

CD86 (B7-2) Can Function to Drive MHC-Restricted Antigen-Specific CTL Responses In Vivo¹

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Activation of T cells requires both TCR-specific ligation by direct contact with peptide Ag-MHC complexes and coligation of the B7 family of ligands through CD28/CTLA-4 on the T cell surface. We recently reported that coadministration of CD86 cDNA along with DNA encoding HIV-1 Ags i.m. dramatically increased Ag-specific CTL responses. We investigated whether the bone marrow-derived professional APCs or muscle cells were responsible for the enhancement of CTL responses following CD86 coadministration. Accordingly, we analyzed CTL induction in bone marrow chimeras. These chimeras are capable of generating functional viral-specific CTLs against vaccinia virus and therefore represent a useful model system to study APC/T cell function in vivo. In vaccinated chimeras, we observed that only CD86 + Ag + MHC class I results in 1) detectable CTLs following in vitro restimulation, 2) detectable direct CTLs, 3) enhanced IFN- γ production in an Ag-specific manner, and 4) dramatic tissue invasion of T cells. These results support that CD86 plays a central role in CTL induction in vivo, enabling non-bone marrow-derived cells to prime CTLs, a property previously associated solely with bone marrow-derived APCs. *The Journal of Immunology*, 1999, 162: 3417–3427.

The generation of the T cell immune response is a complex process that requires the engagement of T cells with professional APCs, such as dendritic cells, macrophages, and B cells. These professional APCs possess large surface areas for interaction with T cells. They also express high levels of MHC class I and II molecules, adhesion molecules, and costimulatory molecules, and produce cytokines that are critical for efficient Ag presentation and T cell activation. Professional APCs initiate T cell activation through binding of antigenic peptide-MHC complexes to specific TCR molecules. In addition, the APCs provide critical secondary signals to T cells through the ligation of the B7 costimulatory molecules with their receptors (CD28/CTLA-4) present on T cells. These costimulatory signals are required for the clonal expansion and differentiation of T cells. The blocking of this additional costimulatory signal leads to T cell anergy (1). Among different costimulatory molecules, CD80 and CD86 have been observed to provide potent immune signals (2, 3). The CD80 and CD86 molecules are surface glycoproteins and are members of the Ig superfamily that are expressed only on professional APCs (2–4). Although both CD80 and CD86 molecules interact with either

CD28 or CTLA-4 molecules on T cells, CD80 and CD86 expression seem to be differentially regulated. CD86 is constitutively expressed by APCs whereas CD80 is expressed only after activation of these cells (5–7). Thus, CD86 may be important in the early interactions between APCs and T cells during the induction phase of the immune response.

We recently reported that CD86 molecules play a prominent role in the Ag-specific induction of CD8⁺ CTL when delivered as vaccine adjuvants (8). Coadministration of CD86 cDNA along with DNA encoding HIV-1 Ags i.m. dramatically increased Ag-specific T cell responses without a significant change to the level of the humoral response. This enhancement of CTL response was both MHC class I restricted and CD8⁺ T cell dependent. Similar results have been obtained by other investigators who also found that CD86, not CD80, coexpression results in the enhancement of T cell-mediated immune responses (9, 10). Accordingly, we speculated that engineering of nonprofessional APCs, such as muscle cells, to express CD86 costimulatory molecules could empower them to prime CTL precursors. On the other hand, the enhancement effect of CD86 codelivery could also have been mediated through the direct transfection of a small number of professional APCs residing within the muscle tissue. Subsequently, these cells could have greater expression of costimulatory molecules and could in theory become more potent.

To investigate this issue, we constructed a set of bone marrow chimeric animals between normal mice and mice bearing a disrupted β_2 -microglobulin (β_2m)⁴ gene. These bone marrow chimeras could respond and develop functional CTL responses following immunization with vaccinia virus. Next, we immunized chimeric

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⁴ Abbreviations used in this paper: β_2m , β_2 -microglobulin; H&E, hematoxylin and eosin; pCEnv, plasmid encoding HIV-1_{MN} envelope protein; pCD80, plasmid encoding CD80 gene; pCD86, plasmid encoding CD86 gene; PCDNA3, control plasmid; WR, wild-type vaccinia virus; vMN462, recombinant vaccinia virus-expressing HIV-1 envelope protein.

animals with a DNA vaccine expressing HIV-1_{MN} envelope protein (pCEnv) and plasmids encoding CD80 or CD86 genes (pCD80 or pCD86). Using this model, we observed that in vivo transfection of only pCEnv and pCD86 could enable non-bone marrow-derived cells to prime and expand CTLs. This study suggests that CD86, and not CD80, plays a central role in the generation of Ag-specific CTL responses. This result has important implications for our understanding of the generation of a primary immune response.

Materials and Methods

Construction of β_2m knockout chimeric mice

Four-week-old female C57BL/6J ($\beta_2m^{+/+}$) and C57BL/6J-B2m^{tm1/Unc} ($\beta_2m^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Both $\beta_2m^{+/+}$ and $\beta_2m^{-/-}$ animals were used for reciprocal bone marrow transplant. The preparation of bone marrow chimeras has been previously described in detail (11). Briefly, recipient mice (both $\beta_2m^{+/+}$ and $\beta_2m^{-/-}$) were depleted of NK cells with i.p. injections of 200 μ g/ml of mAbs PK136 (anti-NK1.1) on day (-2) and (-1). This pretreatment prevents the rejection of bone marrow cells originating from C57BL/6J-B2m^{tm1/Unc} mice by radio-resistant NK cells in C57BL/6J mice (12). On the day of reciprocal bone marrow transplant, recipient mice were lethally irradiated with a total of 1050 rad given in two equally divided doses 3 h apart. Donor mice were sacrificed, and bone marrow was harvested separately by flushing tibias and femurs. Bone marrow cells were depleted of mature T cells by incubation (37°C, 1 h) of cells with Low-Tox-M rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) following incubation (4°C, 45 min) with saturating concentration of a mixture of mAbs anti-CD4 (172.4), anti-CD8 (31M), and anti-Thy1.2 (mmt 1). Recipient mice were reconstituted with reciprocal bone marrow cells with an i.v. injection of 10^7 cells (0.3 ml). All animals were housed in a temperature-controlled, light-cycled facility at the University of Pennsylvania, and their care was under the guidelines of the National Institutes of Health and the University of Pennsylvania.

