Plasmid Vaccine Expressing Granulocyte-Macrophage Colony-Stimulating Factor Attracts Infiltrates Including Immature Dendritic Cells into Injected Muscles

Diana Haddad, Jayanthi Ramprakash, Martha Sedegah, Yupin Charoenvit, Roxanne Baumgartner, Sanjai Kumar, Stephen L. Hoffman and Walter R. Weiss

*J Immunol 2000; 165:3772-3781; *
doi: 10.4049/jimmunol.165.7.3772
http://www.jimmunol.org/content/165/7/3772

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 47 articles, 26 of which you can access for free at:
http://www.jimmunol.org/content/165/7/3772.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
Plasmid Vaccine Expressing Granulocyte-Macrophage Colony-Stimulating Factor Attracts Infiltrates Including Immature Dendritic Cells into Injected Muscles

Diana Haddad,* Jayanthi Ramakrishan,† Martha Sedegah,* Yupin Charoenvit,* Roxanne Baumgartner,# Sanjai Kumar,* Stephen L. Hoffman,* and Walter R. Weiss*#

Plasmid-encoded GM-CSF (pGM-CSF) is an adjuvant for genetic vaccines; however, little is known about how pGM-CSF enhances immunogenicity. We now report that pGM-CSF injected into muscle leads to a local infiltration of potential APCs. Infiltrates reached maximal size on days 3 to 5 after injection and appeared in several large discrete clusters within the muscle. Immunohistological studies in muscle sections from mice injected with pGM-CSF showed staining of cells with the macrophage markers CD11b, Mac-3, IAq/E2 and to the granulocyte marker GR-1 from day 1 through day 14. Cells staining with the dendritic cell marker CD11c were detected only on days 3 to 5. Muscles injected with control plasmids did not stain for CD11c but did stain for CD11b, Mac-3, IAq/E4, and GR-1. No staining was observed with the APC activation markers, B7.1 or CD40, or with markers for T or B cells. These findings are consistent with the infiltrating cells in the pGM-CSF-injected muscles being a mixture of neutrophils, macrophages, and immature dendritic cells and suggest that the i.m. APCs may be enhancing immune responses to co-injected plasmid Ags. This hypothesis is supported by data showing that 1) separation of injections with pGM-CSF and Ag-expressing plasmid into different sites did not enhance immune responses and 2) immune enhancement was associated with the presence of CD11c+ cells in the infiltrates. Thus, pGM-CSF enhancement may depend on APC recruitment to the i.m. site of injection. The Journal of Immunology, 2000, 165: 3772–3781.

The mechanism of action of GM-CSF as an immune enhancer has been studied in the transfected tumor model. Its function is thought to involve a paracrine (local) effect of the cytokine at the injection site that recruits a mixed cellular infiltrate including APCs, eosinophils, and T and B cells capable of recognizing tumor Ags at metastatic sites (1, 18–19). However, it is not certain that the same mechanism is operating when GM-CSF is delivered by other means. A GM-CSF recombinant protein could exert its effects locally at the site of injection or in distant tissues due to circulating levels of GM-CSF in blood or lymph. When pGM-CSF is used to enhance DNA immunization, somatic cells at the site of injection could be transfected and make GM-CSF protein, the plasmid could leave the injection site and transfect distant cells, or APCs moving through the injection site could be transfected (20).

Understanding the mechanism of action of GM-CSF is important both to optimize its immune enhancing effects and for reasons of safety. Recombinant human GM-CSF has been used for several years to stimulate granulocyte production from the bone marrow of neutropenic patients. However, systemic toxicity (21) and flaring of autoimmune disease have been reported (22, 23). If pGM-CSF had effects at distant anatomical sites, toxicity and autoimmunity might limit the usefulness of GM-CSF as a vaccine enhancer. However, if GM-CSF acted locally at the site of vaccine injection, unwanted distant effects would be avoided. Accordingly, before proceeding to clinical trials in humans with pGM-CSF, we initiated studies to define its mechanism of action.

Using our P. yoelii DNA vaccine model, we now show that i.m. injection of GM-CSF plasmid leads to a highly localized influx of cells with APC phenotype, but without T cells or B cells. The immune-enhancing effect requires admixing of GM-CSF plasmid with Ag-encoding plasmids and cannot be replicated by injecting GM-CSF plasmid i.v. or at distant i.m. sites.

*Malaria Program and †Pathology Division, Naval Medical Research Center, Silver Spring, MD 20910

Received for publication March 14, 2000. Accepted for publication July 12, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by Naval Medical Research and Development Command Work Units 611102A, S13.00101-BFX.1431, and 612787A.870.00101.EFX.1432. The opinions and assertions herein are those of the authors and are not to be construed as official or as reflecting the views of the U.S. Navy or Department of Defense.

Abbreviations used in this paper: DC, dendritic cells; ELISPOT, enzyme-linked immunospot; PyCSF, Plasmodium yoelii circumsporozoite protein; H&E, hematoxylin and cosin; SI, stimulation index.
DNA plasmids

The plasmids used in this study have been previously described (17, 24). Briefly, the P. yoelii circumsporozoite protein (PyCSP)-encoding plasmid, PyCP1012, denoted pPyCSP, was created by PCR amplification of the DNA sequence encoding PyCSP from the plasmid nCMVinPyCSP.1 (24) and ligated into the plasmid VR1012 (25). The plasmid encoding murine GM-CSF was produced in the VR1019 plasmid, a derivative version of the VR1012 plasmid with the addition of a leader element from rat preproinsulin II (26). As a negative control, we used a VR1019 plasmid encoding the murine GM-CSF point-mutated at two bases, which has lost the ability to bind to the GM-CSF receptor and lacks bioactivity (17, 27). Plasmids for immunization were purified by double cesium banding and diluted in normal saline. Endotoxin levels were less than 0.6 EU/mg.

