Adjuvanticity of Plasmid DNA Encoding Cytokines Fused to Immunoglobulin Fc Domains

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Abstract Purpose: Plasmid DNAs encoding cytokines enhance immune responses to vaccination in models of infectious diseases and cancer. We compared DNA adjuvants for their ability to enhance immunity against a poorly immunogenic self-antigen expressed by cancer.

Experimental Design: DNAs encoding cytokines that affect T cells [interleukin (IL)-2, IL-12, IL-15, IL-18, IL-21, and the chemokine CCL21] and antigen-presenting cells [granulocyte macrophage colony-stimulating factor (GM-CSF)] were compared in mouse models as adjuvants to enhance CD8+ T-cell responses and tumor immunity. A DNA vaccine against a self-antigen, gp100, expressed by melanoma was used in combination with DNA encoding cytokines and cytokines fused to the Fc domain of mouse IgG1 (Ig).

Results: We found that (a) cytokine DNAs generally increased CD8+ T-cell responses against gp100; (b) ligation to Fc domains further enhanced T-cell responses; (c) adjuvant effects were sensitive to timing of DNA injection; (d) the most efficacious individual adjuvants for improving tumor-free survival were IL-12/Ig, IL-15/Ig, IL-21/Ig, GM-CSF/Ig, and CCL21; and (e) combinations of IL-2/Ig + IL-12/Ig, IL-2/Ig + IL-15/Ig, IL-12/Ig + IL-15/Ig, and IL-12/Ig + IL-21/Ig were most active; and (f) increased adjuvanticity of cytokine/Ig fusion DNAs was not related to higher tissue levels or greater stability.

Conclusions: These observations support the potential of cytokine DNA adjuvants for immunization against self-antigens expressed by cancer, the importance of timing, and the enhancement of immune responses by Fc domains through mechanisms unrelated to increased half-life.

Delivery of genes encoding proinflammatory cytokines with vaccines has the potential to enhance active immune responses. Numerous studies have examined the use of individual molecular adjuvants combined with vaccines targeting infectious pathogens and cancer (1–8). However, the comparative effects of DNA adjuvants for vaccines against cancer antigens, which are generally poorly immunogenic, need to be further investigated.

Most tumor antigens on spontaneous cancers are molecules shared by cancer and normal cells (9). These are poor immunogens due to immune ignorance or tolerance. Recognition of antigens on tumor cells can be induced through the use of homologous proteins closely related to self-antigens, such as proteins encoded by DNA orthologues. Xenogeneic DNA vaccines elicit antibody and T-cell responses that cross-react with the original syngeneic antigen to produce effective immunity (10–12).

We have previously shown that granulocyte macrophage colony-stimulating factor (GM-CSF) DNA enhances antibody responses and antibody-mediated tumor protection induced by DNA immunization against mouse tyrosinase-related protein-1 in a mouse melanoma model (13). Here, we describe a comparison of plasmid DNA adjuvants, using a panel of proinflammatory cytokine genes, to evaluate CD8+ T-cell
responses against a melanocyte-specific self-antigen, gp100, and tumor immunity in a melanoma model. Previous studies have shown that tumor immunity against gp100, including immunity elicited by xenogeneic DNA immunization, is primarily mediated by CD8+ T cells (12, 14–16). Genes encoding proinflammatory cytokines were tested as native full-length cDNAs and as cDNAs encodingFc fusion molecules through creation of chimeric molecules with potential for increased bioavailability and half-life (2, 17–21). In the present report, a comparison of DNAs encoding potential molecular adjuvants shows that an IgG1 Fc domain enhances adjuvant effects of cytokines, but this enhancement is associated with decreased levels of expression in tissues.

Materials and Methods

Plasmid constructs. Human gp100 (hgp100; provided by Nicholas Restifo, National Cancer Institute, Bethesda, MD) and TRP-2 (hTRP-2) plasmid DNAs (provided by S.A. Rosenberg and J.C. Yang, National Cancer Institute, Bethesda, MD; ref. 22) were cloned into the pING vector and WRG/BEN vector, respectively (23). The mouse IL-2 cDNA was provided by PowderMed (Oxford, United Kingdom), and the single-chain mouse IL-12 cDNA used for the fusion molecule was obtained from Dr. Sherie L. Morrison (University of California at Los Angeles, Los Angeles, CA; ref. 24). The mouse GM-CSF construct has been described (13).

To clone the genes for mouse IL-15, IL-18, IL-21, and IgG1 Fc domain, a C57BL/6 mouse was immunized by particle bombardment (gene gun) with hTRP-2 DNA. This method has been shown to induce a local inflammatory response in the skin and regional lymph nodes (13, 25). RNA from lymph nodes was used to clone cytokine and IgG1 Fc domain genes by reverse transcription-PCR. Identity of these genes was confirmed by restriction digestion and complete sequencing. The cytokine genes for mouse IL-2, IL-12, IL-15, IL-18, IL-21, and CCL21 were then cloned into the pING vector. For the Ig fusion constructs, ligation of pING to IgG1 Fc domain was created first, followed by insertion of the appropriate cytokine cDNA 5’ to the Fc domain.

