

# Recruitment of Antigen-Presenting Cells to the Site of Inoculation and Augmentation of Human Immunodeficiency Virus Type 1 DNA Vaccine Immunogenicity by In Vivo Electroporation<sup>∇</sup>

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**In vivo electroporation (EP) has been shown to augment the immunogenicity of plasmid DNA vaccines, but its mechanism of action has not been fully characterized. In this study, we show that in vivo EP augmented cellular and humoral immune responses to a human immunodeficiency virus type 1 Env DNA vaccine in mice and allowed a 10-fold reduction in vaccine dose. This enhancement was durable for over 6 months, and re-exposure to antigen resulted in anamnestic effector and central memory CD8<sup>+</sup> T-lymphocyte responses. Interestingly, in vivo EP also recruited large mixed cellular inflammatory infiltrates to the site of inoculation. These infiltrates contained 45-fold-increased numbers of macrophages and 77-fold-increased numbers of dendritic cells as well as 2- to 6-fold-increased numbers of B and T lymphocytes compared to infiltrates following DNA vaccination alone. These data suggest that recruiting inflammatory cells, including antigen-presenting cells (APCs), to the site of antigen production substantially improves the immunogenicity of DNA vaccines. Combining in vivo EP with plasmid chemokine adjuvants that similarly recruited APCs to the injection site, however, did not result in synergy.**

Plasmid DNA vaccines have proven considerably less immunogenic in clinical studies than in preclinical studies (3, 9, 13, 24, 33), demonstrating the need to improve their potency. Various strategies are currently being pursued, including the use of plasmid cytokine and chemokine adjuvants (5, 6, 11, 19, 26, 30), polymer adjuvants (29), novel transcriptional regulatory elements (7), and improved delivery techniques such as in vivo electroporation (EP) (2, 23, 25). In vivo EP involves the administration of electrical pulses to muscle tissue following intramuscular (i.m.) injection of DNA vaccines and has been shown to enhance the immunogenicity of DNA vaccines in a wide variety of small and large animal models (1, 8, 10, 12, 17, 18, 20, 22, 27, 32). It has been suggested that in vivo EP functions in part by increasing myocyte permeability and thereby facilitating plasmid uptake and antigen expression by host cells (2, 14–16, 25, 28, 34).

We have previously reported that there are very few professional antigen-presenting cells (APCs) in muscles after DNA vaccination (6), and we therefore hypothesized that DNA vaccines may be limited by insufficient APCs at the site of antigen production. Consistent with this hypothesis, we observed that plasmid chemokines and growth factors such as plasmid MIP-1 $\alpha$  and Flt3L were able to recruit dendritic cells (DCs) and macrophages to the site of inoculation and to enhance DNA vaccine-elicited immune responses (26, 30). Whether APCs are similarly recruited by in vivo EP, however, has not previously been investigated. In addition, the phenotype of cellular immune responses elicited by DNA vaccination with in vivo EP has not been assessed in detail. In the present study,

we investigated the magnitude, phenotype, and durability of cellular immune responses elicited in mice by human immunodeficiency virus type 1 (HIV-1) Env DNA vaccination with or without in vivo EP and assessed the extent and nature of cellular inflammatory infiltrates at the site of inoculation.

**In vivo EP augments DNA vaccine-elicited immune responses.** We initiated studies by assessing the immunogenicity of 50, 5, or 0.5  $\mu$ g of a previously described HIV-1 Env IIIB gp120 DNA vaccine (6, 30) either alone or with two different methods of in vivo EP. BALB/c mice (four animals/group) were anesthetized and immunized i.m. in the quadriceps muscles, and in vivo EP was performed according to the manufacturer's protocols (Inovio Biomedical, San Diego, CA). Caliper EP involved application of electric pulses across intact muscle using surface electrodes with conductive gel after DNA vaccination ( $6 \times 100$ - $\mu$ s pulses at 600 V/cm). Needle EP involved delivery of electric pulses from electrodes inserted i.m. flanking the injection site after DNA vaccination ( $2 \times 60$ -ms pulses at 200 V/cm).