Immunization of mice

DNA vaccine construct encoding for the HIV-1_{MN} envelope protein (pC-Env) was prepared as previously described (13, 14). CD80 and CD86 expression cassettes were prepared as we described (8). Each mouse received three i.m. injections (2 wk apart) with 50 μ g of each DNA construct of interest formulated in PBS and 0.25% bupivacaine-HCl (Sigma, St. Louis, MO) as described earlier (8, 15). Fifty micrograms of pCEnv administered in a regimen described above has been shown to induce moderate but positive immune responses in mice (8, 15). This dosage was selected to demonstrate the enhancement of immune responses with the codelivery of costimulatory genes. We also injected animals with recombinant vaccinia virus, which express HIV-1 envelope protein (vMN462) (National Institutes of Health AIDS Research and Reference Reagent Program). Mice were injected i.v. with vMN462 (5×10^6 plaque-forming units (PFU) per mouse). Seven days later, spleens were removed and used for detection of direct CTL assay. Mice were also analyzed for indirect CTL after 4 wk of immunization with the same dose of vMN462.

Flow cytometry

The generation of chimeric mice was confirmed by FACS analysis using mAbs to the $\alpha 3$ domain of H-2D^b molecule as previously described (15). One μ g/ml of mouse mAbs 28-14-8s (IgG2a isotype), which recognized $\alpha 3$ domain of H-2D^b molecule (courtesy of Dr. J. Frelinger, Chapel Hill, NC), was added to PBMC (10×10^5) isolated from individual mice. Data were analyzed by FACScan with CELLQuest data acquisition and software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Immunohistochemical assays on muscle cells

Immunized leg muscle was examined immunohistochemically for the in vivo expression of CD80, CD86, and envelope proteins as previously described (8, 15). Mouse quadriceps muscle was inoculated with 50 μ g of pCEnv + pCD80, pCEnv + pCD86, or control vector. Seven days following inoculation, the mice were sacrificed, and the quadriceps muscles were removed. The fresh muscle tissue was then frozen in O.C.T. compound (Sakura Finetek USA, Torrance, CA). Four micron frozen sections were made using a Leica 1800 cryostat (Leica, Deerfield, IL). The sections were placed onto ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA). The slides were fixed in acetone and blocked with 1.5% goat serum (Vector Laboratories, Burlingame, CA). To detect the coexpression, the slides were

incubated with biotinylated- α -gp120 Abs (Immuno Diagnostics, Bedford MA.) diluted 1:20 along with either FITC-conjugated anti-CD80 or anti-CD86 Abs (PharMingen, San Diego, CA.) diluted 1:5 at 28°C for 12 h. The slides were then incubated with streptavidin Texas Red (NEN Life Sciences, Boston MA) at 1:400 in PBS for 30 min at room temperature. To detect the presence of lymphocytes in muscle, the slides were stained with hematoxylin and eosin (H&E) stain. The slides were viewed with a Nikon OPTIPHOT fluorescing microscope (Nikon, Tokyo, JAPAN) using a $\times 40$ objective (Nikon Fluo $\times 40$ Ph3D2). Slide photographs were obtained using a Nikon FX35DX camera with exposure control by Nikon UFX-II and Kodak Ektachrome 160T slide film.

Infiltration of lymphocytes in muscle was analyzed by preparing frozen muscle sections from DNA-injected animals and stained with H&E stain (Vector Labs). The slides were also stained with anti-CD4 or anti-CD8 Abs (PharMingen).

ELISA

Fifty microliters of recombinant gp120 (ImmunoDiagnostics) diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.5) to 2 μ g/ml concentration was adsorbed onto microtiter wells overnight at 4°C as previously described (8, 15). The plates were washed with PBS-0.05% Tween 20 and blocked with 3% BSA in PBS with 0.05% Tween 20 for 1 h at 37°C. Mouse antisera were diluted with 0.05% Tween 20 and incubated for 1 h at 37°C, then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma). The plates were washed and developed with 3'3'5'5' TMB (Sigma) buffer solution. The plates were read on a Dynatech MR5000 plate reader with the OD at 450 nm. The Ab titer was defined as the highest dilution of serum in which the absorbency of an experimental well exceeded the mean preimmune value by at least two SDs.

Cytotoxic T lymphocyte assay

A 5-h ⁵¹Cr release CTL assay was performed as we described previously (8, 15). Normal and vaccinia-infected EL-4 cells (H-2^b T cell lymphoma) were analyzed by FACS for their ability to express MHC class II molecules. As we expected, EL-4 cells did not express MHC class II molecules in either case (data not shown). The effectors were stimulated nonspecifically for 2 days with CTL culture media consisting of RPMI 1640 (Life Technologies, Grand Island, NY), 10% FCS (Life Technologies), and 10% RAT-T-STIM without Con A (Becton Dickinson) at 5×10^5 cells per ml. The effectors were also stimulated for 4 days by specific target cells. Preparation of specific targets for all CTL experiments was done by infecting EL-4 cells with vMN462 as previously described (8, 15, 16). As a non-specific control for vaccinia virus and DNA immunization experiments, the uninfected EL-4 cells and EL-4 cells infected with WR vaccinia virus (National Institutes of Health AIDS Research and Reference Reagent Program) were used, respectively. A standard chromium release assay was performed, in which target cells were labeled with 100 μ Ci/ml Na₂⁵¹CrO₄ for 2 h and used to incubate with the effector cells for 5 h at 37°C. CTL lysis was determined at E:T ratios ranging from 50:1 to 12.5:1. Supernatants were harvested and counted on an LKB Clinix gamma counter. Percentage specific lysis was determined from the following formula: $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$.

Maximum release was determined by lysis of target cells in 10% Triton X-100 containing medium. An assay was not considered valid if the value for the "spontaneous release" counts are in excess of 20% of the "maximum release." To calculate specific lysis of targets, the percentage lysis of nonspecific (WR-infected) targets was subtracted from the percentage lysis of specific (vMN462-infected) targets. The direct CTL assay was performed as described above except without in vitro stimulation of effector cells (neither specific or nonspecific) (8, 15).

Results

Identification of functional MHC class I molecules in reciprocal β_2m knockout chimeric mice

Expression of β_2m is required for the cell surface expression of MHC class I molecules, which play an important role in the generation of protective cytotoxic immune responses against infectious pathogens (17, 18). These molecules present short peptide fragments derived from foreign Ags synthesized in the cytosol to CD8⁺ cytotoxic lymphocytes. We utilized C57BL/6J-B2m^{tm1/Unc} mice, homozygous for the β_2m knockout gene ($\beta_2m^{-/-}$) along with normal C57BL/6J ($\beta_2m^{+/+}$) animals for the generation of reciprocal bone marrow chimeras of the same haplotype (H-2^b) (Fig. 1).

