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Specific Immune Induction Following DNA-Based Immunization Through In Vivo Transfection and Activation of Macrophages/Antigen-Presenting Cells

Michael A. Chattergoon, Tara M. Robinson, Jean D. Boyer, and David B. Weiner

The initiation of an adaptive immune response requires Ag presentation in combination with the appropriate activation signals. Classically, Ag presentation and immune activation occur in the lymph node and spleen, where a favorable organ architecture and rich cellular help can enhance the process. Recently, several investigators have reported the use of DNA expression cassettes to elicit cellular and humoral immunity against diverse pathogens. Although the immune mechanisms involved are still poorly understood, plasmid inoculation represents a model system for studying immune function in response to invading pathogens. In this report, we demonstrate the presence of activated macrophages or dendritic cells in the blood lymphocyte pool and peripheral tissues of animals inoculated with DNA expression cassettes. These cells are directly transfected in vivo, present Ag, and display the surface proteins CD80 and CD86. Our studies indicate that these cells function as APC and can activate naive T lymphocytes. They may represent an important first step APC in genetic immunization and natural infection.

Intramuscular injection of DNA expression cassettes leads to the in vivo expression of encoded proteins and has been shown to induce specific cellular and humoral immune responses (1–4). The intracellular expression of plasmids leads to the production of biologically active proteins that can be secreted and presented to B cells, facilitating the induction of an Ab response. Secreted proteins may also be ingested by professional APC, degraded within cellular endosomes, and expressed on MHC class II molecules. Transport of these peptide-MHC complexes to the cell surface facilitates Ag presentation to Th cells. The Th cell response and subsequent production of cytokines are critical steps in establishing an immune response. Additionally, finite quantities of all intracellularly produced proteins, including those encoded by DNA expression cassettes, are proteolysed endogenously and the resultant peptides loaded onto newly synthesized MHC class I molecules. Transport of these complexes to the cell surface is needed for the induction of a CTL response.

While potential pathogens may enter an animal through multiple routes, the central tenets of immunology support the induction of an immune response only at the secondary lymphoid tissues. Their architecture, location, and ability to provide the cytokines and co-stimulatory signals needed to activate lymphocytes seem to suggest the secondary lymphoid organs as the most appropriate site for recognition and presentation of Ag. It is generally accepted that the majority of Ag in the blood is processed for Ag presentation in the spleen, and Ags in tissue are transported and then processed and presented in the lymph nodes (LNs). However, studies with DNA expression cassettes indicate that transfection and protein expression are primarily confined to the peripheral tissue. This raises the question of whether these transfected cells also have the ability to prime T lymphocytes (5, 6). Recent experiments suggest that T lymphocyte induction and activation in the context of MHC I is not restricted by transfected peripheral cells but rather by resident bone marrow-derived APC (7, 8). Thus, while transfected somatic tissues may serve as reservoirs of Ag, the initiation of T lymphocyte responses occurs only after the transfer of Ag to professional bone marrow-derived APC. Early results indicate that the activation of CD8+ T cells depends on these bone marrow-derived professional APC.

The APC involved in the induction of the immune response following DNA immunization has also been the focus of recent attention. It seems appropriate that the induced immune response would be mediated by transfected APC at the site of inoculation. Indeed, the migration of transfected skin dendritic cells (DC) to the LNs following cutaneous delivery of plasmid delivery into the skin was reported recently (9). However, the contribution of this small number of transfected cells in immune induction is unclear. The ability of DC to prime immune responses after the direct administration of retroviral vectors using a variety of Ag systems was only recently demonstrated (10). DC might also prime immune responses following i.m. plasmid delivery; however, the relatively low ratio of DC in muscle compared with skin may limit this mechanism as the major mechanism for immune activation following i.m. DNA inoculation.