CD8<sup>+</sup> T-lymphocyte responses to the dominant Env P18 epitope (RGPGRAFVTI) (31) were assessed by D<sup>d</sup>/P18 tetramer binding assays at multiple time points after immunization as previously described (6, 30). Cellular immune responses to a pool of overlapping Env peptides and the P18 epitope peptide were also assessed by gamma interferon (IFN- $\gamma$ ) enzyme-linked immunospot (ELISPOT) assays at week 4 after immunization. As shown in Fig. 1A and B, the DNA vaccine alone elicited potent cellular immune responses at the dose of 50  $\mu$ g and detectable responses at the dose of 5  $\mu$ g, but no responses were observed at the dose of 0.5  $\mu$ g. Caliper EP utilizing these experimental conditions had little adjuvant effect. In contrast, needle EP resulted in a significant threefold enhancement of the magnitude of CD8<sup>+</sup> T-lymphocyte responses at the dose of 50  $\mu$ g ( $P = 0.001$  comparing tetramer

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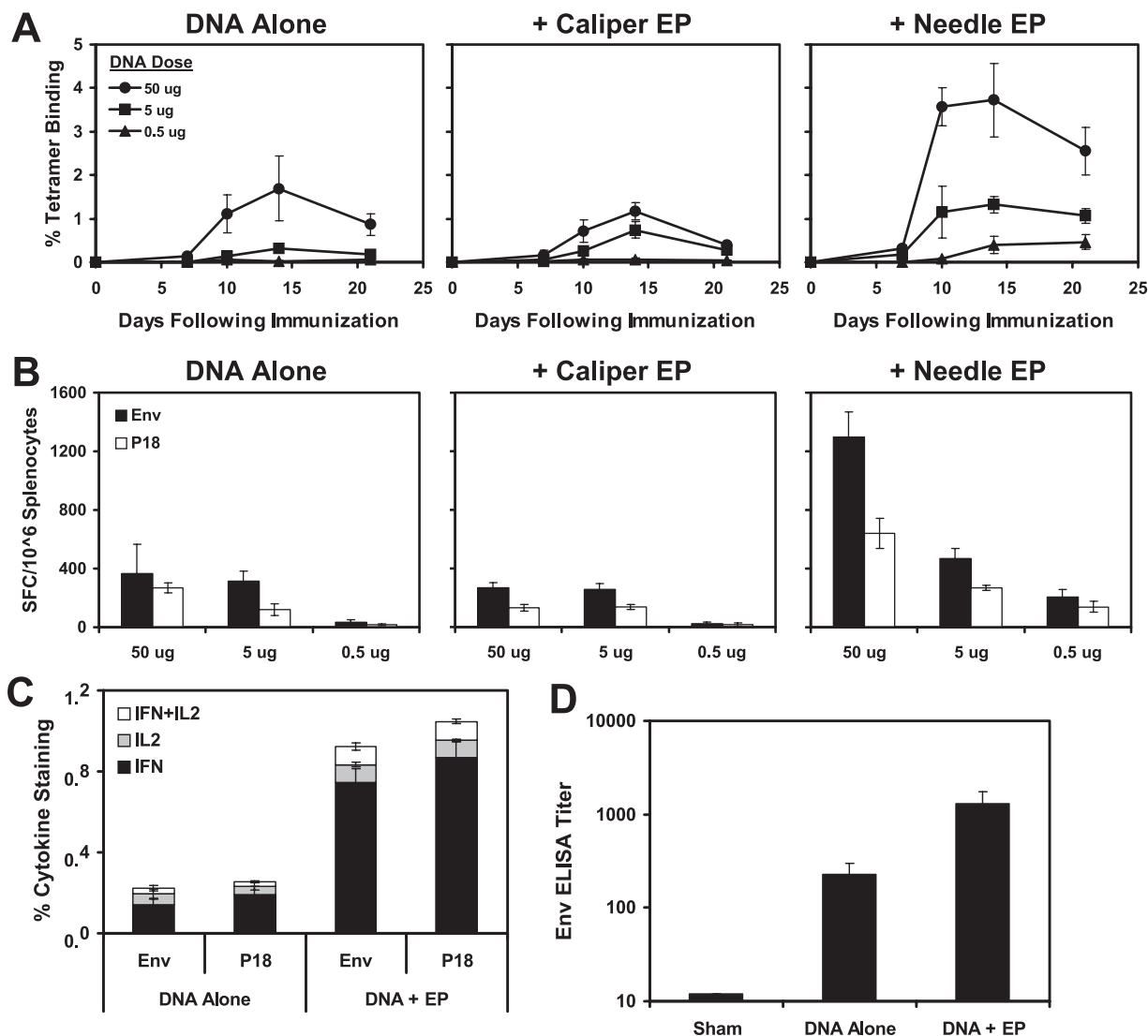