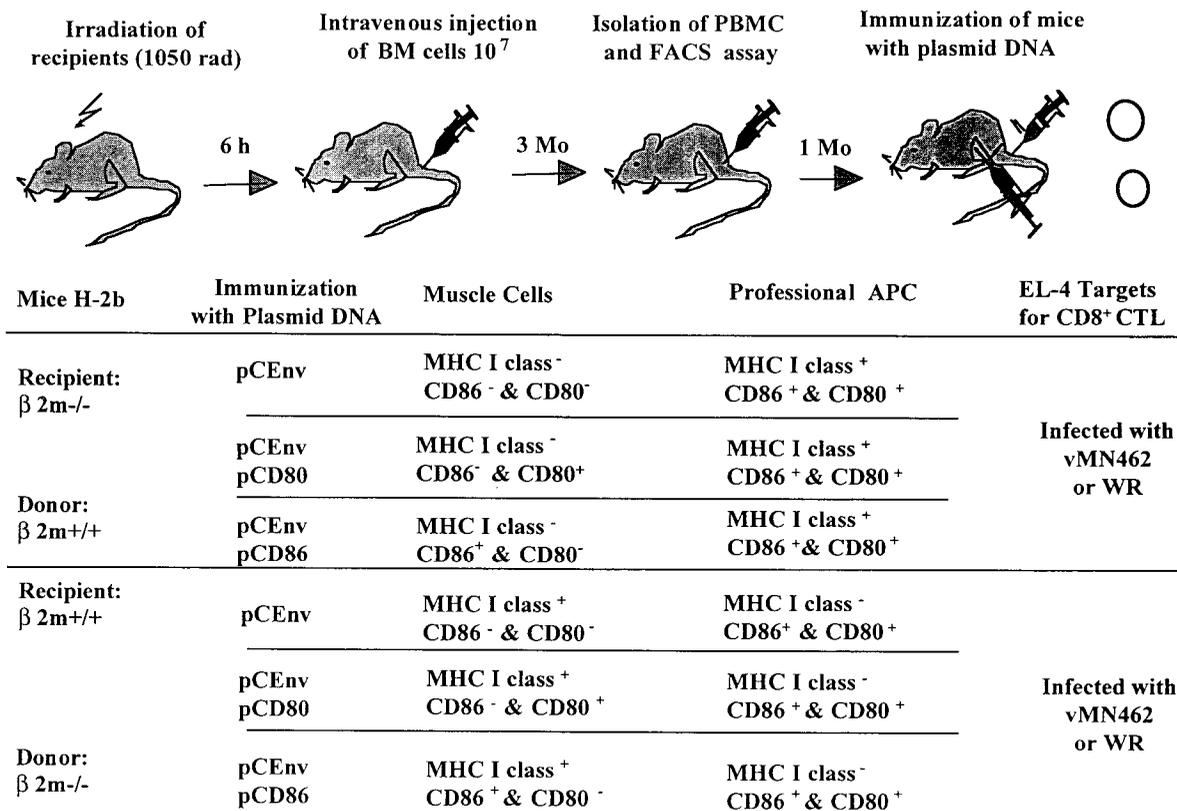


FIGURE 1. Experimental design using reciprocal bone marrow chimeras. Both $\beta_2m^{+/+}$ and $\beta_2m^{-/-}$ animals were used for reciprocal bone marrow transplant. The $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ mice would have bone marrow-derived APCs without MHC class I molecule expression and muscle cells with MHC class I molecule expression. $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeric mice would have MHC class I-positive bone marrow-derived APCs and MHC class I-negative muscle cells. Each mouse received three i.m. injections (2 wk apart) with 50 μ g of each DNA constructs (pCEnv, pCD80, or pCD86) formulated in PBS and 0.25% bupivacaine-HCl.

After the bone marrow transplantation, we confirmed the generation of chimeric mice by FACS analysis using mAbs to the $\alpha 3$ domain of H-2D^b molecule (19). Chimerization of these animals was completed at 3 mo. The resulting chimeric mice displayed a differential expression of MHC class I molecules on the surface of muscle cells and APCs (Fig. 2). The $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ mice possessed bone marrow-derived APCs (donor) without MHC class I molecule expression and muscle cells (recipient) with MHC class I molecule expression. In contrast, $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeric mice possessed MHC class I-positive bone marrow-derived APCs and MHC class I-negative muscle cells. Several reports have suggested that a low level of α -chains (below the level of FACS sensitivity) could be in fact expressed on the surface of $\beta_2m^{-/-}$ cells and that these α -chains could in turn bind free β_2m and cognately present foreign peptides to CD8⁺ T cells (17, 20). On the other hand, $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeric mice, which do not have MHC Class I⁺ thymic epithelial cells, may not generate functional CD8⁺ CTLs during T cell differentiation (21). Even positive selection of these lymphocytes by MHC Class I⁺ bone marrow cells has been demonstrated (22). In fact, it has been shown that both $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ and $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeras generated significant numbers of CD4 and CD8 cells (22). However, we first decided to investigate the ability of the reciprocal chimeric mice to generate anti-viral CD8⁺ CTL immune responses. We immunized normal C57BL/6 ($\beta_2m^{+/+}$) and $\beta_2m^{-/-}$ knockout mice as well as the $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ and $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeric mice with recombinant vaccinia virus (vMN462). Subsequently, we analyzed the anti-viral CD8⁺ CTL immune response generated in these animals. As shown in Fig. 3, we observed that both C57BL/6 and $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ mice generated primary (direct) and secondary

(indirect) CD8⁺ CTL responses. CTL responses in C57BL/6 mice were more potent than in $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeras, and these results agree with earlier observations (22). In contrast, we did not

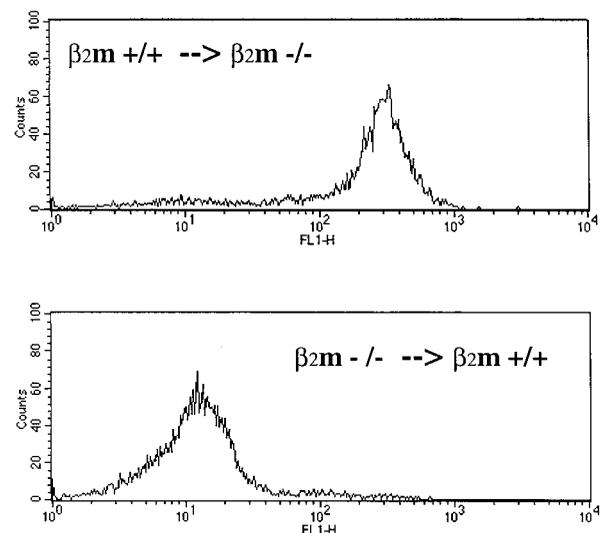


FIGURE 2. Determination of chimerization by flow cytometry. Generation of the chimeric mice was verified by analyzing MHC class I expression 3 mo posttransplant on PBMC by immunofluorescence staining. The PBMC of the $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ mice did not have significant expression of MHC class I molecule whereas the PBMC of the $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeric mice had expression of MHC class I.