We have defined relationships between the in vivo transfected cells following direct i.m. inoculation and immune induction. We examined the kinetics of immune activation following delivery of DNA expression cassettes. The observed kinetics were correlated with the expression and distribution of cell surface proteins. Particular cell surface proteins whose changes on T lymphocytes and APC in the peripheral blood appear to correlate with the kinetics of

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2 Address correspondence and reprint requests to Dr. David Weiner, Department of Pathology and Laboratory Medicine, 505 Stellar-Chance, 422 Curie Blvd., University of Pennsylvania, Philadelphia, PA 19104.
3 Abbreviations used in this paper: LNs, lymph node; DC, dendritic cells, Pr55, protein 55; Pr24, protein 24; GFP, green fluorescent protein.
DNA-BASED IMMUNIZATION THROUGH MACROPHAGE ACTIVATION

Flow cytometry

With PBS and incubated on plates for 1 h at 37°C. The plates were washed twice with PBS-0.05% Tween 20 and blocked with 5% nonfat dry milk (Aldrich). 50 μl of recombinant Pr55 (Quality Biologic, Gaithersburg, VA) MR5000 spectrophotometer.

Immunohistochemistry and immunofluorescence of tissue samples

Protein expression was analyzed using the pcGag/Pol DNA expression cassette encoding the HIV-1 core protein 55 (Pr55) and protease enzyme under CMV promoter control (11). It has been shown that transfection of COS7 cells with this plasmid leads to the production and secretion of the cleavage product, protein 24 (Pr24). The cellular events associated with immune activation were studied with the DNA expression cassette pcEnv, encoding the HIV-1 gp160 envelope protein under CMV promoter control (3). Controls for these experiments included inoculations of 1) normal saline, 2) bupivacaine solution, and 3) vector control. Localization of plasmid and studies of tissue distribution were accomplished with the pcGFP-C1 plasmid (Clontech, Palo Alto, CA). This plasmid encodes a red-shifted mutant green fluorescent protein (GFP) with peak excitation at 488 nm.

Detection of protein expression in vivo

Protein expression was tested by ELISA. Mice were immunized with pcGag/Pol as described; animals were bled and antisera collected and pooled. One hundred microliters of serum and serum dilutions of 1:10, 1:50, and 1:100 were analyzed by capture ELISA using the Coulter HIV-1 Pr24 Ag Assay (Coulter, Miami, FL).

Detection of Ab titers

Animals were inoculated and antisera collected as described previously. Fifteen microliters of recombinant Pr55 (Quality Biologic, Gaithersburg, MD) were diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.5) to a final concentration of 2 μg/ml and absorbed for 1 h at room temperature onto 96-well microrider protein binding plates. The plates were washed twice with PBS-0.05% Tween 20 and blocked with 5% nonfat dry milk, 0.05% Tween 20 in PBS for 1 h at 37°C. Muscle antisera was diluted with PBS and incubated on plates for 1 h at 37°C. The plates were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO), and then washed and developed with 3,3′,5′-TMB solution. OD at 450 nm was read on a Dynatech (Chantilly, VA) MR5000 spectrophotometer.

Flow cytometry

Animals were inoculated as previously described. At the time of harvest, mice were anesthetized with an i.p. injection of 300 μL AVERTIN (5 g 2–2–2 Tribromoethanol [Aldrich, Milwaukee WI] in 5 ml tert-amyl-alcohol [Aldrich], 50× solution). Peripheral blood was collected by cardiocentesis into heparinized blood collection tubes (20 U/ml). Following blood collection, the right inguinal LN was removed and spleen harvested. Spleens and LNs were crushed in a tissue shredder to remove excess tissue and release cells. For flow cytometry analysis, PBMC were isolated from blood by washing three to four times in 1× Geys solution to remove erythrocytes. Following isolation, samples were washed twice in FACS buffer (PBS-1% BSA, 0.1% NaN₃), and then blocked for 15 min at 4°C in FACS buffer supplemented with 10% goat serum (Sigma). Cells were then resuspended to a concentration of 5 × 10⁴ cells/ml, and 100 μL of cells were incubated with Ab combinations for 1 h at 4°C. Unbound Ab was removed by washing in FACS buffer. Samples were analyzed using a Coulter EPICS XL-MCL flow cytometer. Abs specific to the following cell surface proteins were used in the analysis: CD4 and CD8 (Sigma), B220 (12), MAC-3 (13), MAC-1 (14), Ly–49C (15), CD28 (16), CTLA-4 (17), CD80 and CD86 (18), and CD40 and CD40L (19) (PharMingen, San Diego, CA).