Animals and immunization procedure

All experiments were conducted according to published regulations (28). Female BALB/cByJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice received their first immunization at 5–6 wk of age. Unless otherwise specified in the text, mice were immunized twice at 6-wk intervals. Immediately before injection, plasmids were mixed and administered in a total volume of 50 μl in PBS into the tibialis anterior or gastrocnemius muscles of each leg, using a 29-gauge needle (Becton Dickinson, Franklin Lakes, NJ). Unless otherwise specified, each immunizing dose contains 50 μg PyCSP plasmid and 15 μg GM-CSF plasmid or equal amounts of their respective control plasmids, p1012 and p1019, at each site. In some studies, mice were immunized i.v. with 100 μg PyCSP plasmid and 30 μg GM-CSF plasmid in a total volume of 100 μl in PBS.

Synthetic peptides and recombinant proteins

Peptides corresponding to PyCSP57–70(aa) (K1YNRRN1N1RL1LDG) or PyCSP280–288(aa) (SYVPSAEQI) were used in vitro T cell studies (29, 30). Peptides were kindly provided by Dr. G. P. Corradin, University of Lausanne, Epalinges, Switzerland. A recombinant PyCSP protein lacking the secretion and anchor signal, produced in yeast (kindly provided by Dr. Anthony Stowers, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD), was used as solid phase Ag for ELISA.

Lymphocyte proliferation assay

Spleen cells were cultured in complete medium in 96-well flat-bottom plates at a concentration of 2.5 × 10^5 cells/well at 37°C and 5% CO_2. Complete medium was DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (Life Technologies), 200 U/ml penicillin, 200 μg/ml streptomycin, and 200 mM L-glutamine. After 5 days in culture with peptide PyCSP57–70 or at a concentration of 2 μM, cells were pulsed for 16 h with 1 μCi tritiated thymidine, and radioactivity incorporated into DNA was measured by scintillation counting.

IFN-γ assay

Ag-specific IFN-γ secreting cells were evaluated by enzyme-linked immunospot (ELISPOT) in a modification of a previously published assay (31). Briefly, 96-well nitrocellulose-backed microtiter plates (Millipore, Bedford, MA) were coated overnight at 4°C with 10 μg/ml IFN-γ mAb (clone RMMG-1, Bionurate, Camarillo, CA) in PBS (pH 7.2) and then blocked with 5% BSA in PBS. Two-fold serial dilutions of a single-cell suspension, starting from 5 × 10^3 cells/well, were incubated overnight with or without peptide at 37°C in a humidified 5% CO_2 incubator. The plates were then washed with 0.025% Tween 20 in PBS and overlaid with 1 μg/ml biotinylated anti-IFN-γ Ab (clone R46A2, Lee Biochemical, San Diego, CA) for 2 h at room temperature. After washing with 0.025% Tween 20 in H_2O, spots corresponding to the position of cytokine-secreting cells were developed by addition of avidin-conjugated alkaline phosphatase (PharMingen, San Diego, CA) and 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium (Kirkegaard and Perry, Gaithersburg, MD). The dilution of cells producing ~50 spots/well was used to calculate the number of cytokine-secreting cells per 1 × 10^5 cells.

Serum Abs

Abs reactive with the PyCSP yeast recombinant protein were assayed by ELISA as previously described (32).

Histology

For histology and immunohistochemistry, groups of 20 mice were injected with plasmids in both gastrocnemii, and the injected muscles were removed from two mice daily for 7 days and at day 14 after injection. Standard hematoxylin and eosin (H&E) staining was conducted to assess cell morphology and inflammatory infiltrate in the injected muscles. Immediately after removal, muscles were fixed for 24 h in 10% formalin, after which each sample was embedded in paraffin. Sections 6 μm thick at 10 evenly spaced levels were cut through each muscle and adhered onto Superfrost Plus slides (Fisher Scientific, Fair Lawn, NJ) which were stained with Mayer’s H&E (Sigma, St. Louis, MO) following manufacturer’s standard protocol. All histological assessment was conducted by two independent observers in a blinded manner. Grading of muscle infiltrates was done semiquantitatively using the following scale: 0 = no infiltrate; 1+ = one small cell cluster; 2+ = two small or moderate size cell clusters; 3+ = extensive, multifocal cell infiltration.

Immunohistochemistry

Muscles were snap frozen by overlaying with Histo-Prep tissue-embedding medium (Fisher Scientific) and immersing in liquid nitrogen-cooled isopentane. All samples were stored at −70°C until analysis. Serial 6-μm thick frozen sections from each muscle were adhered to Superfrost Plus slides, fixed in ice-cold acetone at −20°C for 10 min, air-dried, and rinsed in distilled water to remove embedding medium. Staining was conducted following standard procedures (33). Briefly, sections were sequentially incubated for 20 min with 0.05% H_2O_2 in PBS and with 5% normal goat serum (Sigma) in PBS. Samples were then incubated for 2 h with primary Abs, CD11b (M1/70), Mac-3 (M3/84), CD11c (HL3), I-A^d/II^d (2G9), GR-1 (SP64), CD4^+ (RM4-5), CD8^+ (53-67), and B220 (RA3-6B2) (all from PharMingen) in accordance with manufacturer’s instructions, followed by a 1-h incubation with 5 μg/ml biotinylated goat anti-rat or anti-hamster secondary Ab (PharMingen). After a 30-min incubation step with streptavidin-peroxidase (PharMingen), Ag-Ab reactions were developed using 3,3′-diaminobenzidine (Dako, Carpinteria, CA) as substrate. Slides were washed twice with PBS between each incubation step. All reagents were added in a volume of 50 μl, and the incubations were conducted at room temperature in a humid chamber. Grading of tissue staining was done semiquantitatively using the following scale: 0 = no cells stained, 1+ = fewer than 10% cells stained, 2+ = 10–50% stained; 3+ = >50% cells stained.

Analysis of CD11c^+ cells in draining lymph nodes by flow cytometry

Expression of DC surface markers in the draining lymph nodes of mice injected with pGM-CSF or p1019 was quantitated by flow cytometry. Plasmids were injected bilaterally into the gastrocnemius muscles, and the popliteal lymph nodes were removed at days 4, 8, 12, and 20. Single-cell suspensions were prepared at 1 × 10^6 cells in 1% BSA in PBS and stained with 1 μg/ml of anti-CD11c (HL3)-CD11b (M1/70), -MHC II (2G9), and -B7.1 (16-10A1) (PharMingen). After 45 min incubation on ice, cells were washed twice in PBS containing 0.1% BSA and 0.02% NaN_3. At least 100,000 cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson).