Mice. C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD) and were kept in a pathogen-free Sloan-Kettering Institute vivarium. All mice entered the study between 7 and 10 weeks of age. All procedures involving animals were conducted under a protocol according to NIH Animal Care guidelines, with approval of the Sloan-Kettering Institutional Animal Care and Use Committee.

DNA administration. Mice received DNA immunizations with hgp100 DNA, with or without the addition of cytokine DNA. DNA was injected into the skin using a helium-driven device, as described (Accell, PowderMed; ref. 23). In brief, plasmid DNA was coated on 1-μm gold microcarriers, mice were depilated over the abdominal skin with Nair depilatory cream (Carter-Wallace, New York, NY), and DNA was administered by gold-DNA complexes into four abdominal quadrants (1 μg plasmid DNA/quadrant) weekly for 3 weeks.

Mouse tumor studies. Tumor challenge experiments were carried out with B16 melanoma cells, as described (26). Briefly, 1 × 10^5 B16F10 melanoma cells (Issaiah Fidler, M.D. Anderson Cancer Center, Houston, TX) were injected into the left flank of mice. Tumor diameter exceeded 10 mm or if tumor became ulcerated or mice showed any discomfort, mice were euthanized.

ELISA for evaluation of cytokine levels. Abdominal skin and blood were collected at designated time points following cytokine DNA administration. To determine the tissue content of cytokines, tissue samples were homogenized in PBS + 0.1% Tween 20 and supernatants were collected after centrifugation as described (25). ELISAs for GM-CSF, IL-2, and IL-12 were done using kits (Endogen, Inc., Woburn, MA). The sensitivity of the cytokine assays was between 2 and 20 pg/ml.

To confirm levels of expression of cytokine DNA constructs, Chinese hamster ovary cells were transfected with DNA using FuGENE 6 (Roche Diagnostics, Indianapolis, IN). Cytokine levels in supernatants were measured at 24 and 48 hours.

IFN-γ ELISPOT assays. Draining inguinal and axillary lymph nodes and spleens of immunized mice (five per group) were harvested and analyzed by ELISPOT and intracellular cytokine assays. IP-Multiscreen plates (Millipore, Burlington, MA) were coated with 100 μl of anti-mouse IFN-γ antibody (10 μg/ml; clone 1-D1K, MabTech, Nacka Strand, Sweden). Purified CD8+ T cells were plated at 1 × 10^5 well. For antigen presentation, 5 × 10^5 irradiated EL-4 lymphoma cells per well were pulsed with 1 μg/ml peptide (unless otherwise noted) to a final volume of 100 μl/well. After incubation for 20 hours, plates were incubated with 100 μl/well biotinylated antibody against mouse IFN-γ (2 μg/ml; clone 7-B6-1, MabTech). Plates were incubated for an additional 2 hours and spot development was done with an Automated ELISPOT Reader System with KS 4.3 software (Carl Zeiss, Thornwood, NY) by an independent scientist not working in the laboratory in a blinded fashion, as described (27).

Intracellular cytokine flow cytometry. Antibodies were purchased from Pharmingen (San Diego, CA). Anti-CD8-peridin chlorophyll protein (clone 53-6.7) was used for cell-surface staining. Intracellular staining for cytokines was done using phycoerythrin anti-IFN-γ (clone XM51.2) and phycoerythrin-anti–tumor necrosis factor (clone MP6-XT220). Isotype controls used irrelevant rat IgG1 (clone R3-34) and rat IgG2b (clone A95-1) conjugated to phycoerythrin. To assess cytokine production, CD8+ T cells were stimulated for 16 hours with 1 μg/ml peptide with irradiated CD8-depleted splenocytes. Brefeldin A (10 μg/ml; Sigma, St. Louis, MO) was added 1 hour after the peptide. Following stimulation, cells were stained for cell-surface markers and intracellular cytokines using the Cytofix/Cytoperm Kit (PharMingen) and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Fluorescence voltages and compensation values were determined using cells single stained with anti-CD8-PE-FTTC, anti-CD8-phycoerythrin, or anti-CD8-peridin chlorophyll protein. Acquisition and analysis were done using CellQuest software (Becton Dickinson Immunocytometry Systems). For each tube, 30,000 events were acquired in a live lymphocyte and CD8 double gate.

Skin biopsies. For histology, cytokine DNAs were delivered by gene gun to the abdomen of depilated C57BL/6 mice. Five days later, skins from the areas with grossly visible gold tattoos were fixed in formalin. Slides were stained with H&E and inflammatory cells quantified by counting cells per 40× field.

Statistics. Tumor-free survival was assessed from the day of tumor challenge. Surviving mice were followed weekly until day 120. Tumor-free survival distributions were generated using the Kaplan-Meier method and comparisons of survival distributions made using the log-rank test. Associations between treatment group and tumor-free survival on day 120 were compared using Fisher’s exact test.