FIG. 1. Immunogenicity of DNA vaccination with in vivo EP. Groups of BALB/c mice (four animals/group) were immunized with 50, 5, or 0.5  $\mu$ g of the DNA vaccine expressing Env IIIIB gp120 alone or with caliper EP or needle EP. Cellular and humoral immune responses were assessed by D<sup>h</sup>/P18 tetramer binding assays at multiple time points after immunization (A) and by Env pooled peptide and P18 epitope peptide IFN- $\gamma$  ELISPOT assays (B). Env pooled peptide and P18 epitope peptide IFN- $\gamma$ /IL-2 CD8<sup>+</sup> intracellular cytokine staining assays (C) and Env-specific ELISAs (D) were performed at week 4 after immunization in mice that received the 50- $\mu$ g dose of the DNA vaccine with or without needle EP.

binding responses on day 14 after immunization using two-tailed *t* tests). Cellular immune responses were also detected at the lowest dose of 0.5  $\mu$ g, indicating that needle EP allowed a 10-fold reduction in the DNA vaccine dose. Needle EP also resulted in a significant fourfold augmentation of both IFN- $\gamma$ <sup>+</sup> and IFN- $\gamma$ <sup>+</sup>/IL-2<sup>+</sup> CD8<sup>+</sup> T-lymphocyte responses by intracellular cytokine staining assays ( $P = 0.0002$ ; Fig. 1C) (21) and a sixfold increase in Env-specific antibody titers by enzyme-linked immunosorbent assay (ELISA) ( $P = 0.01$ ; Fig. 1D) (6, 30) at week 4 after immunization in mice that received the 50- $\mu$ g dose. Needle EP was therefore utilized in subsequent studies, although we do not exclude the possibility that caliper EP could be further optimized.

**In vivo EP does not augment rAd5 vaccine-elicited immune responses.** We next evaluated the capacity of in vivo needle EP to enhance the immunogenicity of a recombinant adenovirus

serotype 5 (rAd5) vector-based vaccine. As shown in Fig. 2, in vivo EP did not significantly augment responses elicited by 10<sup>9</sup>, 10<sup>7</sup>, or 10<sup>5</sup> viral particles (vp) rAd5 expressing Env IIIIB gp120 (4). These data suggest that in vivo EP may prove more useful at improving DNA vaccines rather than certain live vectors, perhaps as a result of the innate inflammatory responses induced by these viral vectors, together with the fact that in vivo EP would not likely increase the infectivity of viral vectors that enter host cells efficiently via specific cellular receptors.