Sorting and immunofluorescence of PBMC

PBMC were collected and treated as described in FACS assays. Sodium azide was excluded from buffers to facilitate cell culture after sorting. After incubation with appropriate Abs, cells were washed and resuspended in RPMI 1640 and 5% FBS growth media. Cells were sorted on Coulter EPICS ELITE and collected in RPMI 1640 and 50% FBS. After sorting, cells were allowed to sit for 30 min, and then pelleted and resuspended in RPMI 1640 and 50% FBS. Cells were placed into chamber slides and photographed with appropriate red and green filters on a Nikon Diaphot microscope.

Immunohistochemistry and immunofluorescence of tissue samples

Muscle samples from the quadriceps at the DNA inoculation site, inguinal LNs, and spleen were removed for immunohistochemistry and immunofluorescence. Samples were fixed in PBS-2% paraformaldehyde for 4 h, washed in 1× PBS, and incubated in PBS-5% sucrose for 2 h. Samples were cryoprotected in PBS-20% sucrose, shock frozen, and sectioned. Tissue sections were incubated with biotinylated Abs specific to mouse CD80 and CD86 at 1 μg/10⁶ cells (PharMingen). Slides were then incubated with Avidin–Texas Red (Vector, Burlingame, CA) for 1 h at 4°C and photographed.

Th cell proliferation assay

Lymphocytes were harvested as previously described. The isolated splenocytes were then resuspended to a concentration of 1 × 10⁶ cells/ml. A 100-μl aliquot containing 1 × 10⁶ cells was immediately added to each well of a 96-well microrider round-bottom plate. Recombinant Pr55 at a final concentration of 5 μg/ml and 0.5 μg/ml was added to wells in triplicate. The cells were incubated at 37°C in 5% CO₂ for 3 days. One micromole of [³H]thymidine was added to each well and the cells were incubated for 12 to 18 h at 37°C. The plate was harvested and the amount of incorporated tritiated thymidine was measured in a β Plate reader (Wallac, Turku, Finland). PHA stimulation was used in this experiment as a polyclonal activator and positive control.

Analysis of APC activity in macrophages

To determine the ability of macrophages to stimulate T lymphocytes in vitro, groups of mice inoculated with pcEnv and plasmid control were compared. Macrophages were isolated by FACS using Abs specific to the Mac-1 and Mac-3 Ag from both groups of mice 14 days postinoculation. Concurrently, lymphocytes were separated from the spleen of naive mice and naive T lymphocytes further isolated by panning with mouse IgG-specific Ab (Sigma). The efficiency of T lymphocyte separation was tested by flow cytometry using a mAb to CD3 (Sigma). Naive T lymphocytes and macrophages were incubated at ratios of 10:1, 4:1, and 2:1. Cells were harvested after 3 days of stimulation, stained, and analyzed by flow cytometry. Macrophages and T lymphocytes were incubated together for 1 day, followed by the addition of recombinant protein for 3 days in a proliferation experiment. Cells were harvested and analyzed as previously described in a Th cell proliferation assay.

Results

The relationship between protein Ag expression and immune induction by DNA vaccines is not yet clear. Accordingly, we evaluated the relationship between Ag expression and the development of humoral and cellular immune responses.

Protein expression

The kinetics of protein expression following inoculation with pcGag/Pol was tested by ELISA. Mice were immunized with 50
μg of pcGag/Pol plasmid DNA and plasmid control as described in Materials and Methods. Serum was collected on days 0–6, 9, 14, and 30 postinoculation and tested for the presence of free Pr24 (HIV-1 core proteins). Protein expression followed the kinetics usually seen during in vitro transfection. Following a short lag phase, Pr24 was detected in the serum of mice 2 days after inoculation. Protein levels increased, and then peaked at day 5 to 6; levels then decreased slowly in a roughly exponential manner (Fig. 1A). Pr24 could not be detected in the serum of naive and plasmid control mice; these groups showed little reactivity throughout the study.

Humoral immune response

Humoral immune responses were detectable in the experimental animals following plasmid delivery. Mice inoculated with pcGag/Pol DNA expression cassettes developed observable Pr24-specific Abs as early as 7 days after a single inoculation. The Ab response continued to rise through day 30 (Fig. 1B). The rise in the anti-Pr24 Ab level accompanied a concurrent fall in free Pr24 levels detectable in the serum of the immunized mice, suggesting that the immune response may have inhibited our ability to detect Pr24 in the serum. The control mice did not develop humoral response to Pr24 during the study.