Results

Tissue levels and inflammation following injection of cytokine DNAs. Immunization of C57BL/6 mice with hgp100 DNA induces CD8+ T-cell–dependent responses against the dominant D2-restricted human epitope hgp10025-33 and the corresponding syngeneic mouse epitope mgp10025-33. This T-cell response is necessary and sufficient for tumor immunity (12, 14–16). To compare potential DNA adjuvants, we developed a panel of plasmid DNAs encoding proinflammatory proteins that influence activation, proliferation, survival of T cells [T-helper-1 (Th1) interleukin (IL)-2, IL-12, IL-15, IL-18, IL-21], chemotaxis of T cells (CCL21), and growth and
activation of antigen-presenting cells (GM-CSF). In addition, cytokine DNA constructs incorporating the Fc domain of mouse IgG1 at the COOH terminus were created for each cytokine.

*In vivo* expression of cytokine molecules following DNA administration was measured by cytokine levels in injected skin using the technique previously reported (25). Expression kinetics were compared following injection of DNA encoding IL-2, IL-12, and GM-CSF, and the corresponding cytokine/Ig DNAs. Cytokines were expressed over 6 to 96 hours, with each cytokine reaching peak levels around 24 hours. Notably, tissue levels of cytokine/Ig fusion molecules were substantially lower at all time points compared with non-Ig cytokines (Fig. 1A). To address whether decreased *in vivo* gene expression of the Ig fusion constructs was a result of inherent differences of individual vectors, cytokine secretion was quantified after *in vitro* transfection of Chinese hamster ovary cells. Expressions of IL-12 and IL-12/Ig DNAs were not different, showing that differences were not simply a result of vector properties; on the other hand, GM-CSF/Ig secretion was 2- to 3-fold lower than GM-CSF DNA, implicating differences in vectors (data not shown).

To further characterize cytokine effects and confirm bioactivity, H&E-stained sections from injected skin were assessed for inflammatory cell infiltrates 5 days following administration of DNA. Control DNA vector induced a mild mononuclear cell infiltration, presumably due to local trauma and proinflammatory effects of bacterial plasmid DNA (Fig. 1B; Table 1). Inflammatory responses for GM-CSF, previously reported, were characterized by recruitment of myeloid mononuclear cells, including dendritic cells (25). Injection of other cytokine DNAs led to 2- to 8-fold increases in densities of myeloid mononuclear cell infiltrates, predominantly macrophages (Fig. 1B; Table 1). The most prominent inflammatory responses were observed following IL-12, IL-15, IL-21, and CCL21 DNAs. In particular, CCL21/Ig induced the most extensive cutaneous infiltration of mononuclear cells, with a high proportion of lymphocytes, consistent with its chemotactic activity for naive T cells.

Timing of adjuvant DNA delivery influences CD8+ T-cell responses. Proinflammatory molecules provide adjuvant effects by delivering signals to antigen-presenting cells and T cells before, during, and/or after presentation of antigen to T cells. The temporal sequence of cytokine signals to

Fig. 1. Cutaneous cytokine levels and inflammation following delivery of cytokine DNA. A, C57BL/6 mice (three per group) were injected with DNA encoding IL-2, IL-12, or GM-CSF or the corresponding cytokine/Ig fusion molecules. Levels of the relevant cytokine were measured in the skin at 6, 24, 48, and 72 or 96 hours, as described in Materials and Methods and in ref. 25. Bars, SE. B, histology of inflammatory cell infiltrates in the skin following delivery of cytokine DNA. Skin samples from mice were harvested on day 5 after DNA delivery by gene gun with the indicated cytokine, Ig fusion cytokine, or vector control (pING). H&E staining is shown at × 40 magnification. Descriptions of the histology are in Table 1. Bar, 50 μm.
antigen-presenting cells or T cells is potentially critical, and timing of administration of antigen and proinflammatory genes might have substantial consequences on subsequent T-cell responses. We previously reported that delivery of GM-CSF DNA is optimal 5 to 7 days before antigen delivery for maximum recruitment of antigen-presenting cells (13).

CD8+ T-cell responses in draining lymph nodes against the mouse gp100 peptide mpg10025-33 were evaluated by IFN-γ ELISPOT assays, assessing DNA adjuvants delivered at days −2, −1, 0, +1, and +2 in relation to delivery of antigen. The frequency of CD8+ T cells was greatest (Fig. 2A) when (i) IL-2 was administered at day +2; (ii) IL-12 at day +2; (iii) IL-12/Ig at days +1 and +2; (iv) IL-15 at day 0; (v) IL-18 and IL-21 at day +2 (data not shown); and (vi) CCL21 at day −1, 0, or +1. IL-12/Ig induced the greatest enhancement of CD8+ T-cell responses, with comparable T-cell enhancement seen at days +1 and +2. These observations are consistent with the capacity of IL-2, IL-12, IL-15, and IL-21 to enhance differentiation, activation, and/or expansion of primed T cells following initial recognition of cognate antigen; yet, they also suggest that different times of administration are needed for optimal responses. Adjuvant effect of CCL21 was observed even when administered 1 day before antigen, presumably acting through recruitment of antigen-presenting cells (13).