**Comparison of in vivo EP with plasmid chemokine adjuvants.** Previous studies have suggested that in vivo EP may function in part by increasing the permeability of myocytes and enhancing plasmid uptake and antigen expression (2, 25, 28, 34). We have previously reported that coadministration of plasmid chemokines and growth factors, particularly the combination of plasmid MIP-1 $\alpha$  and Flt3L, recruited APCs to the

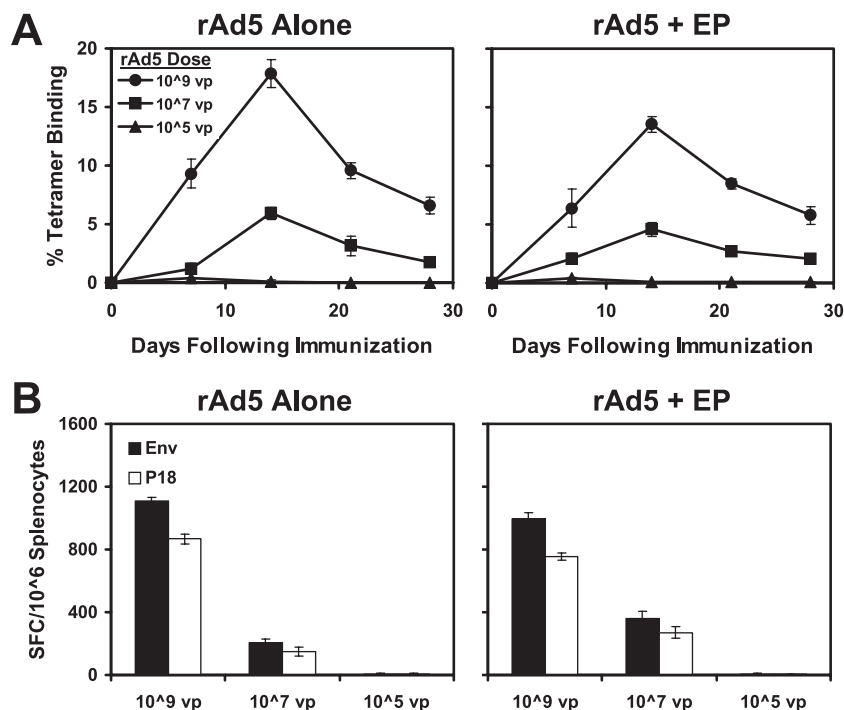


FIG. 2. Immunogenicity of rAd5 vaccination with in vivo EP. Groups of BALB/c mice (four animals/group) were immunized with  $10^9$ ,  $10^7$ , or  $10^5$  vp gp120 rAd5 expressing Env IIB gp120 alone or with needle EP. Cellular immune responses were assessed by D<sup>d</sup>/P18 tetramer binding assays (A) and Env pooled peptide and P18 epitope peptide IFN- $\gamma$  ELISPOT assays (B) at week 4 after immunization.

site of inoculation and enhanced DNA vaccine-elicited immune responses (30). We therefore hypothesized that combining plasmid chemokine adjuvants with in vivo EP might result in synergistic effects by simultaneously recruiting APCs to the injection site and improving transfection of these cells. To explore this hypothesis, groups of mice (four animals/group) were immunized with 50  $\mu$ g (Fig. 3A) or 5  $\mu$ g (Fig. 3B) of the gp120 DNA vaccine alone or with plasmid MIP-1 $\alpha$ /Flt3L, in vivo EP, or both. The plasmid chemokines and in vivo EP resulted in similar threefold enhancements of peak responses on days 10 to 14 after immunization, although the effects of in vivo EP proved more durable. The combination of plasmid chemokines and in vivo EP led to a transient additive effect on day 10, but these responses contracted quickly, and no long-term synergy was observed by combining these two adjuvant strategies.