Th response

T lymphocyte proliferation responses were also analyzed in animals that received the pcGag/Pol plasmid DNA. Mice were inoculated and spleens harvested every day for 1 wk, then at 2 and 4 wk postinoculation. T lymphocytes incubated in a mixed lymphocyte reaction and stimulated with recombinant Pr55 show increased thymidine incorporation compared with cells stimulated with an irrelevant Ag (data not shown) or the cells similarly stimulated from naive and plasmid control mice (Fig. 1C). The results of the T lymphocyte proliferation assay indicated that the cellular response follows a pattern similar to that of protein expression with a lag of some 8 to 9 days. Proliferation was not observed in the first 4 days postinoculation, increased on day 5, and reached a maximum on day 14 postinoculation. The levels of thymidine incorporation after Pr55 stimulation returned to normal levels by day 30, and was indistinguishable from control animals shortly thereafter. Control mice groups showed no response to Pr24 stimulation throughout the study. These results suggest that in vivo expression is transient and the duration of expression may have implications for immune boosting.

Screening of PBMC and splenocytes

To further characterize immune activation, splenocytes and PBMC were surveyed for changes in the expression of activation markers following inoculation with DNA expression cassettes. Multicolor FACS analysis with Abs to distinguish cell subtype and combinations of Abs to known immune activation markers was performed. In initial experiments animals (n = 5) were inoculated with 50 μg of pcEnv as described in Materials and Methods. Splenocytes and PBMC were examined for changes in cell surface markers 14 days postinoculation; mice were analyzed individually and the results averaged to determine the mean change.

Changes in the expression of activation markers are indexed by cell type and summarized in Table I. On T lymphocytes we examined CD28, a costimulatory signal and ligand for CD80 and CD86; CD40-L, the natural ligand for CD40; and a signal of T lymphocyte activation. CTLA-4, found on activated T cells and involved in the down-regulation of T cells, was also followed. An up-regulation of CD28, CD40-L, and CTLA-4 activation signals on both the CD4+ and CD8+ T cells was observed. Macrophages in the peripheral blood showed increases in CD80 and CD86 expression suggesting a relationship with APC ability. Furthermore, the numbers of macrophages now expressing the costimulatory ligands represents a significant percentage of the total macrophage pool (20). B cells and NK isolated from the PBMC showed very small changes in the activation markers examined.

The changes in T cells and macrophages observed in PBMC were not observed among the splenocytes. To ensure that small changes in spleen T cells and macrophages were not obscured by
the large B cell population in the spleen (21), additional experiments with purified CD4⁺ and CD8⁺ T cells were done. With T cells isolated from the spleen of pcEnv-immunized mice, the result of our survey did not change. Among splenocytes, only B cells show some activation in CD40 expression with changes of 5% or more in comparison to the control groups (Table I).

As described in Materials and Methods, there were multiple controls to these experiments, the most significant of which is the vector control. In the saline and bupivicaine control groups, there were minimal changes at various time points. The vector control group was very similar to the previous control groups except for some time points, where a 1 to 3% deviation from the saline control was observed.
Analysis of cell activation

Based on the changes noted in our initial survey, a more extensive analysis of T lymphocytes and macrophages was designed to illustrate the relationship between the induced immune response and changes in activation at the cellular level. Animals receiving 50 μg of pcEnv and vector control were divided into three groups (n = 6) and analyzed at 3, 14, and 28 days postinoculation. PBMC and splenocyte samples were collected, and analyzed by FACS as described in Materials and Methods. Following pcEnv immunization, an increase in the percentage of CD3^+ cells was observed at day 14 in the spleen and PBMC (data not shown). Concurrent with the increase in CD3^+ cells, macrophages in the spleen and peripheral blood