Statistical analysis

An unpaired, two-tailed Student t test was used for evaluating statistical significance between the immunization groups. One-way ANOVA with Scheffé post hoc tests was used to evaluate differences among the mean immune responses in the groups of mice with CD11c^+ indexes of 3, 2, 1, or 0.

ANOVAs was performed in SPSS for Windows, version 8 (SPSS, Chicago, IL.).

Results

Intramuscular but not i.v. pGM-CSF boosts Ag-specific immune responses

Torres et al. (34) reported that plasmid DNA injections into mouse muscles resulted in Ag-specific immune responses even when the muscles were surgically removed minutes after injection. One possible explanation for those findings is that a small amount of plasmid might have leaked into the blood stream after i.m. injection, leading to a systemic immune response. To address this possibility, we injected plasmid pGM-CSF into mice separately or together with pPyCSP via the i.m. or i.v. routes and evaluated their immune responses. Injections were at week 0 and 6 with a total of 100 μg pPyCSP and 30 μg pGM-CSF or equal amounts of corresponding
control plasmids, p1012 and p1019, as described in Fig. 1. Experiments were performed on 3 separate occasions. Data from a representative experiment are shown in Fig. 1.

The number of cells secreting IFN-γ in response to the defined CD8 T cell epitope, PyCSP\textsubscript{280–288 aa}, at 3 and 8 wk after the final immunization is presented in Fig. 1A. Intramuscular coinjection of pPyCSP + pGM-CSF (group 1) significantly enhanced the response at both times compared with pPyCSP + p1019 control injections (group 2) \((p < 0.05)\). Injection of pPyCSP i.m. combined with an i.v. injection of either pGM-CSF (group 6) or p1019 (group 7) resulted in a transient boost to the response at 3 wk after injection which disappeared by week 8 \((p < 0.05)\).

**FIGURE 1.** Immune responses to PyCSP elicited in BALB/c mice by injecting i.m. and/or i.v. with pPyCSP and pGM-CSF DNA plasmids. Groups of five BALB/c mice were injected as noted, and the immune responses measured at indicated time points. A, IFN-γ secretion after immunization. Spleen cells were cultured overnight with the PyCSP\textsubscript{280–288 aa} CD8 T cell epitope and the number of IFN-γ-producing cells was determined by ELISPOT. B, Proliferation of T lymphocytes after immunization. Splenocytes were cultured for 5 days with the PyCSP\textsubscript{57–70 aa} CD4 T cell epitope. Results shown in A and B represent the means (± SD) for five individual mice. C, Serum Ab responses to recombinant PyCSP measured 3 wk after the second immunization. Sera were tested as a pool of five individual sera. Displayed are the endpoint titers with OD values >0.2. *, Immunization regimens that were statistically better than i.m. pPyCSP + p1019 \((p < 0.05)\). **, Immunization regimens that were statistically better than i.v. pPyCSP + p1019 \((p < 0.05)\).
The T cell proliferation response to the PyCSP$_{57–70}$ aa CD4+ T cell epitope in the same experiment is shown in Fig. 1B. Again, i.m. injection of the mixture of pPyCSP + pGM-CSF (group 1) gave an enhanced stimulation index (SI) at 3 (SI = 11) and 8 (SI = 10) wk after the last immunization ($p < 0.05$). Interestingly, i.v. injection with pPyCSP + pGM-CSF (group 3) gave a much stronger response at the early time point (SI = 48), compared with i.v. pPyCSP + p1019 control injection (group 4) (SI = 5) ($p < 0.05$), but this response diminished substantially (SI = 3) by 8 wk. The serum Ab responses to the recombinant PyCSP protein 3 wk after the second immunization are shown in Fig. 1C. As noted for T cell responses to the 280–288 aa epitope, i.m. coinjection of pPyCSP + pGM-CSF elicited the highest titers with almost a 2-log difference compared with the corresponding control injections (group 2) ($p < 0.05$). No difference in Ab titers was observed when an i.m. injection of pPyCSP was given combined with an iv dose of either the pGM-CSF (group 6) or the p1019 control plasmid (group 7).

In summary, these data indicate that i.v. pGM-CSF may result in nonspecific immune enhancement due to the DNA backbone (Fig. 1, A and C) as well as in specific enhancement elicited by the encoded GM-CSF (Fig. 1B). Yet, the specific enhancement effects of i.v. pGM-CSF appear to be short lived as compared with the prolonged enhancement observed with i.m. pGM-CSF.

**Separating the site or time of i.m. injection with pPyCSP and pGM-CSF abrogates enhancement of immune responses**

Next, experiments were designed to test whether colocalization of pGM-CSF with pPyCSP in muscles was required for immune enhancement. Specifically, we wished to compare the effects of a single i.m. injection with mixed plasmids in one muscle with 1) injections of each plasmid into separate limbs, 2) injections of each plasmid into adjacent muscles within the same lymphatic drainage area, or 3) consecutive injections of pPyCSP and pGM-CSF plasmids given 10 min apart in the same muscle. Representative data from one of four experiments are shown in Table I. All mice received a constant total amount of DNA split between three different muscles: the right tibialis anterior; the left tibialis anterior; and the right gastrocnemius. Some groups received pGM-CSF and others received p1019 as a negative control. In groups 1–4, each of the three muscles was injected with a total volume of 50 μl. Groups 5 and 6 differed from the other groups in that they received two injections of 25 μl given 10 min apart into the right tibialis anterior. An attempt was made to place the second injection in the same location as the first. Six weeks after injection, mice were bled for serum Abs, and spleens were harvested for T cell studies. Ab assays were performed on pooled serum, and T cell studies were performed on individual animals.