We next evaluated the memory phenotypes and recall responses after priming with 50  $\mu$ g of the gp120 DNA vaccine alone, the DNA vaccine with in vivo EP, or the DNA vaccine with both plasmid MIP-1 $\alpha$ /Flt3L and in vivo EP. Env P18-specific responses in total, effector memory/effector (EM/E; CD62L<sup>-</sup>), central memory (CM; CD44<sup>+</sup> CD62L<sup>+</sup>), and naive (N; CD44<sup>-</sup> CD62L<sup>+</sup>) CD8<sup>+</sup> T-lymphocyte subpopulations were evaluated by multiparameter tetramer binding assays. As shown in Fig. 3C to F, in vivo EP resulted in a durable 2.9-, 3.7-, and 2.0-fold augmentation of total, EM/E, and CM CD8<sup>+</sup> T-lymphocyte responses for 168 days after the priming immunization ( $P = 0.007$ ,  $0.007$ , and  $0.01$ , respectively). In vivo EP did not skew the relative proportions of memory phenotypes compared to DNA vaccination alone at this time point.

At 6 months after the priming immunization, all groups of mice, including the sham-primed mice, were boosted with 50

$\mu$ g of the gp120 DNA vaccine without any adjuvants (Fig. 3C to F, arrows). After the boost immunization, a robust 10-fold expansion of EM/E responses and a 3-fold increase in CM responses were detected in all groups. Mice that were primed with in vivo EP 168 days previously exhibited significantly threefold-higher anamnestic EM/E responses and twofold-higher CM responses after the boost immunization compared to mice that received the DNA vaccine alone ( $P = 0.01$ ), demonstrating the durability of the effects of in vivo EP administered during the priming immunization. The addition of plasmid MIP1 $\alpha$ /Flt3L to in vivo EP led to transiently greater peak responses but did not result in long-term synergistic effects.

**In vivo EP recruits large cellular infiltrates to the site of inoculation.** We next evaluated the extent and nature of cellular infiltrates at the site of inoculation after DNA vaccination alone or with plasmid MIP-1 $\alpha$ /Flt3L, in vivo EP, or both. As shown in Fig. 4A and B, only mild inflammation was observed in sections of injected muscles on day 7 after DNA vaccination alone, a finding consistent with our prior studies (6, 26, 30). In contrast, as depicted in Fig. 4C and D, large cellular infiltrates were detected after DNA vaccination with in vivo EP. These infiltrates consisted of large clusters of mixed inflammatory cells both in perivascular spaces and throughout muscle tissue and consisted of both polymorphonuclear and mononuclear cells. The extent of these infiltrates was greater than that observed with plasmid MIP-1 $\alpha$ /Flt3L (30).

To evaluate the nature and composition of these infiltrates, we extracted cells from injected muscles on day 7 after immunization and evaluated them by multiparameter flow cytometry (30). Compared to DNA vaccination alone, DNA vaccination