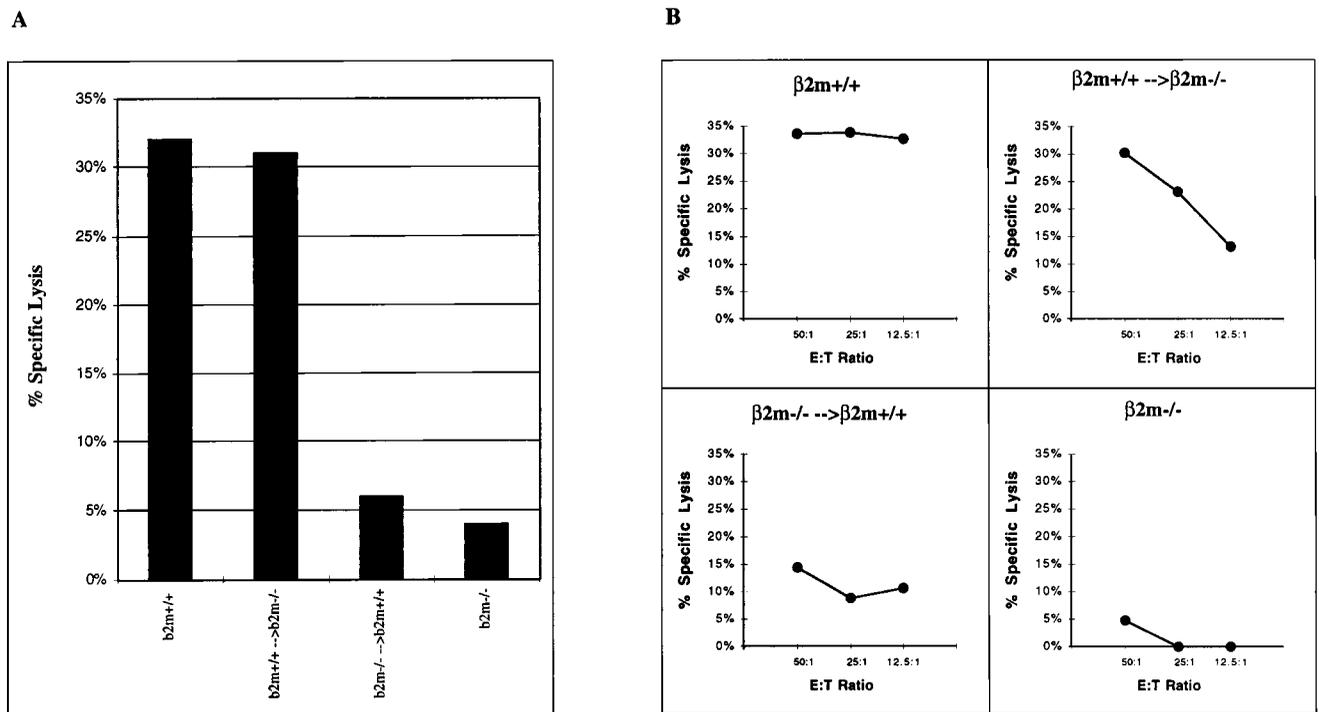


FIGURE 3. Vaccinia virus (vMN462)-specific direct (A) and indirect (B) CTL responses in $\beta_2m^{+/+}$, $\beta_2m^{-/-}$, $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$, and $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ mice. The direct vaccinia-specific CTL responses were analyzed 7 days after vaccinia immunization without in vitro stimulation of effector cells. The indirect vaccinia-specific CTL assay was conducted 4 wk after vaccinia immunization with in vitro stimulation of effector cells. To calculate specific lysis of targets, the percentage lysis of nonspecific (noninfected) targets was subtracted from the percentage lysis of specific (vMN462-infected) targets. These experiments have been repeated two times with similar results.

observe anti-viral CD8⁺ CTL responses in $\beta_2m^{-/-}$ knockout or $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ chimeric mice. Therefore, these results demonstrate that the chimeras between normal and β_2m -knockout mice provide a clean model to examine the role of muscle cells in MHC class I-restricted T cell responses following DNA immunization.

Coexpression of costimulatory molecules with viral protein on muscle cells

We had previously reported that i.m. injection of mice with plasmids encoding for CD80 and CD86 costimulatory molecules resulted in expression of CD80 and CD86 molecules in muscle with similar transfection efficiencies (8). We further investigated whether the codelivery of two expression constructs (one encoding for HIV-1 envelope protein and one encoding for a costimulatory molecule) results in coexpression of these proteins in the same cell. We coimmunized $\beta_2m^{+/+}$ mice with a DNA vaccine expressing HIV-1_{MN} envelope protein (pCEnv) and plasmids encoding CD80 or CD86 genes (pCD80 or pCD86) or control plasmid (pCDNA3). We immunohistochemically examined the expression of CD80, CD86, and envelope proteins in the injected leg muscles (Fig. 4). We observed that coimmunization with pCEnv + pCD80 or pCEnv + pCD86 resulted in coexpression of these proteins in muscle cells. We also observed that the coexpression levels of envelope and CD80 or envelope and CD86 from the mice injected with pCEnv + pCD80 and pCEnv + pCD86, respectively, were similar. In contrast, control legs did not show expression of these proteins.

H-2D^b-positive nonhemopoietic cells can be engineered to activate precursors of MHC class I restricted CTLs

We next analyzed both humoral and cellular immune responses in chimeric and control mice immunized with plasmids encoding viral Ag and costimulatory molecules. Humoral immune responses in sera collected from experimental mice before and after immunization were analyzed by ELISA. As shown in Table I, HIV-1 envelope-specific humoral responses were generated in both types of chimeras. Humoral immune responses of $\beta_2m^{-/-}$ mice were similar to those of the chimeric mice (data not shown). These results demonstrate that Ag-specific humoral immune responses could be generated in the β_2m knockout mice after plasmid DNA immunization and agree with results previously reported in this model system following protein immunization (23). Furthermore, these results indicate that the coimmunization of reciprocal chimeras with either pCD80 or pCD86 had little effect on the specific Ab endpoint titer induced by pCEnv immunizations, as we had previously observed in normal BALB/c mice (8).