Table I shows that coinjection of pPyCSP and pGM-CSF gave a substantial boost to the Ab response to PyCSP, as well as to the IFN-γ responses to the CD4+ (PyCSP$_{57–70}$ aa) and CD8+ (PyCSP$_{280–288}$ aa) T cell epitopes (group 1 vs group 2). T cell proliferation to the 57- to 70-aa epitope was also enhanced in group 1 (data not shown). Comparison of responses in groups 1–4 indicates that separating the pPyCSP and pGM-CSF injection sites into muscles on separate limbs (groups 1 vs 3) or into separate muscles on the same limb (group 1 vs group 4) completely eliminated the immune enhancing effects of pGM-CSF. Statistical analysis of these data using Student’s $t$ test indicated that for each T cell epitope, the responses from group 1 were significantly higher than those of groups 2–6 ($p < 0.02$ for both CD4 and CD8 T epitopes). Interestingly, although there is no significant difference in responses between groups 3 and 4 ($p > 0.1$), which received pGM-CSF separate from pPyCSP, both these groups had lower T cell responses to the 57- to 70-aa epitope than group 2, which received no pGM-CSF ($p < 0.02$). Responses to the 280-288-aa epitope in groups 3 and 4 were also lower than in group 2 but not statistically different ($p > 0.1$). Thus, it appears that the physical separation of the pGM-CSF and pPyCSP injection sites decreased T cell and Ab responses to pPyCSP (groups 3 and 4 vs group 1).

Interestingly, the data from groups 5 and 6 showed that when the pPyCSP and pGM-CSF are injected 10 min apart into the same muscle, there is only a limited enhancing effect (small increase in Ab and no change in T cell responses). This result was reproduced three of five times. In two experiments, the second injection of pGM-CSF did enhance the T cell and Ab responses to PyCSP (data not shown). Thus, even within the same muscle, admixing the two plasmids in the same syringe is the optimal way of boosting by GM-CSF.

**Kinetics of cell infiltration into muscle after injection of pGM-CSF**

To investigate the local effects of plasmid-expressed GM-CSF in muscle, mice were injected once in both gastrocnemius muscles with pGM-CSF + pPyCSP, with the GM-CSF plasmid in decreasing amounts (30, 3, and 0.3 μg; in Fig. 2, groups 1, 2, and 3, respectively). Control mice were injected with pGM-CSF + p1012 plasmid (group 4), mutated pGM-CSF + pPyCSP (group 5), p1019 + pPyCSP plasmid (group 6), p1019 + p1012 (group 7), or p1012 (group 8). Six weeks after injection, mice were bled for serum Abs, and spleens were transferred for T cell studies. Ab assays were performed on pooled serum, and T cell studies were performed on individual animals.

$^a$ Groups of six BALB/c mice were injected in the indicated muscles with 50 μg pPyCSP and 15 μg pGM-CSF and/or of p1019 plasmids as described in Materials and Methods. Six weeks after injection, mice were bled for serum Abs, and spleens were harvested for T cell studies. Ab assays were performed on pooled serum, and T cell studies were performed on individual animals.

$^b$ Titer is here defined as the reciprocal of the serum dilution at which the OD reading was 0.5.

$^c$ CD4 T cell epitope.

$^d$ CD8 T cell epitope.

$^e$ Groups 5 and 6 differ from the rest in that the second injection was given 10 min after the first injection.

### Table I. Immune responses to PyCSP obtained by separating the location and time of injection of the pPyCSP and pGM-CSF plasmids

<table>
<thead>
<tr>
<th>Group</th>
<th>Right Tibialis Anterior Injection</th>
<th>Left Tibialis Anterior Injection</th>
<th>Serum Ab Titer to PyCSP$^a$</th>
<th>IFN-γ ELISPOT Spots/10^6 Cells to PyCSP$^{57–70}$ aa (SD)</th>
<th>IFN-γ ELISPOT Spots/10^6 Cells to PyCSP$^{280–288}$ aa (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pPyCSP + pGM-CSF</td>
<td>p1019</td>
<td>p1019</td>
<td>3277$^*$</td>
<td>914 (433)$^*$</td>
</tr>
<tr>
<td>2</td>
<td>pPyCSP + p1019</td>
<td>p1019</td>
<td>p1019</td>
<td>239</td>
<td>425 (135)</td>
</tr>
<tr>
<td>3</td>
<td>pPyCSP + p1012</td>
<td>p1019</td>
<td>p1019</td>
<td>281</td>
<td>24 (14)$^*$</td>
</tr>
<tr>
<td>4</td>
<td>pPyCSP + p1019</td>
<td>pGM-CSF</td>
<td>p1019</td>
<td>219</td>
<td>51 (32)$^*$</td>
</tr>
<tr>
<td>5</td>
<td>1st pPyCSP, 2nd pGM-CSF$^c$</td>
<td>p1019</td>
<td>p1019</td>
<td>790$^{**}$</td>
<td>91 (47)</td>
</tr>
<tr>
<td>6</td>
<td>1st pPyCSP, 2nd p1012$^c$</td>
<td>p1019</td>
<td>p1019</td>
<td>239</td>
<td>87 (33)</td>
</tr>
</tbody>
</table>

$^a$ Groups of six BALB/c mice were injected in the indicated muscles with 50 μg pPyCSP and 15 μg pGM-CSF and/or of p1019 plasmids as described in Materials and Methods. Six weeks after injection, mice were bled for serum Abs, and spleens were harvested for T cell studies. Ab assays were performed on pooled serum, and T cell studies were performed on individual animals.

$^b$ Titer is here defined as the reciprocal of the serum dilution at which the OD reading was 0.5.

$^c$ CD4 T cell epitope.

$^d$ CD8 T cell epitope.

$^e$ Groups 5 and 6 differ from the rest in that the second injection was given 10 min after the first injection.
The gastrocnemius muscle was used in these studies because of the ease with which it can be removed and handled. The injected muscles were removed at different time points and examined by histology and immunohistochemistry.