FIGURE 3. FACS analysis of T lymphocyte subsets. The expression of CD28 on peripheral blood CD4^+ and CD8^+ cells from plasmid control and pcEnv immunized was analyzed. CD4/CD28-positive T cells show small increases as early as 3 days (D) postinoculation and peak 14 days after inoculation (E) in pcEnv-immunized mice. Expression of CD4 returns to pre-inoculation levels (not shown; similar to day 3) 28 days after inoculation (F). CD8/CD28-positive T cells were also monitored in mice receiving DNA expression cassettes. Mice inoculated with the pcEnv plasmid showed increased expression of CD28 on CD8 T cells at day 14 (K) compared with pre-immunization levels, day 3 (J) and day 28 (L) after inoculation. Control plasmid mice did not show changes over the course of the study. Day 3 CD4 (A), day 14 CD4 (B), and day 28 CD4 (C) show little change throughout the experiment. Similarly, CD8 control groups day 3 (G), day 14 (H), and day 28 (I) showed little change through the observation period.
The most dramatic population changes were observed in the PBMC. The changes in the CD8/CTLA-4 population were less dramatic. There were minimal changes in the CD4/CTLA-4 in the PBMC. The changes in the splenocyte population were less dramatic. There were minimal changes in the number of CD8/CD28-, CD4/CD28-, CD8/CTLA-4-, and CD4/CTLA-4-positive cells show a DC morphology. The frequency of green loci was calculated for mice inoculated with GFP. In general only the LN proximal to the site of inoculation showed significant GFP signal (Fig. 5B). Both experimental and control plasmid mice LN show areas of background CD80 and CD86 expression not associated with green loci (Fig. 5A). This is in agreement with previous reports of CD80 and CD86 expression in LN and spleen (22, 23).

Identification of DNA-transfected APCs

GFP encoding vectors were used to determine whether the observed activation of macrophages was due to the direct transfection of cells in vivo or due to endocytosis of secreted GFP. Intramuscular inoculation of the pEGFP-C1 plasmid leads to in vivo transfection and production of the GFP, which is detected by conventional fluorescence microscopy. In the muscle, expression of GFP follows the time course of expression observed for Pr24. The GFP expression vector was used as an immunogen with Abs to detect activation markers expressed on transfected PBMC. GFP expression can be seen in myocytes 3 to 4 days postinoculation (data not shown) and is still present in isolated muscle fibers at day 14. No expression of CD80 or CD86 was observed on muscle fibers stained with specific Ab 14 days after inoculation (Fig. 4). Samples from plasmid control groups showed no GFP expression or CD80 or CD86 staining.

Three to four days following immunization, very few scattered individual cells with GFP expression can also be seen in the spleen (data not shown); however, expression in spleen is undetectable 14 days after inoculation. Staining for CD80 and CD86 shows no coexpression of these proteins on the surface of these cells. In contrast to these results, GFP expression is detectable in the proximal LN 14 days (Fig. 5A) postinoculation. Within the proximal LN, but not in LN taken from plasmid control mice, transfected cells are arranged in small clusters in approximately one to two areas per LN. These transfected cells showed lower levels of CD80 expression and relatively high levels of CD86 expression (Fig. 5A). Many of these cells are macrophages; however, some B7-2-positive cells show a DC morphology. The frequency of green loci was calculated for mice inoculated with GFP. In general only the LN proximal to the site of inoculation showed significant GFP signal (Fig. 5B). Both experimental and control plasmid mice LN show areas of background CD80 and CD86 expression not associated with green loci (Fig. 5A). This is in agreement with previous reports of CD80 and CD86 expression in LN and spleen (22, 23).

Macrophages were sorted from the pooled splenocytes and PBMC of animals immunized with 50 μg of pEGFP-C1 and control plasmid 14 days after inoculation. These cells were analyzed by immunofluorescence microscopy and transfected macrophages photographed. Among cells sorted from the spleen, no green cells were found by immunofluorescence, indicating a lack of transfected cells in the spleen. In contrast, there were transfected/green cells observed in the peripheral blood (Fig. 6). The cells were then restained and analyzed with anti-CD80 (PharMingen) and anti-CD86 (PharMingen) Ab. Importantly, these transfected macrophages from the peripheral blood compartment were found to express CD86.