We next investigated the generation of CTL responses in reciprocal chimeras and $\beta_2m^{-/-}$ animals. Earlier we demonstrated that coimmunization with CD86, not CD80, genes resulted in dramatic enhancement of MHC-class I-restricted anti-viral CTLs in MHC normal animals (8). Using EL-4 T lymphoma cells as targets, which do not express MHC-II class molecules, we analyzed MHC class I-restricted CD8⁺ CTL responses. To calculate specific lysis of targets, the percentage lysis of nonspecific (WR-infected) targets was subtracted from the percentage lysis of specific (vMN462-infected) targets. A background level of specific killing was observed from the $\beta_2m^{-/-}$ control and $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$

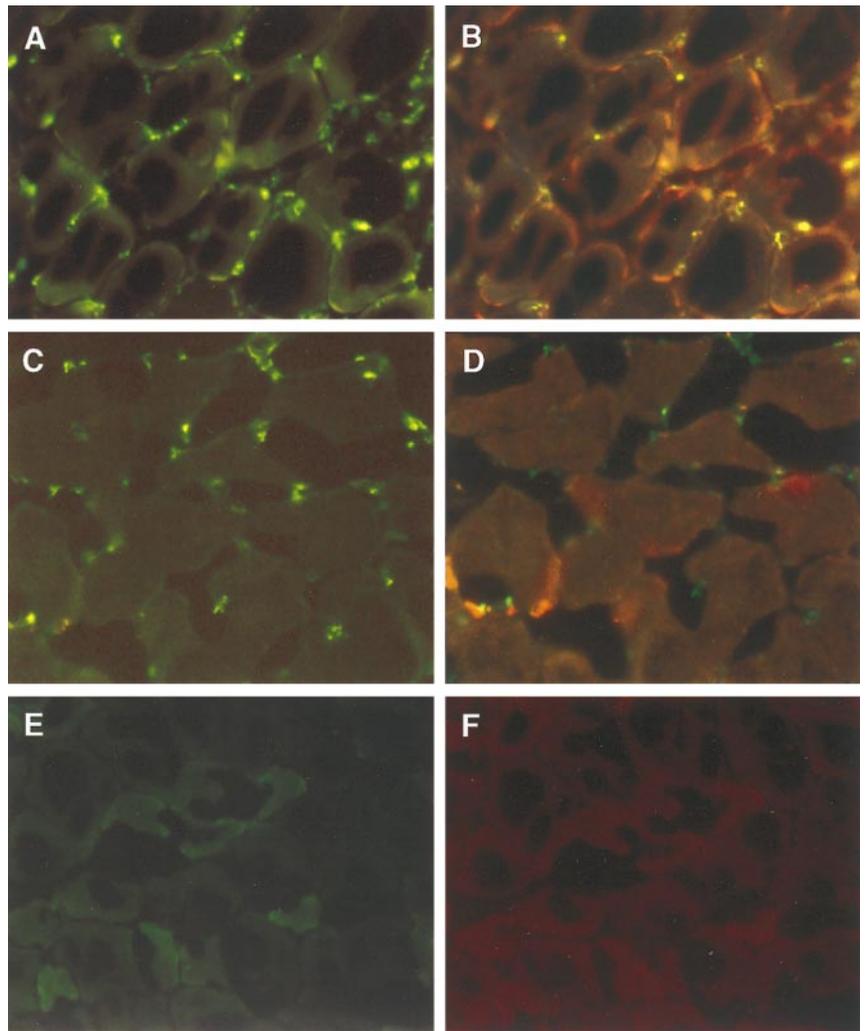


FIGURE 4. Coexpression of HIV-1 envelope gp120 protein with CD80 or CD86 on muscle cells. Frozen muscle sections were prepared from DNA-injected animals and stained with FITC-labeled (green) anti-CD80 or anti-CD86 Abs and Texas Red-labeled (red) anti-gp120 Abs. A slide from a leg immunized with pCEnv + pCD80 was stained with anti-CD80 (A) or anti-CD80 and anti-gp120 Abs (B). A slide from a leg immunized with pCEnv + pCD86 was stained with anti-CD86 (C) or anti-CD86 and anti-gp120 Abs (D). A slide from a leg immunized with pCDNA3 (control vector) was stained with anti-CD80 and anti-CD86 Abs (E) or with anti-CD80, anti-CD86, and anti-gp120 Abs (F).

chimeric mice immunized with control plasmid, pCEnv, pCEnv + pCD80 or pCEnv + pCD86 (Fig. 5). However, $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ mice coimmunized with pCEnv + pCD86, but not pCEnv or pCEnv + pCD80, resulted in a high level of envelope-specific CTL (37% at E:T ratio of 50:1). To further examine the potency of CTL induction, we analyzed the ability to induce direct, unstimulated CTL responses in $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ chimeric mice (Fig. 6) (8). The mice immunized with pCEnv did not induce specific killing. In contrast, specific lysis of 23% was observed from the pCEnv + pCD86 immunization group at an E:T ratio of 50:1 and

titered out to 11% at the 12.5:1 E:T ratio. Since the bone marrow-derived cells in $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ mice could not generate substantial anti-viral CD8⁺ CTLs (Fig. 3), these results suggest that coexpression of HIV envelope and CD86 molecules on nonhemopoietic cells can enable them to prime anti-HIV-1-specific CTL responses. In addition, the results from the immunologically normal $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeras (Fig. 3) indicate that the enhancement effect of CD86 molecules on CTL expansion is not observed through conversion of the small number of APCs, but rather is more prevalent on the nonhemopoietic cells.