The results from H&E-stained muscle sections are presented in Fig. 2. Data represent the average infiltration index for four muscles at each time point, with SDs ranging between 0 and 0.6. Coinjection i.m. of control plasmids p1019 pPyCSP (group 6) or p1019 p1012 (group 7) led to small infiltrates which persisted for 7 days. A similarly small infiltrate was seen after injection of pPyCSP plasmid mixed with a plasmid encoding the murine GM-CSF gene with two point mutations that lead to production of GM-CSF without bioactivity (group 5). However, a significantly larger infiltrate was seen when plasmid encoding the native GM-CSF was injected either alone (data not shown), with pPyCSP (group 1) \( (p < 0.002) \) or with control p1012 (group 4) \( (p < 0.006) \). Doses of 30 or 3 \( \mu g \) of pGM-CSF + pPyCSP produced infiltrates similar in duration and size (groups 1 and 2) which were significantly larger than 0.3 \( \mu g \) pGM-CSF (group 3) \( (p < 0.02) \). The latter dose showed infiltrates that were indistinguishable from pPyCSP alone (not shown) or control plasmid injections (group 4, 5, or 6) \( (p > 0.1 \) in all cases). Interestingly, in most cases, the infiltration induced by pGM-CSF was found at one area within the muscle but with several foci of inflammation in the vicinity (Fig. 3A). This infiltrate reached its largest size at days 3, 4, and 5; diminished by day 7; but was still detected at day 14 after injection. Muscles excised 1 or 8 h after injection with pGM-CSF showed little infiltration which was seemingly limited to the needle site (group 8). The gastrocnemius muscle was used in these studies because of the ease with which it can be removed and handled. The injected muscles were removed at different time points and examined by histology and immunohistochemistry.

The results from H&E-stained muscle sections are presented in Fig. 2. Data represent the average infiltration index for four muscles at each time point, with SDs ranging between 0 and 0.6. Coinjection i.m. of control plasmids p1019 pPyCSP (group 6) or p1019 p1012 (group 7) led to small infiltrates which persisted for \( \sim \)7 days. A similarly small infiltrate was seen after injection of pPyCSP plasmid mixed with a plasmid encoding the murine GM-CSF gene with two point mutations that lead to production of GM-CSF without bioactivity (group 5). However, a significantly larger infiltrate was seen when plasmid encoding the native GM-CSF was injected either alone (data not shown), with pPyCSP (group 1) \( (p < 0.002) \) or with control p1012 (group 4) \( (p < 0.006) \). Doses of 30 or 3 \( \mu g \) of pGM-CSF + pPyCSP produced infiltrates similar in duration and size (groups 1 and 2) which were significantly larger than 0.3 \( \mu g \) pGM-CSF (group 3) \( (p < 0.02) \). The latter dose showed infiltrates that were indistinguishable from pPyCSP alone (not shown) or control plasmid injections (group 4, 5, or 6) \( (p > 0.1 \) in all cases). Interestingly, in most cases, the infiltration induced by pGM-CSF was found at one area within the muscle but with several foci of inflammation in the vicinity (Fig. 3A). This infiltrate reached its largest size at days 3, 4, and 5; diminished by day 7; but was still detected at day 14 after injection. Muscles excised 1 or 8 h after injection with pGM-CSF showed little infiltration which was seemingly limited to the needle site (group 8). The gastrocnemius muscle was used in these studies because of the ease with which it can be removed and handled. The injected muscles were removed at different time points and examined by histology and immunohistochemistry.

The results from H&E-stained muscle sections are presented in Fig. 2. Data represent the average infiltration index for four muscles at each time point, with SDs ranging between 0 and 0.6. Coinjection i.m. of control plasmids p1019 pPyCSP (group 6) or p1019 p1012 (group 7) led to small infiltrates which persisted for \( \sim \)7 days. A similarly small infiltrate was seen after injection of pPyCSP plasmid mixed with a plasmid encoding the murine GM-CSF gene with two point mutations that lead to production of GM-CSF without bioactivity (group 5). However, a significantly larger infiltrate was seen when plasmid encoding the native GM-CSF was injected either alone (data not shown), with pPyCSP (group 1) \( (p < 0.002) \) or with control p1012 (group 4) \( (p < 0.006) \). Doses of 30 or 3 \( \mu g \) of pGM-CSF + pPyCSP produced infiltrates similar in duration and size (groups 1 and 2) which were significantly larger than 0.3 \( \mu g \) pGM-CSF (group 3) \( (p < 0.02) \). The latter dose showed infiltrates that were indistinguishable from pPyCSP alone (not shown) or control plasmid injections (group 4, 5, or 6) \( (p > 0.1 \) in all cases). Interestingly, in most cases, the infiltration induced by pGM-CSF was found at one area within the muscle but with several foci of inflammation in the vicinity (Fig. 3A). This infiltrate reached its largest size at days 3, 4, and 5; diminished by day 7; but was still detected at day 14 after injection. Muscles excised 1 or 8 h after injection with pGM-CSF showed little infiltration which was seemingly limited to the needle site (group 8). The gastrocnemius muscle was used in these studies because of the ease with which it can be removed and handled. The injected muscles were removed at different time points and examined by histology and immunohistochemistry.

The results from H&E-stained muscle sections are presented in Fig. 2. Data represent the average infiltration index for four muscles at each time point, with SDs ranging between 0 and 0.6. Coinjection i.m. of control plasmids p1019 pPyCSP (group 6) or p1019 p1012 (group 7) led to small infiltrates which persisted for \( \sim \)7 days. A similarly small infiltrate was seen after injection of pPyCSP plasmid mixed with a plasmid encoding the murine GM-CSF gene with two point mutations that lead to production of GM-CSF without bioactivity (group 5). However, a significantly larger infiltrate was seen when plasmid encoding the native GM-CSF was injected either alone (data not shown), with pPyCSP (group 1) \( (p < 0.002) \) or with control p1012 (group 4) \( (p < 0.006) \). Doses of 30 or 3 \( \mu g \) of pGM-CSF + pPyCSP produced infiltrates similar in duration and size (groups 1 and 2) which were significantly larger than 0.3 \( \mu g \) pGM-CSF (group 3) \( (p < 0.02) \). The latter dose showed infiltrates that were indistinguishable from pPyCSP alone (not shown) or control plasmid injections (group 4, 5, or 6) \( (p > 0.1 \) in all cases). Interestingly, in most cases, the infiltration induced by pGM-CSF was found at one area within the muscle but with several foci of inflammation in the vicinity (Fig. 3A). This infiltrate reached its largest size at days 3, 4, and 5; diminished by day 7; but was still detected at day 14 after injection. Muscles excised 1 or 8 h after injection with pGM-CSF showed little infiltration which was seemingly limited to the needle site (group 8). The gastrocnemius muscle was used in these studies because of the ease with which it can be removed and handled. The injected muscles were removed at different time points and examined by histology and immunohistochemistry.
path and was not different from that seen with PBS or control plasmid (data not shown).