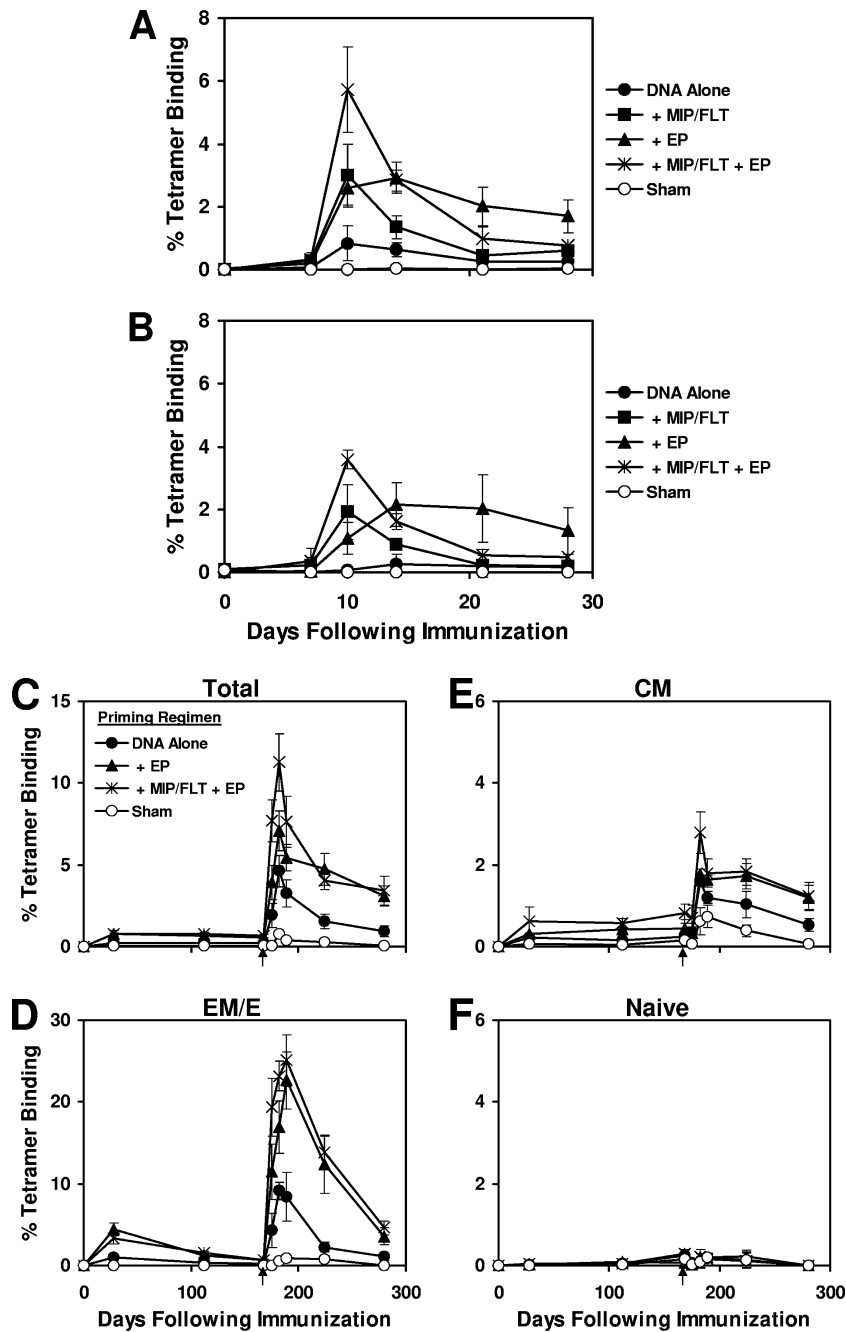


FIG. 3. Durability and phenotype of DNA vaccination with in vivo EP and plasmid chemokine adjuvants. Groups of BALB/c mice (four animals/group) were immunized with 50 µg (A) or 5 µg (B) of gp120 DNA vaccine alone or with plasmid MIP-1 $\alpha$ /Flt3L, in vivo EP, or both. CD8<sup>+</sup> T-lymphocyte responses were assessed by D<sup>d</sup>/P18 tetramer binding assays. In a separate experiment, mice were primed with 50 µg of gp120 DNA vaccine alone, the DNA vaccine with in vivo EP, or the DNA vaccine with both plasmid MIP-1 $\alpha$ /Flt3L and in vivo EP. Env P18-specific responses in total (C), EM/E (CD62L<sup>-</sup>) (D), CM (CD44<sup>+</sup> CD62L<sup>+</sup>) (E), and N (CD44<sup>-</sup> CD62L<sup>+</sup>) (F) CD8<sup>+</sup> T-lymphocyte subpopulations were evaluated by multiparameter D<sup>d</sup>/P18 tetramer binding assays. All groups of mice, including the sham-primed mice, were boosted on day 168 with 50 µg of gp120 DNA vaccine alone (arrows).