Analysis of macrophages as APCs

Based on the evidence that there were transfected circulating activated macrophages with Ag-presenting capability, we tested the ability of macrophages separated from pcEnv-immunized mice to activate naïve T lymphocytes. Purified naïve T lymphocytes were incubated with macrophages separated from pcEnv, naive, and plasmid control mice. Inactivation was measured by FACS and proliferation assays. Activation was evident early in T lymphocytes stimulated with macrophages from immunized mice. An increase in expression of CD69 (Very Early Activation Ag) on CD4+ cells, and to a lesser extent on CD8+ T lymphocytes, indicates the macrophage capacity to present Ag following DNA immunization (Fig. 7A). The Very Early Activation Ag molecule (CD69) is found at very low levels on all lymphocytes; enhanced expression is known to occur within hours of in vivo and in vitro activation of T lymphocytes (24). CD28 expression, which was unchanged at day 1, was observed to increase primarily on CD4+
FIGURE 5. A. Ag expression in the LN. The inguinal LNs proximal and isolateral to the site of injection were harvested, sectioned, and photographed from mice 14 days after inoculation with pEGFP-C1 plasmid saline, bupivacaine, and control plasmid. Control plasmid mice show no GFP expression (data not shown). Cells expressing GFP can be seen in the proximal inguinal LN after inoculation with pEGFP-C1 (B and D) (×100). Immunofluorescence costaining with mAb to CD80 (A) and CD86 (C) shows colocalized B7 and GFP expression. All control mice were negative for GFP expression. B. GFP localization in the LN following DNA inoculation. The inguinal LNs proximal and isolateral to the site of injection were harvested, sectioned, and scored from mice 14 days after inoculation with pEGFP-C1 plasmid, plasmid control, and saline control mice. LN were sectioned to 5-μm thickness. Data shown indicate the result of four randomly chosen section planes.
T lymphocytes at day 3 (Fig. 7B). However, by day 3, CD69 expression had returned to preimmunization levels.

The functional relevance of these activated macrophages was further evaluated in a T lymphocyte proliferation assay. Here, naive T lymphocytes primed by activated macrophages derived from animals immunized with pEnv showed significant incorporation of thymidine in response to incubation with recombinant gp120, while macrophages derived from plasmid control mice did not show this result (Fig. 8) (naive mice not shown). Polyclonal activation with PHA was used as a positive control in these assays (data not shown). These data demonstrate that transfected APC, which are activated and expressing increased levels of B7,2, can be important primary APC in immune activation following DNA vaccination. It is important to note that activation was only noted at a 2:1 ratio of T lymphocytes to macrophages, indicating the very small number of transfected macrophages obtained after immunization.

Discussion

Genetic immunization is an attractive method for inducing adaptive immunity

The induction of humoral and cell-mediated immune responses has been demonstrated following the delivery of plasmid DNA, through a number of routes, and via multiple delivery systems (1, 2, 11, 25, 26). Genetic immunization offers several real and conceptual advantages over traditional vaccination strategies, including rapid construction and testing of vaccines. The technique of DNA immunization is likely safer than other live vaccination strategies. DNA expression cassettes pose little threat to the immunocompromised host. No significant immune response against the expression cassette has been observed, and pathogenic or immunomodulatory elements can be removed from the plasmid, further enhancing vector safety (27).

Currently, our understanding of immune activation is incomplete. In this regard, understanding immune activation following DNA immunization may provide insight into immune mechanisms. This understanding may also aid in vaccine and immune therapeutic design. Recently, several groups have begun to investigate the mechanism(s) by which DNA expression cassettes induce immune responses. Early results indicate that this pathway is more complex than originally thought (7, 8). Here we have investigated the changes in surface proteins related to immune activation following plasmid DNA immunization in an effort to define the events associated with immune activation and T lymphocyte priming.

The kinetics of immune activation following plasmid inoculation indicate that protein expression precedes and drives immune activation (Fig. 1A). The lag time observed between plasmid delivery and protein detection in the serum distinguishes the time of in vivo infection from translation and secretion of encoded plasmid proteins. The humoral immune response further lagged behind protein expression by several days (Fig. 1B). This is not surprising as it is not unreasonable to expect that some minimum protein concentration is needed to surpass the activation threshold of B cells and develop a functional Th cell response. T cell proliferation after inoculation was present following Ag expression. Following transfection and Ag expression, specific T lymphocyte activation occurs (Fig. 1C), as evidenced by T cell proliferation. The activation of CD4+ T lymphocytes peaks and falls in a pattern similar to the expression of Ag and coincident with the induction of Ab responses.