**Immunohistochemical analysis of cells at the site of injection of pGM-CSF**

To identify the cells recruited by pGM-CSF to the site of injection, muscle sections were analyzed by immunohistochemistry using Abs with specificities for different cell markers. The results from muscles analyzed at day 3 after plasmid injection are presented in Table II. The predominant cell type found in all infiltrates after injection with any of the DNA plasmids was a population with a macrophage phenotype, staining strongly with Abs to CD11b$^+$ (Fig. 3B), Mac-3$^-$, and IA$^+$E$^+$$. Cells with this staining were observed throughout the entire 14 days of the study (data not shown). Appreciable numbers of granulocytes, as detected by Abs specific to GR-1 (Fig. 3C), were also present in all infiltrated muscles, regardless of the plasmid used for injection (Table II). H&E staining revealed that these granulocytes were mostly neutrophils, with few eosinophils (not shown). Perhaps the most striking finding was the presence of cells expressing the DC marker CD11c in muscles injected with 30 or 3 μg of pGM-CSF (Fig. 3D) but not in muscles injected with 0.3-μg doses or lower of pGM-CSF or with any of the control plasmids (Table II). These CD11c$^+$ cells were detected only on days 3, 4, and 5 after pGM-CSF injection, appearing later and disappearing earlier than the cells with CD11b$^+$, Mac-3$^-$, IA$^+$E$^+$$, or GR-1 phenotypes. Serial sections from muscles displaying CD11c$^+$ staining did not present any reactivity with B7-1 or CD40-specific Abs (not shown), which are both markers of APC activation and DC maturation. Neither was there staining with CD4+, CD8-, or B220-specific Abs in any of the injected muscles (not shown), indicating the absence of T or B lymphocytes in the infiltrates. Immunostaining of normal spleen sections with anti-CD4-, -CD8-, -B220, B7-1, or -CD40 Abs, however, displayed a typical reactivity (not shown). No reactivity was observed with isotype control primary Abs in any muscle (Fig. 3E). Thus, these data indicate that the effect of pGM-CSF is both to increase the infiltrate size and duration (as seen in H&E sections) and to participate in the recruitment of CD11c$^+$ cells.

**Analysis of CD11c$^+$ DC in the draining lymph nodes**

Immature CD11c$^+$ B7-1$^+$ CD40$^-$ DC are recruited into muscles by GM-CSF but are detected for only a short period after injection. Either these cells are dying or they must be migrating out of the muscle tissue, possibly to become mature DC. To investigate whether these DC leave the muscle, the draining popliteal lymph nodes were analyzed by flow cytometry. Thus, increases in numbers of CD11c/CD11b, CD11c/B7-1, or CD11c/MHC II double-positive mature DC were assessed at different time points in the draining lymph nodes of mice injected with pGM-CSF compared with control p1019. Significant increases were found in the percentages of CD11c/CD11b (p < 0.01) and CD11c/B7.1 (p < 0.06) but not those of CD11c/MHC II (p > 0.1) (data not shown) double-positive cells at days 4 (Fig. 4), 8, and 12 after i.m. injection with pGM-CSF. No significant differences were found at day 20 after injection (data not shown). Fig. 4 shows the FACS profiles from representative animals at day 4 after injection with pGM-CSF or p1019, where a 1.5- to 3.0-fold difference in CD11c/B7.1 or CD11c/CD11b double-positive cells was consistently found between the two immunization groups.

**The histological profile and the immune responses from low doses of pGM-CSF**

To determine the lowest dose of pGM-CSF that would boost immune responses and to assess whether the immune enhancement effect could be dissociated from the presence of the i.m. infiltrate, groups of mice were injected with decreasing amounts (60, 6, or 0.6 μg) of GM-CSF plasmid mixed with 50 μg pPyCSP. Injections were split into both gastrocnemius muscles, each muscle receiving 30, 3, or 0.3 μg of pGM-CSF together with 25 μg pPyCSP. Control groups of mice received injections with 60 μg control plasmids, and/or 50 μg pPyCSP alone, also split bilaterally. Three days after injection, two mice from each dose group were sacrificed, and their injected muscles (two from each mouse) were studied for cellular infiltrates using immunohistochemistry and H&E staining. Other mice were maintained for 6 wk, when their T cell and Ab responses to pPyCSP were measured.

The immune responses from mice injected with pPyCSP plus varying doses of pGM-CSF are shown in Fig. 5. Data shown are typical of three separate experiments using five mice per group. Fig. 5A shows the numbers of spleen cells producing IFN-γ in an ELISPOT assay after in vitro exposure to the PyCSP$^{57–70}$ aa peptide, CD8 epitope. Compared with control injections with pPyCSP alone or admixed with p1019 control plasmid, a dose of 0.6 μg of pGM-CSF or higher boosted the response to the CD8 (p < 0.01) epitope. Fig. 5B shows the T cell proliferation responses to the PyCSP$^{57–70}$ aa CD4 epitope. As noted for the IFN-γ results, higher

---

**Table II. Immunohistochemical analysis of muscles from BALB/c mice at day 3 after coinjection with DNA plasmids encoding GM-CSF and PyCSP**