The level of various cytokines released by immune cells reflects the direction and magnitude of the immune response. IFN- γ and IL-4 cytokines are produced not only by CD4⁺ but also by CD8⁺ T cells (24). IFN- γ is intricately involved in the regulation of T cell-mediated cytotoxic immune responses (25, 26) while IL-4 plays a dominant role in B cell-mediated immune responses (27). Therefore, in addition to our CTL analysis, we collected supernatant from the effector cells stimulated in vitro for CTL assay and tested them for the release of IFN- γ and IL-4. As shown in Fig. 7, the level of IFN- γ release corresponded with the level of CTL response seen in Fig. 5. In fact, the level of IFN- γ released from $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ mice immunized with pCEnv + pCD86 (45 ng/ml) was at least three times those of the other groups. On the other hand, the levels of IL-4 released from all groups were similar. Therefore, IFN- γ release data supports that CD86 expression on nonhemopoietic cells could prime cytokine induction

Table I. Endpoint Ab titer against HIV-1 envelope protein (gp120)^a

Chimeric Mice	Immunization Group	Titer of Ab (wk postimmunization)	
		3 wk	7 wk
$\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$	pCEnv	1:1024	1:1024
$\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$	pCEnv + pCD80	1:2048	1:512
$\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$	pCEnv + pCD86	1:2048	1:1024
$\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$	pCEnv	1:1024	1:1024
$\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$	pCEnv + pCD80	1:1024	1:512
$\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$	pCEnv + pCD86	1:1024	1:512

^a HIV-1 envelope-specific Ab response following coimmunization with pCD80 or pCD86 (four mice per group). The mouse sera were tested for envelope-specific Ab response by ELISA using HIV-1 gp120 protein. The serial dilutions were 1/64, 1/128, 1/256, 1/512, 1/1024, 1/2048, and 1/4096. The background OD level for ELISA was <0.015. The experiments were repeated two times with similar results.

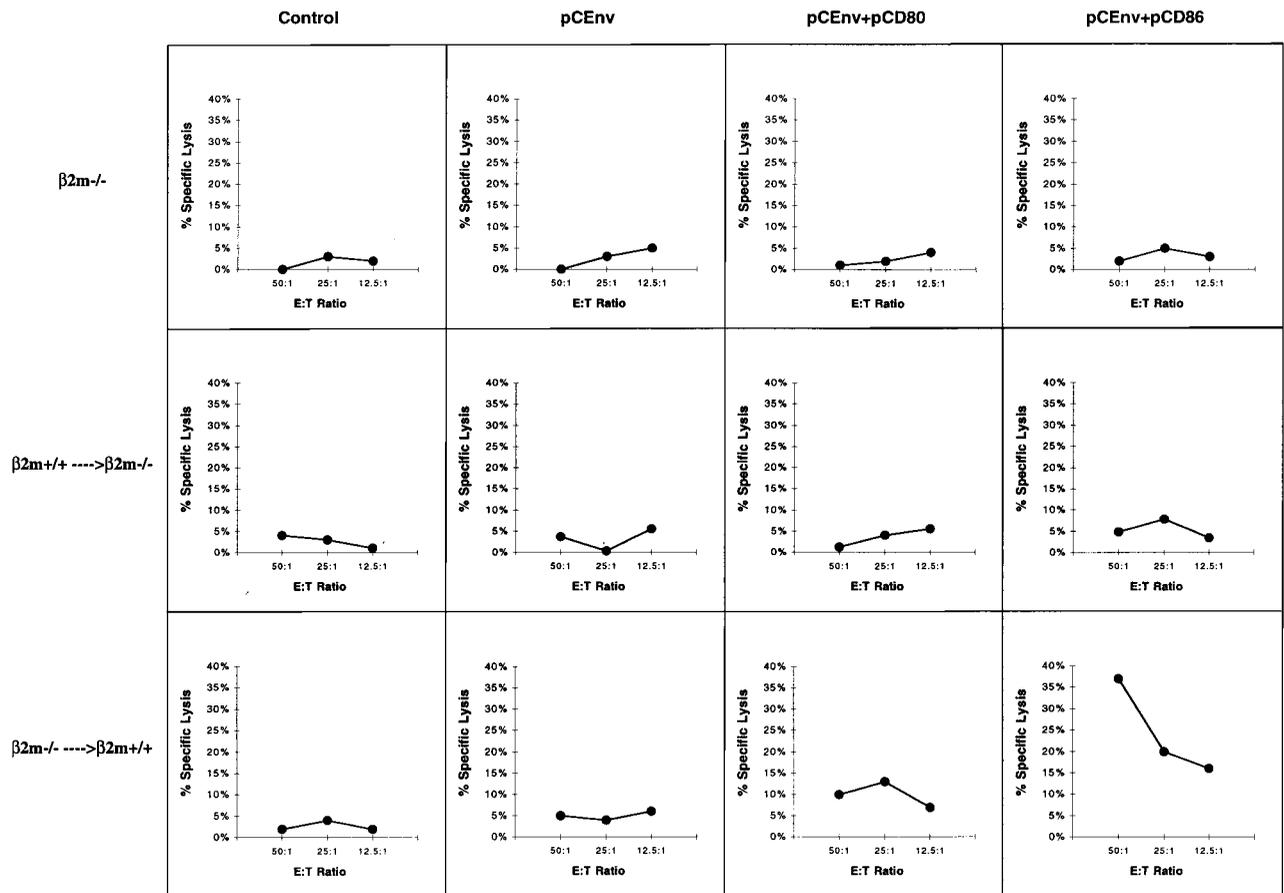


FIGURE 5. HIV-1 envelope-specific CTL responses in $\beta_2m^{-/-}$ mice (A), $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ (B), and $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ (C) chimeras immunized with pCDNA3, pCEnv, pCEnv + pCD80, or pCEnv + pCD86 (four mice per group). The HIV-1 Env-specific CTL responses were analyzed against vaccinia-infected targets after in vitro stimulation of effector cells. To calculate specific lysis of targets, the percentage lysis of nonspecific (WR-infected) targets was subtracted from the percentage lysis of specific (vMN462 infected) targets. The maximum level of nonspecific lysis was 6.5%. These experiments have been repeated two times with similar results.

primarily in the context of MHC class I expression, supporting direct TCR coligation by nonprofessional APCs.

Expression of CD86-induced infiltration of lymphocytes into the muscle of immunized animals

To further clarify the ability of non-bone marrow cells transfected with CD86 to directly drive T cells, we looked for direct evidence of T cell ligation to transfected muscle cells in vivo. We observed much more infiltration of lymphocytes into the muscle of $\beta_2m^{+/+}$

mice immunized with pCEnv + pCD86 than in the muscle of control or pCEnv + pCD80-immunized $\beta_2m^{+/+}$ mice at 7 days postimmunization (Fig. 8). These numerous infiltrating lymphocytes at the site of Ag and CD86 expression seem likely to attack the presenting muscle cells. We stained the slides immunohistochemically for T cells and observed that the infiltrating T cells included both CD4⁺ and CD8⁺ T cells (Fig. 9). The lymphocyte infiltration in the immunized muscle was observed to clear within 1 mo, correlating to the duration of Ag expression following