It is noteworthy that the Ab response did not diminish over the short observation period of this study. In studies with other vectors (data not shown), the Ab response persisted for several months. This is in agreement with the results of several authors that have found persistent Ab responses following DNA inoculation (2). CD8+ CTL was not analyzed in this study; previous reports have indicated that CTL activity is very low after one inoculation but appears 14 days following a boost, suggesting that the sensitivity of the assay would limit this initial study (28).

These results indicate that T lymphocytes help both humoral and cellular responses, and activation appears to be coordinated with protein expression. We further investigated specific T cell activation: the T cell subsets were studied in more detail by following the activation markers and costimulatory molecules on T lymphocytes. The roles of the CD28 and CTLA-4 (CD152) proteins have recently become the focus of much investigation (29). Additionally, the role of CD40-L, as another costimulatory molecule to T lymphocytes has recently received attention (30). The expression of the costimulatory molecule CD28, both by mean fluorescence and percent positive cells, increased in both the CD4+ and CD8+ T cell populations following DNA inoculation (Fig. 3). Our results indicate that DNA immunization 1) increases the number of circulating T lymphocytes expressing the CD28 molecule, and 2) increases expression of CD28 in both T lymphocyte subsets. The elevation of CD28 expression has been linked to a stronger immune response, and the development of resistance to HIV infection in human cells (31). Furthermore, in experiments performed by our group, the development of a protective immune response to HIV-1 correlated with an increase in CD28 expression in the PBMC of immunized chimpanzees (11). The correlation of CD28 expression and induced immune responses indicates that this may be an important early predictor of the effectiveness of a particular immunization cassette.

Plasmid DNA also induced an increased expression of CTLA-4 and CD40-L. CTLA-4 is known to be transiently expressed in T lymphocytes following immune activation and is thought to provide a down-modulatory signal to T lymphocytes.
It is a powerful inhibitor of T cells. CTLA-4 knockout mice exhibit lymphoproliferative disorders and develop fatal spontaneous autoimmune disease (33). The increased expression of CTLA-4 followed the same time course as CD28. It is possible that this competing ligand is induced to control the active T cell or to suppress the threshold activation of other T cell clones. More extensive analysis is needed to determine this.

Another important finding clearly observed here is that the activation of T cells by plasmid DNA inoculation is an inducible and transient phenomenon. The activation of T cells requires specific TCR-MHC complexes in addition to a costimulatory signal provided by the B7 family of molecules. T lymphocytes costimulated by B7 through the CD28 receptor and TCR complex in the absence of other stimulatory signals may up-regulate pleiotropic immune response.

![Graph A](image1.png)

**Day 1 in vitro stimulation**
- Media Control
- Mφ from Saline control
- Mφ from plasmid control mice

**Day 3 in vitro stimulation**
- Media Control
- Mφ from Saline control
- Mφ from plasmid control mice

**Percent Positive CD69**
- Media Control
- Mφ from Saline control
- Mφ from plasmid control mice

![Graph B](image2.png)

**Day 1 in vitro stimulation**
- Media Control
- Mφ from Saline control
- Mφ from plasmid control mice

**Day 3 in vitro stimulation**
- Media Control
- Mφ from Saline control
- Mφ from plasmid control mice

**Percent Positive CD28**
- Media Control
- Mφ from Saline control
- Mφ from plasmid control mice
The earliest reports on DNA immunization showed that in vivo transfection and Ag presentation following gene delivery was mainly confined to the immediate somatic tissue (5, 35). However, our improved understanding of cytokine and costimulatory molecule distribution makes the muscle an unlikely primary APC. Muscle APC activity is limited to MHC I expression, and we have found no evidence of B7 expression on muscle cells (Fig. 5). The inefficiency of the muscle cell as APC was further demonstrated by Kim et al. (28). In an investigation of the codelivery of plasmids, it was found that CD86 codelivery and expression increased CD8\(^+\) T lymphocyte-restricted CTL responses to HIV-1 targets (28). In elegant studies by Doe et al. (7), the importance of a second APC in plasmid DNA-induced immune responses was further reinforced. Adoptive transfer experiments in which bone marrow cells from an F\(_1\), H\(_2^d\) and H\(_2^k\) hybrid mouse were transplanted into H\(_2^k\) mice, it was reported that CTL to H\(_2^k\)-restricted epitopes could be measured (7). These studies indicated the importance of a second bone marrow-derived APC in priming T lymphocyte responses. The identification of this second APC has become the focus of much attention (36). It is thought that this cell acts as an intermediary between the muscle and CD8\(^+\) T lymphocyte, engulfing Ag and presenting it on MHC-I molecules, facilitating T lymphocyte priming. The mechanism by which immunogens expressed by transfected myocytes are processed and presented as a peptide-MHC I complex has not been fully elucidated.