with in vivo EP led to a substantial increase in total extracted cells ( $P = 0.02$ ; Fig. 4E), as well as two- to sixfold increases in CD19<sup>+</sup> B lymphocytes, CD3<sup>+</sup> CD4<sup>+</sup> T lymphocytes, and CD3<sup>+</sup> CD8<sup>+</sup> T lymphocytes ( $P = 0.02$ , 0.002, and 0.004, respectively; Fig. 4F). Interestingly, in vivo EP also led to dramatic 45-fold increases in CD3<sup>-</sup> CD19<sup>-</sup> CD11b<sup>+</sup> CD11c<sup>-</sup>

macrophages and 77-fold increases in CD3<sup>-</sup> CD19<sup>-</sup> CD11c<sup>+</sup> DCs compared to DNA vaccination alone ( $P = 0.006$  and 0.007, respectively; Fig. 4G and H), although the absolute number of recruited professional APCs was lower than the number of recruited lymphocytes and acute inflammatory cells. In vivo EP also led to larger DC infiltrates compared to plas-

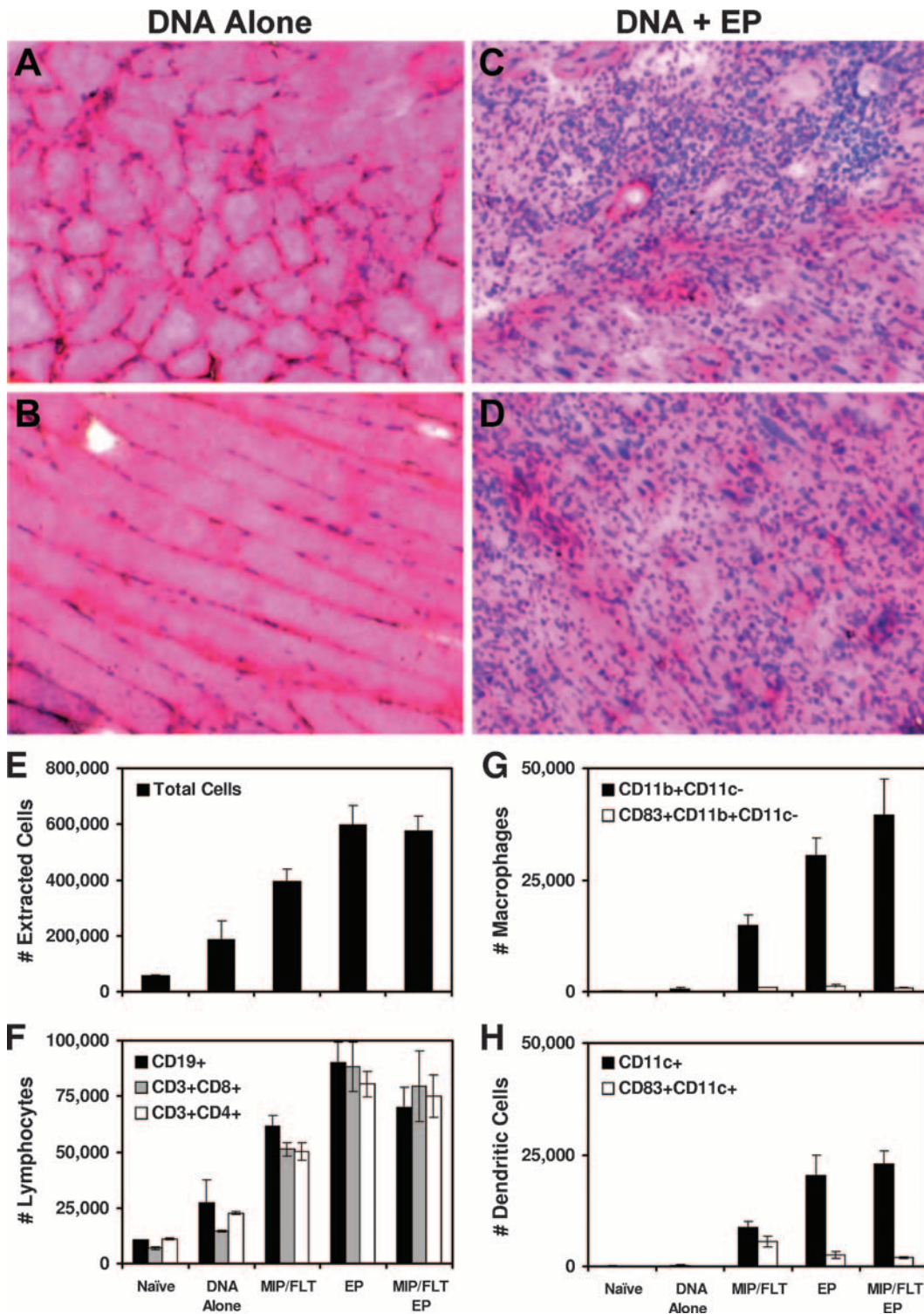


FIG. 4. Cellular infiltrates after DNA vaccination with in vivo EP. Groups of BALB/c mice (four animals/group) were immunized with 50 µg of gp120 DNA vaccine alone (A and B) or with in vivo EP (C and D). Cellular infiltrates were assessed by hematoxylin and eosin staining of 5-µm transverse (A and C) and longitudinal (B and D) sections of injected muscle harvested on day 7 after immunization. Representative sections are shown. Cells were then extracted from muscles injected with the various regimens and assessed by flow cytometry to determine the numbers of total cells (E), CD19<sup>+</sup> and CD3<sup>+</sup> lymphocytes (F), CD3<sup>-</sup> CD19<sup>-</sup> CD11b<sup>+</sup> CD11c<sup>-</sup> macrophages (G), and CD3<sup>-</sup> CD19<sup>-</sup> CD11c<sup>+</sup> DCs (H).

mid MIP-1 $\alpha$ /Flt3L, although a smaller percentage of DCs recruited by in vivo EP expressed the activation marker CD83 (Fig. 4H). The infiltrates observed with the combination of plasmid MIP-1 $\alpha$ /Flt3L and EP, however, were only marginally greater than those induced by EP alone. These data are consistent with the lack of substantial immunologic synergy observed with these two adjuvant modalities (Fig. 3). No differences in cell populations were observed in blood, draining lymph nodes, and spleen in mice after DNA vaccination with or without in vivo EP (data not shown), suggesting that the mechanism of in vivo EP likely involves local rather than systemic factors. The cellular inflammatory infiltrates in all groups proved transient and were largely resolved by day 14 after immunization (data not shown).