Enhancement of tumor rejection by cytokine DNA adjuvants. Enhancement of in vivo tumor immunity by cytokine DNAs was also influenced by the timing of cytokine DNA administration. Immunization with hgp100 was given thrice at weekly intervals, with or without cytokine DNAs, followed by tumor challenge with a supralethal dose of gp100-positive B16 melanoma 5 days after the last immunization. Hgp100 alone led to long-term (≥4 months) tumor-free survival in only ~10% to 25% (mean, 17%) of mice (Table 2). When each cytokine gene was administered alone without hgp100, no detectable long-term tumor-free survival was seen (data not shown), similar to the previously reported GM-CSF results (25).

Comparison of adjuvant effects for CD8+ T-cell responses. The adjuvant effects of the cytokine DNAs on the generation of mpg10025-33-specific CD8+ T cells from draining lymph nodes were compared. Most cytokine DNAs augmented CD8+ T-cell responses against gp100 (Fig. 2B), with the highest responses observed for IL-12, IL-12/Ig, IL-15/Ig, and CCL21. Four cytokine/lg DNAs were more potent than cytokine DNA alone (IL-2/Ig, IL-12/Ig, IL-15/Ig, and GM-CSF/lg; not shown), with IL-12/Ig inducing the highest CD8+ T-cell responses. Potencies of IL-18, IL-21, and CCL21 lg constructs were lower than the corresponding non-lg cytokine DNAs. Relative increases in T-cell responses in the spleen by ELISPOT assays corresponded to those for draining lymph node T cells, but at a ~10-fold higher level (data not shown). Cytokine and cytokine/lg DNAs alone, without DNA encoding antigens, did not elicit any detectable gp100-specific CD8+ T-cell response (data not shown).

CD8+ T-cell responses following immunization with hgp100 DNA alone showed a dose-response between 10 nmol/L and 10 μmol/L of mpg10025-33 peptide (Fig. 2C). Avidity of CD8+ T-cell responses to mpg10025-33 peptide was enhanced with IL-18 and IL-21, with higher apparent avidity responses for non-lg DNAs (Fig. 2D). No enhanced avidity was detected for IL-12, IL-12/Ig, IL-15, or IL-15/Ig compared with hgp100 DNA alone (data not shown).

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**Table 1. Histology of cutaneous inflammation induced by cytokines**

<table>
<thead>
<tr>
<th>DNA construct</th>
<th>Inflammatory cells/hpf* [mean ± SEM (range)]</th>
<th>Description of inflammatory infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>63 ± 8 (39-83)</td>
<td>Myeloid mononuclear cells within papillary dermis; 0-2 PMN/hpf</td>
</tr>
<tr>
<td>IL-2</td>
<td>149 ± 11 (115-191)</td>
<td>Myeloid mononuclear infiltrate within papillary dermis; 1-3 PMN/hpf</td>
</tr>
<tr>
<td>IL-2/Ig</td>
<td>132 ± 15 (78-158)</td>
<td>Myeloid mononuclear infiltrate within papillary dermis; 0-5 PMN/hpf</td>
</tr>
<tr>
<td>IL-12</td>
<td>252 ± 21 (130-336)</td>
<td>Myeloid mononuclear infiltrate extending into reticular dermis; 1-2 PMN/hpf</td>
</tr>
<tr>
<td>IL-12/Ig</td>
<td>254 ± 36 (130-336)</td>
<td>Mixed mononuclear infiltrate, predominantly myeloid, within papillary dermis; 1-3 PMN/hpf</td>
</tr>
<tr>
<td>IL-15</td>
<td>209 ± 10 (179-244)</td>
<td>Mixed mononuclear infiltrate, predominantly myeloid, extending into reticular dermis; 0-6 PMN/hpf</td>
</tr>
<tr>
<td>IL-15/Ig</td>
<td>237 ± 24 (171-328)</td>
<td>Myeloid mononuclear infiltrate extending into reticular dermis and s.c. tissue; 3-6 PMN/hpf</td>
</tr>
<tr>
<td>IL-21</td>
<td>336 ± 34 (264-483)</td>
<td>Mixed mononuclear infiltrate, predominantly myeloid, extending into reticular dermis; 6-10 PMN/hpf</td>
</tr>
<tr>
<td>CCL21</td>
<td>287 ± 18 (247-342)</td>
<td>Mixed mononuclear infiltrate, myeloid more than lymphoid, extending into reticular dermis and s.c. tissue; 6-10 PMN/hpf</td>
</tr>
<tr>
<td>CCL21/Ig</td>
<td>504 ± 37 (417-671)</td>
<td>Mixed mononuclear infiltrate, lymphoid more frequent than for CCL21, with inflammatory cells permeating into the s.c. tissue; 20-40 PMN/hpf</td>
</tr>
</tbody>
</table>

Abbreviations: hpf, high power field; PMN, polymorphonuclear leukocyte.