The direct transfection of professional APC is another method by which Ags encoded by plasmid DNA could be presented on MHC I molecules and prime CD8\(^+\) T lymphocytes. We have observed plasmid in the muscle, LN, peripheral blood, and spleen following i.m. inoculation of a GFP-expressing plasmid. These transfected cells showed diffuse cytoplasmic GFP signals, consistent with intracellular GFP expression as opposed to a localized vesicle pattern that would suggest phagocytosis of secreted GFP. GFP localization in the LN and spleen could be the result of direct transfection or the trafficking of transfected cells into these organs. In genetic immunization, very short half-life of extracellular DNA in lymph and blood makes it unlikely that plasmids directly transfact the LN or spleen in significant numbers (37). However, studies of DNA elimination kinetics in the thyroid (38) and synovial fluid (39) indicate that plasmid may in general be long lived in tissue and interstitial fluids with a t\(_{1/2}\) of 10 to 40 h. The longevity of DNA expression cassettes in the tissue increases the likelihood that cells are transfected at the site of inoculation, and then these transfected cells travel to other organs (Fig. 9). It seems most likely that the localization of plasmid in the LN and spleen would be the result of immigrating transfected cells.

The migration of transfected DC to the LN following DNA immunization in the skin was recently shown (9). Transfected skin-derived DC can be detected in the LN 24 h after immunization. While transfected DC may be important in i.m. inoculation, the density of DC in muscle is expected to be lower than in skin. We have also observed transfected cells in the regional LN 14 days following i.m. immunization (Fig. 5). The majority of these cells have been identified as macrophages; they are transfected, and these small clusters of transfected cells express the B7 ligands necessary for T cell activation. Based on morphology, a smaller number of transfected cells may be DC’s. Our result suggests that transfected macrophages at the site of immunization migrate to the LN following i.m. inoculation and may be an important APC, possibly playing a role similar to the bone marrow APC.

Macrophages express both MHC class I and II and the costimulatory ligands for T lymphocyte activation. In our experiments, transfected macrophages have the necessary capabilities to prime both Ab- and cell-mediated immune responses. The transfection of macrophages is itself an interesting observation: the lack of transfected lymphocytes in the pool of cells exposed to plasmid DNA suggests that macrophages may have a specific receptor for free nucleic acids. Ligation of this receptor may lead to transport of DNA across the cell membrane and activation of the cell (40). Surprisingly, the DNA is not degraded in this process and in the case of plasmid DNA may be expressed. Selective DNA uptake may be a natural scavenger function of
macrophages. The macrophage would be protected in the process, since either the free DNA would be too small to be expressed, or transcription of DNA from cells leads to innocuous self-protein production. Bacterial plasmids may use this mechanism to gain entry of DNA into cells. However, because they lack mammalian promoters, they would normally not be expressed. The observation of specific nucleic acid uptake by macrophages would on the surface appear counterintuitive and will certainly require additional investigation. The finding of capable APCs in the peripheral compartment was also intriguing and raises questions of possible peripheral T cell activation. The peripheral activated APC would form a formidable barrier to further pathogen invasion from the periphery.

There are similarities between DNA immunization and other traditional live vaccine strategies. However, there are several unique differences, among which is the extended period of plasmid survival in transfected cells and selective uptake of nucleic acid by APC. The traditional centers of Ag presentation appear to be important for immune activation; however, with relatively little plasmid localizing in the regional LN questions of the efficacy and efficiency of this location arise. Recently, the method or pathway by which DNA expression cassettes induce immune responses has been the focus of many authors’ studies. It is hoped that with a clearer understanding of the cells involved in this process, more directed and possibly more potent vaccines and therapeutics may be developed.

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References