After DNA vaccination in the absence of adjuvants, antigen is primarily expressed in muscle tissue (35). The typical paucity of professional APCs at the site of inoculation and antigen production may therefore represent a critical limitation of DNA vaccines. Our data demonstrate that in vivo EP recruited large mixed cellular infiltrates that contained a proportionally concentrated number of APCs to the site of inoculation. It is likely that the increased numbers of APCs at the site of antigen production contributed substantially to the enhanced immunogenicity of the DNA vaccine. Consistent with this model, the lack of synergy between plasmid chemokines and in vivo EP may reflect their similar mechanisms of action. It is also possible that local cellular inflammation after in vivo EP may have provided an improved cytokine milieu that favored antigen presentation and immune priming.

In vivo EP significantly enhanced the magnitude and durability of DNA vaccine-elicited cellular and humoral immune responses. Qualitative aspects of CD8<sup>+</sup> T-lymphocyte responses elicited by DNA vaccination with or without in vivo EP, however, appeared similar in terms of cytokine secretion profiles (Fig. 1C) and memory phenotypes (Fig. 3C to F). Our data extend previous studies by evaluating the durability and phenotype of CD8<sup>+</sup> T-lymphocyte responses, as well as the extent and nature of cellular inflammatory infiltrates after DNA vaccination with in vivo EP. We propose that in vivo EP may function by a combination of mechanisms, including not only recruitment of APCs but also improved transfection of cells and increased antigen expression as previously reported (2, 14–16, 25, 28, 34). The potency of in vivo EP thus may reflect its capacity to facilitate multiple steps of the immune priming process.

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