*For each DNA construct, ≥6 high-power 40× fields were counted.*
of tumor rejection was noted for IL-12/Ig, IL-15/Ig, GM-CSF/Ig, and CCL21. IL-12/Ig, IL-15/Ig, GM-CSF/Ig, and CCL21 also induced the greatest increases in CD8\(^+\) T-cell responses. DNA encoding IL-12/Ig, IL-15/Ig, GM-CSF/Ig, and CCL21 increased tumor-free survival at day 60 from 26% to 58%, 44%, 53%, and 52%, respectively (\(P < 0.05\), versus hgp100; log-rank analysis). IL-12/Ig, IL-15/Ig, and CCL21 improved survival at day 120 to 52%, 39%, and 52%, respectively, compared with 17% for hgp100 DNA alone (\(P < 0.001\), versus hgp100).

However, the magnitude of CD8\(^+\) T-cell responses to mgp10025-33 did not always correspond to the increase of in vivo tumor rejection (Fig. 2B; Table 2), with a disconnect between peak cytokine levels, T-cell responses, and tumor-free survival: (a) no tumor-free survival advantage was observed with IL-12 (10%), IL-15 (6%), IL-18 (5%), or IL-18/Ig (9%), despite enhanced mgp100-specific CD8\(^+\) T-cell responses; (b) IL-12, IL-15, and IL-18 DNAs without Fc domains enhanced CD8\(^+\) T-cell responses, but had no adjuvant effects; (c) IL-21/Ig did not enhance gp100-specific T-cell responses, and yet induced a significant improvement in tumor-free survival at day 120 (38%; \(P = 0.02\), versus hgp100), suggesting an effect that was independent of CD8\(^+\) T cells (possibly providing adjuvanticity through IL-18 effects on natural killer (NK) cells); (d) IL-12 produced a substantially higher peak and sustained...
IL-12 and IL-12/Ig DNAs increase autoimmune hypopigmentation. We and others have previously reported that DNA immunization against melanoma differentiation antigens results in autoimmunity, visualized as vitiligo (hypopigmentation; refs. 10, 11). However, we have observed that the degree of hypopigmentation does not consistently correspond to the level of tumor immunity. In contrast to DNA immunization against the melanoma differentiation antigens TRP-2 and TYRP1, DNA immunization against gp100 leads to minimal vitiligo (12). In the present study, we observed strong hypopigmentation when hgp100 DNA was administered with IL-12 or IL-12/Ig DNA, but not with other cytokine or cytokine/Ig DNAs (results not shown). The most marked vitiligo was observed with the combination of IL-2/Ig + IL-12/Ig, whereas there was minimal hypopigmentation with IL-2/Ig alone or when IL-2/Ig was combined with IL-15/Ig. This discrepancy between tumor immunity and autoimmunity is notable because overall tumor-free survivals for these combinations were very similar, showing that amplifying tumor immunity with cytokine adjuvants does not necessarily increase autoimmunity.

Discussion

The present study is the first to systematically compare adjuvant effects of plasmid DNAs encoding cytokines and their Fc fusion counterpart. A large number of studies have shown that the immune response to antigens expressed by cancer and infectious microorganisms can be enhanced by individual genes encoding proinflammatory cytokines, although space constraints limit the ability to review these studies.

Because CD8+ T cells are the predominant arm of the antitumor immune response following hgp100 DNA immunization, we examined the role of DNA encoding proinflammatory cytokines as molecular adjuvants in an attempt to improve tumor-free survival. Building on the weak immunogenicity of hgp100 DNA, plasmid DNA adjuvants were added to potentiate CD8+ T-cell responses and tumor immunity. We selected Th1 cytokines that influence T-cell activation, differentiation, and survival through direct effects on T cells and indirect effects with antigen-presenting cells. IL-2 acts directly on T cells to induce their proliferation, eliciting a robust response early in the course of T-cell activation, but eventually leading to activation-induced cell