<table>
<thead>
<tr>
<th>Plasmid Injected (μg)</th>
<th>Infiltrate Size$^b$ (SD)</th>
<th>CD11b$^+$/Mac-3$^-$ (SD)</th>
<th>IA$^+$E$^+$ (SD)</th>
<th>CD11c$^+$ (SD)</th>
<th>GR-1$^+$ (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGM-CSF-30 + pPyCSP-50</td>
<td>3+ (0)</td>
<td>3+ (0)</td>
<td>1.25+ (0.5)</td>
<td>2+ (0)</td>
<td></td>
</tr>
<tr>
<td>pGM-CSF-30 + p1012-50</td>
<td>3+ (0)</td>
<td>3+ (0)</td>
<td>1.5+ (0.58)</td>
<td>1.25+ (0.5)</td>
<td></td>
</tr>
<tr>
<td>pGM-CSF-3 + pPyCSP-50</td>
<td>3+ (0)</td>
<td>3+ (0)</td>
<td>2.2+ (0.5)</td>
<td>2.25+ (0.5)</td>
<td></td>
</tr>
<tr>
<td>pGM-CSF-0.3 + pPyCSP-50</td>
<td>1.5+ (0.58)</td>
<td>2.5+ (0.58)</td>
<td>0</td>
<td>0.75+ (0.5)</td>
<td></td>
</tr>
<tr>
<td>Mutated pGM-CSF-30 + pPyCSP-50</td>
<td>1.0+ (0)</td>
<td>2+ (0.58)</td>
<td>0</td>
<td>1.5+ (0.5)</td>
<td></td>
</tr>
<tr>
<td>p1019-30 + pPyCSP-50</td>
<td>1.75+ (0.5)</td>
<td>3+ (0)</td>
<td>0</td>
<td>1.25+ (0.5)</td>
<td></td>
</tr>
<tr>
<td>p1019-30 + P1012-50</td>
<td>1.75+ (0.5)</td>
<td>3+ (0)</td>
<td>1.25+ (0.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPyCSP-50</td>
<td>1.5+ (0.58)</td>
<td>2.75+ (0.50)</td>
<td>0</td>
<td>1.25+ (0.5)</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0.25+ (0.5)</td>
<td>0.5+ (0.58)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Groups of two BALB/c mice were injected in each gastrocnemius muscle with indicated amounts of pGM-CSF + pPyCSP or with corresponding control plasmids, p1019 or p1012, respectively. Results are given as an average of four muscles.

$^b$ Infiltrate size was graded on H&E sections as described in Fig. 2 and in Materials and Methods. Cell markers predominantly expressed by: $^c$ macrophages, $^d$ dendritic cells, $^e$ granulocytes, or $^f$ MHC class II APCs.

Staining of CD11b, Mac-3, IA$^+$E$^+$, CD11c, and GR-1-expressing cells was graded on a 0–3 scale: 0, no positive staining; 1, fewer than 10% stained cells; 2, between 10 and 50% stained cells; and 3, 50–100% stained cells.
amounts of pGM-CSF lead to more robust proliferative responses. Stimulation indexes obtained from mice injected with 60, 6, or 0.6 μg of pGM-CSF were significantly higher than those evoked by the control injections with 60 μg p1019 or 50 μg pPyCSP (p < 0.01). An increase in Ab responses occurred at doses of 60 or 6 μg pGM-CSF but not at lower doses (Fig. 5C). In summary, there was an association, albeit imperfect, between enhancement of immune responses and the presence of CD11c+ cells in the injected muscles. Infiltrates containing CD11c+ cells were detected with doses of 3 μg pGM-CSF per muscle but not with doses of 0.3 μg or lower. Likewise, enhancement of Ab responses was observed with bilateral doses of 3 but not with 0.3 μg pGM-CSF. However, both CD4 and CD8 T cell responses to defined peptide epitopes were enhanced when mice were given a bilateral dose of 0.3 μg of pGM-CSF, despite the absence of CD11c+ in muscles injected with this dose. A one-way ANOVA indicated, however, that the T cell responses of mice presenting i.m. infiltrating CD11c+ cells (injected with 30 or 3 μg pGM-CSF) were significantly different from the T cell responses of those animals lacking these infiltrating cells (0.3 μg pGM-CSF or control plasmid injections) (p < 0.005).

Discussion
In the present study, we have shown that 1) pGM-CSF induces an i.m. infiltrate of cells with phenotypes consistent with APC; and 2) for sustained immune enhancement, the pGM-CSF and pPyCSP plasmids must be injected into the same site.

Our study is the first description of the immunohistology in muscle associated with pGM-CSF injection. We found large and multifocal conglomerates of cells that were initially MHC class II+Mac-3+CD11b+CD11c+B7.1+CD40+. During the peak of the infiltrate, a subset of cells expressing CD11c+ appeared. Numerous GR1+ cells that were mostly neutrophils with few eosinophils were also present within this infiltrate, but there were no T cells or B cells at any time point examined.

The infiltrates we identified in muscles injected with pGM-CSF plasmid are different from those observed in the skin surrounding irradiated tumor vaccines either transduced with the GM-CSF gene (18, 19, 35) or infected with viral vectors expressing GM-CSF (36, 37). Whereas plasmid encoded GM-CSF seems to have induced a relatively homogenous infiltrate of APCs and neutrophils in muscle, the tumor-expressed GM-CSF generated infiltrates in skin that included T cells, B cells, and eosinophils. The differences with regard to plasmid GM-CSF may be due to the complexity of the antitumor immune response at the site of injection, to the intrinsic cell composition of each tissue (muscle vs skin), or perhaps to the Th1 immunomodulatory effects of the DNA altering the local cytokine milieu (38, 39). At present, it is unclear whether the GM-CSF in our system is directly acting to recruit the infiltrating cells or is working through the induction of other lymphokines or chemokines (36).
Our results show that after pGM-CSF injection, the incoming cells are a mixture of macrophages, neutrophils, and immature DC. It has been well established that in vitro culture of bone marrow or CD34⁺ PBMC with GM-CSF and IL-4 leads to the outgrowth of immature DC with a CD11b⁺CD11c⁻/low B7.1⁻/low B7.2⁻/low CD40⁻ phenotype, which can mature into CD11b⁺CD11c⁺B7.1⁺B7.2⁺CD40⁺ DC after a variety of inflammatory stimuli (40, 41). It is possible that after i.m. pGM-CSF injection, in vivo expressed GM-CSF may have similar effects on circulating DC precursors recruited into the injection site, with DC maturation resulting from the low grade inflammatory response seen after any DNA injection. Immature DC are especially efficient at Ag uptake, whereas mature DC are more adapted for Ag presentation (40, 41). The appearance in muscle of CD11c⁺ cells lacking the activation markers B7.1 or CD40 on days 3–5 after pGM-CSF injection, followed by an increase of CD11c⁺CD11b⁺B7.1⁺ cells in lymph nodes on days 4–12, is consistent with egression of these immature DC from the muscle and migration to the draining lymph nodes, where they become mature DC (42).

