specific responses by CD4\(^+\) T cells from mice immunized with plasmid DNA-Luc (Figure 5A). To examine whether the higher frequency immune responses with persistent antigen expression in FasL KO mice might be the consequence of a functional defect outside of the Fas pathway, we performed intercellular cytokine staining on splenocytes from these animals after immunizing with plasmid DNA-Luc. We observed no significant differences in the interleukin-2 production by splenocytes of the immunized FasL KO and wild-type mice (Figure 5A).

We then sought to determine whether CD4\(^+\) T cells alone were sufficient to damp plasmid DNA vaccine antigen. CD4\(^+\) T cells were isolated from FasL KO or wild-type mice 14 days after immunization with the plasmid DNA-Luc. These CD4\(^+\) T cells

![Figure 3: Damping of luciferase expression from a plasmid DNA vaccine construct does not require NK cells or macrophages but does require T cells.](image)

![Figure 4: Damping of luciferase expression from a plasmid DNA vaccine construct requires MHC class II molecules and CD4\(^+\) T cells.](image)
Accruing evidence suggested that the kinetics of antigen expression of a plasmid DNA vaccine is linked to its immunogenicity. To enhance the immunogenicity of DNA vaccines, we thought to examine the mechanisms by which the kinetics of DNA antigen clearance is regulated. Therefore, we sought to define the role of different components of the immune system in the clearance of plasmid DNA vaccine antigen expression. We previously hypothesized that CD8^+ T cells might be the primary mediator of vaccine antigen clearance, based on the observation that potent MHC class I–restricted cellular immune responses emerge coincidently with the damping of plasmid DNA vaccine antigen expression. Consistent with a role for T cells in vaccine antigen clearance, we observed that depletion of NK cells and macrophages did not affect the damping of antigen expression in myocytes but that elimination of TCR-β/TCR-δ and MHC class I/MHC class II molecules prolonged antigen expression. Surprisingly, depletion of CD8^+ T cells and knockout of MHC class I expression did not affect plasmid DNA vaccine antigen clearance. However, disruption of MHC class II expression or depletion of functional CD4^+ T cells led to persistent antigen expression, suggesting that CD4^+ T cells are necessary for clearance of plasmid DNA vaccine antigen expression. Most importantly, depletion of MHC class II signaling and CD4^+ T cells led to abolishment of the CD8^+ T-cell immune response. Adoptive transfer studies demonstrated that CD4^+ T cells were sufficient to clear the expression of plasmid DNA antigen. Thus, CD4^+ T cells, but not the components of innate immune system tested, or CD8^+ T cells, or antibodies attenuate in vivo plasmid DNA antigen expression in mice and play the dominant role in modulating the magnitude of the CD8^+ T-cell immune response.

CD4^+ T cells have previously been shown to have cytotoxic effector function. Although CD4^+ T cells use many of the cytotoxic effector mediators used by CD8^+ T cells, including Fas/FasL, perforin, and interferon-γ, there is evidence that Fas/FasL–mediated apoptosis is the predominant killing mechanism used by CD4^+ T cells. To explore the contribution of the Fas/FasL interaction in the plasmid DNA vaccine antigen expression, we extended our adoptive transfer experiments to include CD4^+ T cells derived from FasL KO mice inoculated with a plasmid DNA vaccine construct. We found that there was a requirement for FasL expression on these adoptively transferred CD4^+ T cells for the cells to mediate clearance of plasmid DNA vaccine antigen clearance. These findings therefore indicate that plasmid DNA vaccine antigen expression is cleared primarily through CD4^+ T cell and FasL–mediated apoptosis.

These findings define the mechanism by which vaccine antigen expression is regulated after intramuscular plasmid DNA vaccination. Although Fas-mediated destruction of myocytes by CD4^+ T-cell immune responses has been reported in inflammatory myopathies, including polymyositis, the present findings indicate that this mechanism can play a role in the down-regulation of immune responses. Indeed, we have observed that plasmid DNA vaccines generate higher magnitude and more persistent CD8^+ T-cell immune responses in FasL KO than in wild-type mice (Figure 1). This finding suggests that increased magnitude and duration of local vaccine antigen expression may lead to higher magnitude immune responses. Together, these findings suggest that damping of T cell–mediated apoptosis may lead to more potent DNA vaccines and that CD8^+ T-cell immune response needs a...
higher magnitude of CD4+ T-cell help than anticipated in earlier studies.23 Thus far, it is not clear whether direct antigen presentation of myocytes to T cells, direct antigen presentation of professional antigen-presenting cells to T cells, or cross-priming is the most important mechanism to achieve a strong T-cell immune response.23-28 Although it has been demonstrated that myocytes can present MHC class II molecules,26,27 our data suggest that direct antigen presentation through myocytes to CD4+ T cells might offer an important interaction to manipulate to improve DNA-induced T-cell immune responses. It is, however, possible that the importance of CD4+ T cells in vaccine antigen clearance might not be seen for all antigens. The present studies were using a luciferase-gag epitope construct. The T-cell immune response is the predominant immune response to this vaccine construct. For surface-expressed proteins, antigen clearance might be more dependent on antibodies.29

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Authorship

Contribution: R.G.-L. designed and performed the research, analyzed data, and wrote the paper; J.R.G. designed and performed research and analyzed data; K.F.-B. performed research; N.v.R. contributed vital new reagents; A.-H.H. performed research; and N.L.L. designed the research, analyzed data, and wrote the paper.

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