### Table 2. Tumor-free survival following immunization with hgp100 DNA in combination with cytokine DNA

<table>
<thead>
<tr>
<th>Group (hgp100+)*</th>
<th>No. expt†</th>
<th>Day 30 †</th>
<th>Day 60</th>
<th>Day 90</th>
<th>Day 120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alone</td>
<td>36</td>
<td>111 of 312 (36)</td>
<td>82 of 312 (26)</td>
<td>74 of 302 (24)</td>
<td>54 of 247 (17)</td>
</tr>
<tr>
<td>IL-2</td>
<td>2</td>
<td>7 of 29 (24)</td>
<td>7 of 29 (24)</td>
<td>4 of 17 (24)</td>
<td>4 of 17 (24)</td>
</tr>
<tr>
<td>IL-2/Ig</td>
<td>6</td>
<td>53 of 91 (58)</td>
<td>36 of 91 (40)</td>
<td>34 of 91 (37)</td>
<td>20 of 62 (32)</td>
</tr>
<tr>
<td>IL-12</td>
<td>2</td>
<td>7 of 27 (26)</td>
<td>4 of 27 (15)</td>
<td>2 of 20 (10)</td>
<td>2 of 20 (10)</td>
</tr>
<tr>
<td>IL-12/Ig</td>
<td>6</td>
<td>65 of 91 (71)</td>
<td>53 of 91 (58)</td>
<td>49 of 91 (54)</td>
<td>34 of 66 (52)</td>
</tr>
<tr>
<td>IL-15</td>
<td>1</td>
<td>2 of 17 (12)</td>
<td>1 of 17 (6)</td>
<td>1 of 17 (6)</td>
<td>1 of 17 (6)</td>
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<tr>
<td>IL-15/Ig</td>
<td>4</td>
<td>37 of 57 (65)</td>
<td>25 of 57 (44)</td>
<td>23 of 57 (40)</td>
<td>22 of 57 (39)</td>
</tr>
<tr>
<td>IL-18</td>
<td>2</td>
<td>5 of 20 (25)</td>
<td>2 of 20 (10)</td>
<td>1 of 20 (5)</td>
<td>1 of 20 (5)</td>
</tr>
<tr>
<td>IL-18/Ig</td>
<td>2</td>
<td>9 of 35 (26)</td>
<td>4 of 35 (11)</td>
<td>3 of 35 (9)</td>
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<tr>
<td>IL-21</td>
<td>3</td>
<td>14 of 40 (35)</td>
<td>11 of 40 (28)</td>
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</tr>
<tr>
<td>IL-21/Ig</td>
<td>3</td>
<td>27 of 55 (49)</td>
<td>23 of 55 (42)</td>
<td>21 of 55 (38)</td>
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<tr>
<td>CCL21</td>
<td>2</td>
<td>13 of 25 (52)</td>
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<tr>
<td>CCL21/Ig</td>
<td>1</td>
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<td>5 of 15 (33)</td>
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</tr>
<tr>
<td>GM-CSF</td>
<td>1</td>
<td>6 of 15 (33)</td>
<td>5 of 15 (33)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GM-CSF/Ig</td>
<td>1</td>
<td>9 of 15 (60)</td>
<td>8 of 15 (53)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE: Mice (10-20 per group) were immunized with hgp100 DNA thrice with or without cytokine DNAs. Abbreviation: ND, not determined.

* Cytokine DNAs were administered day +1 after each hgp100 DNA injection, except for GM-CSF and GM-CSF/Ig DNAs, which were administered 7 days before the first hgp100 DNA injection and 4 days before subsequent hgp100 injections.

† Number of experiments. Results compiled from 36 different experiments.

§ Day after tumor challenge.

Because gp100-specific T-cell responses and tumor rejection could be enhanced by individual cytokine genes, we evaluated combinations of interleukin/Ig DNAs (Table 3). Coadministration of several combinations of interleukin-Fc fusions increased tumor-free survival, including IL-2/Ig combined with either IL-12/Ig (60%) or IL-15/Ig (53%). For these combinations, protection was higher than that observed for each interleukin/Ig DNA alone, corresponding to increased gp100-specific T-cell responses. In contrast, addition of IL-21/Ig did not increase tumor rejection when combined with IL-2/Ig, IL-12/Ig, or IL-15/Ig, and the combination of IL-12/Ig and IL-15/Ig was not superior to IL-12/Ig alone. Although enhanced T-cell responses were detected for IL-21/Ig + IL-15/Ig and IL-12/Ig + IL-15/Ig combinations (data not shown), there was no enhancement in tumor protection. Finally, addition of IL-18/Ig to IL-12/Ig decreased the level of tumor protection from that observed with IL-12/Ig alone.

Discussion

The present study is the first to systematically compare adjuvant effects of plasmid DNAs encoding cytokines and their Fc fusion counterpart. A large number of studies have shown that the immune response to antigens expressed by cancer and infectious microorganisms can be enhanced by individual genes encoding proinflammatory cytokines, although space constraints limit the ability to review these studies.

Because CD8+ T cells are the predominant arm of the antitumor immune response following hgp100 DNA immunization, we examined the role of DNA encoding proinflammatory cytokines as molecular adjuvants in an attempt to improve tumor-free survival. Building on the weak immunogenicity of hgp100 DNA, plasmid DNA adjuvants were added to potentiate CD8+ T-cell responses and tumor immunity. We selected Th1 cytokines that influence T-cell activation, differentiation, and survival through direct effects on T cells and indirect effects with antigen-presenting cells. IL-2 acts directly on T cells to induce their proliferation, eliciting a robust response early in the course of T-cell activation, but eventually leading to activation-induced cell
death, presumably as a protective mechanism against autoimmunity. IL-12 (28) and IL-18 (29) are type I cytokines that lead to production of IFN-γ by T cells and NK cells, as well as directly stimulate T cells. IL-12 is the best studied of the two, and it is primarily produced by dendritic cells and monocytes to form a link between the innate and adaptive immune systems (30).