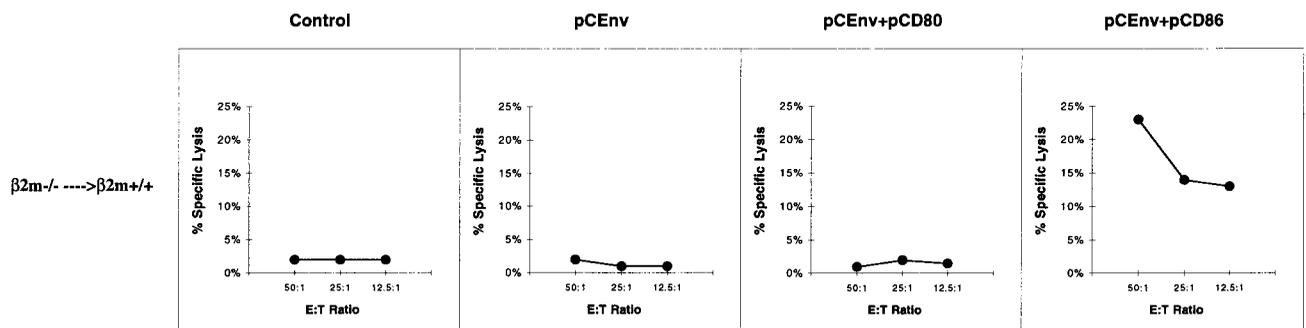
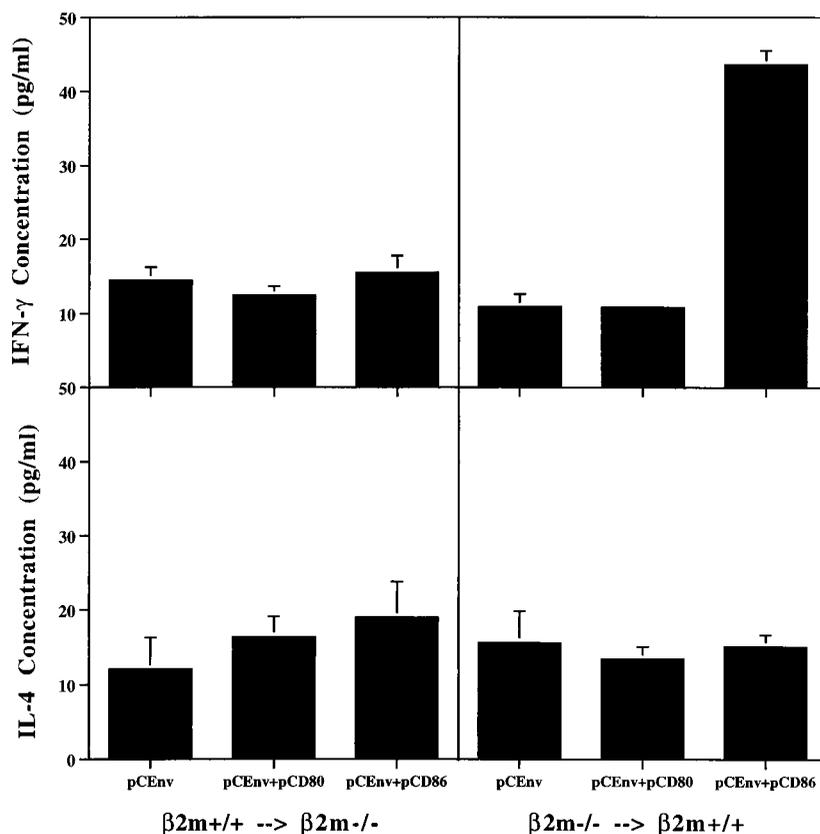


FIGURE 6. Direct HIV-1 envelope-specific CTL responses in $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ chimeric mice immunized with pCDNA3, pCEnv, pCEnv + pCD80, or pCEnv + pCD86. The HIV-1 env-specific CTL responses were analyzed against vaccinia-infected targets without in vitro stimulation of effector cells. In these experiments maximum level of nonspecific lysis was less than 5%.

FIGURE 7. Expression of cytokines by stimulated effector cells. Supernatants from effectors stimulated for CTL assay were collected at day 6 and tested for cytokine profile using ELISA kits for IFN- γ and IL-4 (both kits from Biosource International, Camarillo, CA). These experiments have been repeated two times with similar results.



cDNA expression (data not shown). Animals exhibited no clear phenotypic effects of this invasion compared with nonvaccinated animals (data not shown). Examination of muscle sections at later time points demonstrated a normal muscle phenotype without lymphocyte invasion. It is interesting that even during the early phase of lymphocyte infiltration, the mice behaved normally. These results suggest that muscle cells that are engineered to express viral Ag along with MHC class I and CD86, but not CD80, molecules could effectively attract lymphocytes and directly interact with them. These data clearly distinguish that attraction, per se, is not the function of CD80.

Discussion

DNA inoculation is an important vaccination technique that delivers DNA constructs encoding a specific immunogen directly into the host (28–34). These expression cassettes transfect the host cells, which become the *in vivo* protein source for the production of Ag, inducing an immune response. Although this novel vaccination technique is being investigated as a potentially effective strategy in infectious diseases and cancer, the mechanism of Ag processing and presentation following DNA immunization is not clearly understood. Even though muscle is a major producer of Ag from DNA inoculation, muscle cells do not express the costimulatory molecules required for appropriate Ag presentation and activation of T cells (35–37). Using bone marrow chimeric mice generated from SCID mice with two different haplotypes (H-2^b or H-2^d), Doe et al. have shown that Ag presentation from *i.m.* DNA injection is restricted by the bone marrow-derived professional APCs (38). Independently, Corr et al. came to a similar conclusion using chimeric mice generated using H-2^{bxd} recipients and H-2^b or H-2^d donors (39). Nevertheless, it is still unclear whether the CTL priming by APCs is due to direct transfection of the bone marrow-derived cells, as has been reported (40, 41), processing by APCs of

Ag shed from peripheral cells, or “hand-off” of Ag produced from non-APCs to professional APCs in the draining lymph nodes and presented molecule to T cells in association with MHC class I molecule and costimulatory molecule.

Recently, three groups (including ours) independently reported that coadministration of CD86 cDNA along with DNA Ags *i.m.* dramatically increased Ag-specific CTL responses (8–10). In contrast, this enhancement of CTL responses was not observed with CD80 coexpression (8–10). More recently, CD80 has been reported to enhance CTL responses when it was coexpressed with plasmid DNA encoding a peptide minigene (42). This result is different from three other recent papers suggesting that free epitopes rather than natural Ags can behave uniquely. While further work will be required to investigate the differences, the similarities of the results from different groups are instructive.