Ongoing efforts to isolate and purify the cells from the i.m. pGM-CSF infiltrates as well as from the corresponding draining lymph nodes should shed more light on the functional attributes of these cells.

The pGM-CSF dose titration experiments showed that infiltrate size and appearance of CD11c⁺ cells are associated with enhancement of Ab responses to an admixed malaria DNA vaccine. Yet, T cell responses were boosted at low doses of pGM-CSF where no CD11c⁺ cells are detected. It is possible, however, that the detection of the relatively scarce CD11c⁺ cells may have been missed by our sectioning procedure in infiltrates of small size and reduced depth.

The plasmid backbone of the DNA vaccines used in this study is of bacterial origin and contains some immunostimulatory non-methylated CpG sequences (38, 39). It is unlikely, however, that the inflammatory reaction we observe with pGM-CSF as well as the adjuvant effect of this plasmid is due to its CpG content. Injection of several different control plasmids not only evoked smaller infiltrates lacking CD11c⁺ DC but also elicited statistically lower immune responses than did pGM-CSF. The most appropriate control for immunostimulatory effects of DNA is the plasmid encoding murine GM-CSF with two point mutations (not at CpG sites), producing inactive GM-CSF protein (17, 27). Injections with this plasmid also gave small infiltrates that lacked DC and did not enhance the immune responses. Thus, although we acknowledge that in some circumstances CpGs can have immunostimulatory activity, we believe that this cannot explain the effects of pGM-CSF. Our findings suggest that events occurring in the muscle after pGM-CSF injection may be contributing to the immune enhancement. Direct transfection of the infiltrating APC with the coinjected pPyCSP plasmid or improved cross-priming by APCs taking up PyCSP immunogens from myocytes would be predicted to contribute to a better immune response (43–45). However, this...
premise has two important caveats. Firstly, the enhancement of immune responses by pGM-CSF may be multifactorial and some of these factors may be independent of DC (10). Secondly, the importance of Ag production by the plasmid-transfected muscle is still controversial. It has been reported that Ag expression by nonlymphoid somatic cells may be responsible for the majority of antigenicity of an OVA-encoded DNA vaccine (45). However, surgical ablation of injected muscles minutes after receiving plasmid was reported to have no effect on immunogenicity, implying that the muscle itself may not be the site of Ag expression during DNA vaccination (34). Leakage of plasmid from the muscle into the blood or the lymph, or direct transfection of an APC in transit through the injected muscle, might represent the true mode of immunization (46). Notably, Klinman et al. (47) showed that migratory cells at the site of vaccination were indispensable for induction of primary immunity as well as immunological memory. Our results indicate that leakage of pGM-CSF into the bloodstream does not contribute significantly to immune enhancement, because an i.v. injection of pGM-CSF combined with pPyCSP administered either i.v. or i.m. evoked at best only transient immune responses.

Separation of injections with pGM-CSF and pPyCSP, either into two adjacent muscles in the same leg or into muscles in opposite legs gave interesting results. We reasoned that if plasmid can leak into the lymphatic circulation, separating the injection of each plasmid into two different muscles drained by the same lymph nodes could result in a response equivalent to that evoked by injection of both plasmids into the same muscle. However, not only was there no boosting of responses when pGM-CSF and pPyCSP were given into separate muscles, as previously noted for rabies-encoding plasmids (12), but there was a decrease in T cell responses to pPyCSP. This is not unprecedented, because weaker T cell responses from PBMC have been reported to occur when recombinant GM-CSF protein was injected s.c. preceding peptide immunization in humans (48). It is possible that the production of GM-CSF after plasmid injection into one muscle was able to draw away circulating APCs from the location where pPyCSP was injected, thereby decreasing the immune responses.

As a second part of this experiment, the i.m. injections with pPyCSP and pGM-CSF were given 10 min apart in the same muscle. We expected to reproduce the boosting effects of a single injection of admixed plasmids. Instead, we found that the second injection with pGM-CSF had very little effect. This lack of response could be due to several factors. Perhaps the first injection transiently changes the character of the muscle so that the second injection given within minutes of the first is not as effective. The results of an experiment in progress support this idea. We injected pGM-CSF i.m. 3 days before injecting pPyCSP into the same muscle. Specific Abs were boosted when pGM-CSF and pPyCSP injections were separated by several days or when the plasmids were coinjected. This 3-day time lag is similar to that found by Disis et al. (6) using recombinant GM-CSF protein to boost protein Ags. However, Disis found very poor immunogenicity when she mixed recombinant GM-CSF protein together with protein Ag, in sharp contrast to our results with plasmid immunization. We think the kinetics of protein production from plasmid-transfected cells as well as the multifocal and highly localized character of the infiltrates seen after pGM-CSF injection could account for this difference. There is still much to be learned about the mechanisms and timing of boosting by GM-CSF.

The exclusively local activity of i.m. pGM-CSF is encouraging for its safe use as a vaccine adjuvant. It would seem extremely unlikely that pGM-CSF injected into one site would enhance an ongoing pathological T cell response in a different anatomic location. Thus, pGM-CSF plasmid merits serious consideration as a DNA vaccine enhancer in humans.

Acknowledgments

We thank Noelle Patterson for advice, Henry Fleetwood for expert technical assistance with the immunohistology studies, Victoria Fallarme for performing the Ab assays, David Miles for diligent assistance with the digital images, Dr. Denise Doolan for critically reading the manuscript, and Dr. Trevor Jones for assistance with the statistical analyses.

References