IL-12 promotes Th1 differentiation, enhances the proliferation of preactivated T cells and NK cells, and induces the production of downstream cytokines (including IFN-γ, tumor necrosis factor, and GM-CSF) by antigen-presenting cells (31–33). IL-15 influences CD8+ T-cell growth and survival, promoting memory CD8+ T-cell responses (34, 35). IL-21, structurally related to IL-2 and IL-15, enhances T-cell proliferation alone and in combination with IL-2 and IL-15 and augments activation of NK cells (36, 37).

CCL21 is a chemokine that recruits naïve T cells and activated dendritic cells via the CCR7 receptor to orchestrate priming of naïve T cells by dendritic cells (29, 30). Kirk et al. (38, 39) showed antitumor effects against B16 melanoma following administration of CCL21 or dendritic cells modified to express CCL21, with activation of T cells within 24 hours and detection of effector T cells within 7 days after administration.

The disparities between optimal timing for different cytokines in our study are not clear but must relate to differences in primary target cells and their activation states, divergent downstream signaling pathways for different receptors, and distinct spatial and temporal constraints on mechanisms of actions. For instance, IL-18 and IL-21 can affect T-cell responses at least partially through indirect events involving helper NK cells (29, 40, 41). Furthermore, administration of the adjuvant DNAs before antigen delivery generally diminished gp100-dependent tumor-free survival. This suggests that premature delivery of cytokines to a naïve immune system may lead to aborted immune activation in response to antigen or trafficking of target cells out of the administration site before antigen is delivered. Our observations reveal that the timing of the adjuvant cytokine DNA relative to the antigen is critical in determining the magnitude of the immune response and the clinical effect.

The importance of timing of molecular adjuvants has been noted in our previous study of GM-CSF DNA and in the results of Barouch et al. (17, 42) with IL-2/Ig DNA. For GM-CSF DNA, sensitivity to timing is related to the effects of GM-CSF DNA on the growth and maturation of dendritic cells and production of secondary cytokines and chemokines in relation to antigen presentation (13, 17, 25, 42). For cytokines that act directly on T cells, such as IL-2, it is likely that the timing of the cytokine in relation to priming is critical. For example, excessive IL-2 may cause activation-induced cell death. In a related study, Yamano et al. (43) showed optimal immune response to TRP-2 DNA delivered in liposomes when CCL21 protein was administered at the same site 24 hours before TRP-2 DNA. We observed that CCL21 plasmid DNA enhanced CD8+ T-cell responses maximally also when delivered 1 day before DNA immunization (Fig. 2A), but day +1 was most effective for immunity against B16 melanoma challenge. The differences between our results and those of Yamano et al. may relate to different forms of adjuvant delivery (protein versus DNA) as well as different strategies for DNA delivery.

The mechanism for enhanced efficacy for cytokine-Ig constructs is not clear. The most extensive studies to date have been carried out by Barouch et al. (2, 17–20), who have shown in both mouse and monkey HIV models that IL-2/Ig DNA is a more potent immune stimulant than IL-2 DNA. Based on these prior reports, mouse IgG1 Fc fusion constructs were systematically tested with multiple cytokines. We originally postulated
that the Ig fusion constructs would have a longer \textit{in vivo} half life. Interestingly, enhancement of T-cell responses and tumor immunity was not related to improved kinetics or higher peak tissue levels of the proinflammatory gene products. Unexpectedly, efficacy was associated with a decrease (not increase) in cytokine levels and half-life \textit{in vivo}. Barouch et al. (44) recently reported, after we had finished our experiments, lower cytokine levels with IL-2/Ig DNA and showed that lower IL-2 concentrations were not related to neutralizing antibodies.

Lower bioavailability (e.g., due to immunogenicity to accelerate clearance) is unlikely to translate into increased effectiveness. We propose that potency is based on interactions with activating Fc receptors (e.g., on dendritic cells, macrophages, or NK cells), with interactions with Fc receptors amplifying adjuvant effects through downstream Fc receptor signaling, leading to production of secondary cytokines/chemokines.

An alternative hypothesis is that the Fc domain provides a passive effect by binding to Fc receptors (without a role for signaling), pulling soluble cytokine/Ig molecules out of solution in three-dimensional space onto the surface of Fc receptor-positive cells (onto more constrained two-dimensional surfaces). The increased density and immobility at the cell surface would produce cross-linking of cytokine receptors on target cells. We favor the first hypothesis that signaling by activating Fc receptors is involved because the rapid disappearance of cytokine-Fc could occur following phagocytosis and degradation by activated Fc receptor-positive cells. In addition, injection of cytokine/Ig constructs creates greater inflammatory responses that penetrated deeper into the dermis compared with cytokine DNA alone, although cytokine/Ig DNA does not increase density of inflammatory cells with the exception of CCL21/Ig (Table 1). A scenario involving Fc receptor signaling would have important implications for selecting appropriate Fc domains and for engineering molecular adjuvants by manipulation of Fc structures.

When single cytokine adjuvants were investigated, IL-12/Ig elicited the highest tumor-free survival at 120 days. However, autoimmunity, revealed as hypopigmentation, was also much more apparent than for other cytokine DNAs. Combinations of fusion cytokines were subsequently evaluated in an attempt to further improve tumor-free survival. A combination of IL-2 plus IL-12 provided the maximum tumor-free survival (Table 3). The improved survival of this combination, however, was at the expense of substantially increased hypopigmentation, which was associated with weight loss (not shown). Interestingly, the combination of IL-2/Ig and IL-15/Ig elicited a similar degree of enhanced tumor protection (Table 3) with limited or no hypopigmentation and no detectable weight loss. These observations suggest that the combination of IL-2/Ig and IL-15/Ig is the optimal combination adjuvant when balancing antitumor effects versus autoimmunity.

A number of other reports have examined coadministration of plasmid cytokines with DNA vaccines. The present study differs from these previous reports in several ways. First, it uses the most comprehensive panel of plasmid DNA adjuvants. Second, it is the most complete study evaluating the timing of these proinflammatory immune adjuvants relative to the antigen in terms of T-cell functional assays and antitumor effects. Third, we compared cytokine/Ig fusion constructs for each adjuvant to assess a broader role for the strategy. Fourth, combinations were identified that improved tumor immunity while minimizing autoimmunity.

For some cytokines, there was a lack of correlation between the results of \textit{in vitro} T-cell studies and \textit{in vivo} tumor studies. This disengagement has previously been observed in other mouse studies as well as in clinical trials of peptide vaccines in patients with melanoma (45, 46). Although higher levels of CD8+ T cells were detected \textit{ex vivo}, these cells may have been defective or short-lived effector function \textit{in vivo}. Thus, results of \textit{in vitro} assays may not reflect the avidity for antigen of effector cells \textit{in vivo} (47, 48). For instance, in the case of IL-21/Ig DNA, where increased tumor-free survival was observed in the absence of increased T-cell responses, it is possible that IL-21 leads to improved effector function (e.g., through increased avidity; Fig. 2) and/or enhanced memory CD8+ T-cell responses. In fact, recent studies have shown that IL-21 is an important regulator of CD8+ T-cell expansion and effector function and that it acts in synergy with IL-15 (37).

### Acknowledgments

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**Table 3. Tumor-free survival following immunization with hgp100 DNA with combinations of cytokine DNA**

<table>
<thead>
<tr>
<th>Group (hgp100+)</th>
<th>No. expt</th>
<th>Day 30*</th>
<th>Day 60</th>
<th>Day 90</th>
<th>Day 120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alone</td>
<td>36</td>
<td>111 of 312 (36)</td>
<td>82 of 312 (26)</td>
<td>74 of 302 (24)</td>
<td>54 of 247 (17)</td>
</tr>
<tr>
<td>IL-2/Ig + IL-12/Ig</td>
<td>2</td>
<td>30 of 35 (86)</td>
<td>21 of 35 (60)</td>
<td>21 of 35 (60)</td>
<td>21 of 35 (60)</td>
</tr>
<tr>
<td>L-2/Ig + IL-15/Ig</td>
<td>2</td>
<td>26 of 40 (65)</td>
<td>23 of 40 (58)</td>
<td>21 of 40 (53)</td>
<td>21 of 40 (53)</td>
</tr>
<tr>
<td>IL-2/Ig + IL-21/Ig</td>
<td>2</td>
<td>18 of 35 (51)</td>
<td>14 of 35 (40)</td>
<td>13 of 35 (37)</td>
<td>13 of 35 (37)</td>
</tr>
<tr>
<td>IL-12/Ig + IL-15/Ig</td>
<td>1</td>
<td>15 of 20 (75)</td>
<td>10 of 20 (50)</td>
<td>10 of 20 (50)</td>
<td>10 of 20 (50)</td>
</tr>
<tr>
<td>IL-12/Ig + IL-18/Ig</td>
<td>1</td>
<td>9 of 15 (60)</td>
<td>6 of 15 (40)</td>
<td>4 of 15 (27)</td>
<td>4 of 15 (27)</td>
</tr>
<tr>
<td>IL-12/Ig + IL-21/Ig</td>
<td>1</td>
<td>11 of 15 (73)</td>
<td>8 of 15 (53)</td>
<td>8 of 15 (53)</td>
<td>8 of 15 (53)</td>
</tr>
<tr>
<td>IL-15/Ig + IL-21/Ig</td>
<td>1</td>
<td>12 of 20 (60)</td>
<td>10 of 20 (50)</td>
<td>8 of 20 (40)</td>
<td>8 of 20 (40)</td>
</tr>
</tbody>
</table>

*NOTE: Results compiled from 10 experiments. Mice (10-20 per group) were immunized with hgp100 DNA thrice with or without cytokine DNAs. Cytokine DNAs were administered +1 day after each hgp100 DNA injection. Each mouse received 2 μg of each adjuvant for these combinations. *Days post tumor challenge.
References

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