To investigate whether the bone marrow-derived professional APCs or muscle cells were responsible for the enhancement of CTL priming following CD86 coadministration, we developed a set of bone marrow chimeric animals using β_2m knockout mice. The β_2m chimeric animals represent an appropriate model to conduct our studies. These chimeric animals provide a straightforward way to study CTL responses, which are restricted by MHC class I molecules of the bone marrow-derived cells or the muscle cells. The $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ mice possessed bone marrow-derived APCs (donor) without MHC class I molecule expression and muscle cells (recipient) with MHC class I molecule expression. In contrast, $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeric mice possessed MHC class I-positive bone marrow-derived APCs and MHC class I-negative muscle cells (Fig. 1). β_2m , peptide, and α -chain (heavy chain) are all necessary for effective transport and expression of functional MHC class I molecules. Therefore, the cells that do not express β_2m do not express MHC class I molecules on the cell surface (43). Many studies have confirmed that the α -chains of MHC class

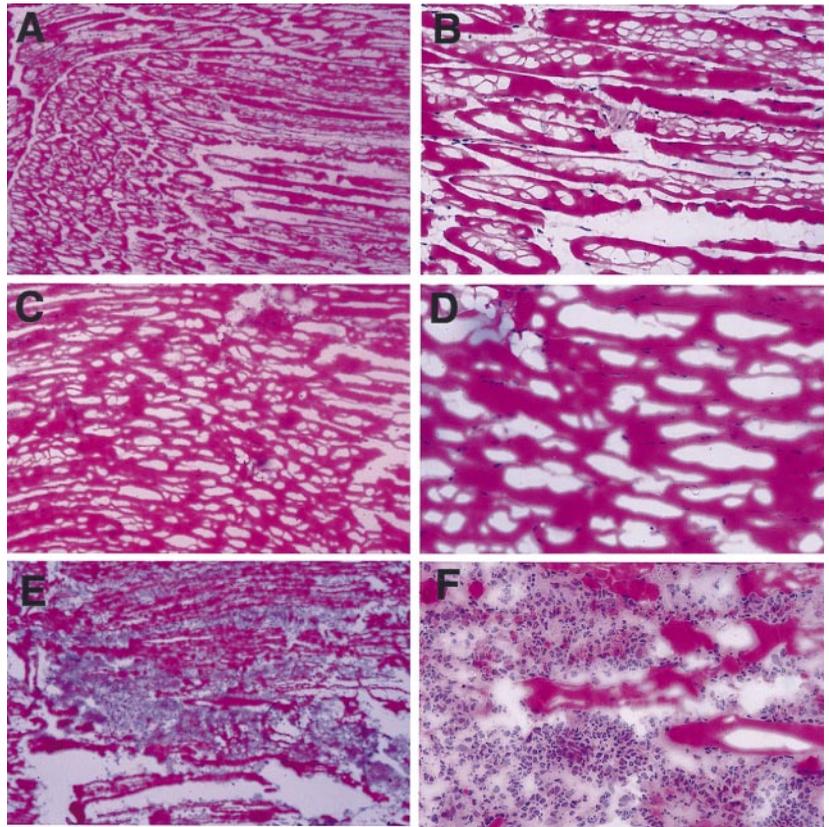


FIGURE 8. Infiltration of lymphocytes in muscle following coimmunization with pCD86. Frozen muscle sections were prepared from DNA-injected $\beta_2m^{+/+}$ animals and stained with H&E stain. Slides from control mice (A) and (B), pCEnv + pCD80-immunized mice (C) and (D), and pCEnv + pCD86-immunized mice (E) and (F) are shown. A, C, and E are shown at $\times 20$ magnification, and B, D, and F are shown at $\times 40$ magnification.

I are not detectable by FACS analysis in $\beta_2m^{-/-}$ animals (20, 23, 43–45). As expected, our FACS analysis failed to detect a significant presence of MHC class I α -chains on the PBMC of $\beta_2m^{-/-}$ or $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ chimeric mice (Fig. 2).

There was a concern that $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeric mice may not possess functional CD8⁺ T cells without MHC Class I⁺ thymic epithelial cells to educate them (21). It has been reported, however, that in $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeric mice, which have $\beta_2m^{-/-}$ thymic epithelial cells and $\beta_2m^{+/+}$ hemopoietic cells, CD8⁺ T cells are educated by MHC class I⁺ bone marrow cells (22, 23). In an elegant set of experiments, Bix and Raulet demonstrated that $\beta_2m^{+/+}$ hemopoietic cells grafted into the $\beta_2m^{-/-}$ host direct positive selection of functional CD8⁺ T cells (22). They also found that this positive selection of CD8⁺ T cells was provided by the bone marrow cells and not by the potentially reconstituted thymic epithelial MHC class I molecules ($\beta_2m^{-/-}$ thymic epithelial cells reconstituted by donor β_2m) (22). Earlier, the rate of positive selection of CD8⁺ T cells by $\beta_2m^{+/+}$ hemopoietic cells in $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeric mice was observed to be less than that of normal mice (about 1/6 of that in normal mice) (22). Nevertheless, these chimeric mice possessed functional CD8⁺ T cells that mounted strong CD8 and MHC class I-restricted CTL responses (22). The responses were comparable to those observed in normal mice (22). We also analyzed the immunocompetency of our $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeras and observed that these mice produced functional CD8⁺ CTL response against vaccinia virus, even though it was at a lower level than that of $\beta_2m^{+/+}$ mice (Fig. 3).

Several reports have suggested that a low level of α -chains (below the level of FACS sensitivity) could be expressed on the surface of $\beta_2m^{-/-}$ cells and that these α -chains could in turn bind free β_2m and cognately present foreign peptides to CD8⁺ T cells (17, 20). These studies, however, were performed in vitro, and the

activity detected was significantly less than that induced by wild-type β_2m -expressing cells. Although in vitro β_2m -independent presentation of endogenous self H-2D^b peptides by MHC class I restricted α -chains has been reported, such effects were not observed in in vivo experiments (22, 23, 46). More importantly, we did not observe antiviral CTL response after inoculation of $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ animals with vMN462 (Fig. 3). Therefore, even if a low level of the MHC-class I molecules was expressed on the bone marrow-derived APCs of these mice, these molecules were functionally ineffective.

One should also note that MHC-class I alloantigen-specific CD8⁺ CTL responses in β_2m -deficient mice has been reported (47–49). It has been shown that both $\beta_2m^{+/+}$ and $\beta_2m^{-/-}$ allospecific CD8⁺ CTL could lyse $\beta_2m^{+/+}$ and $\beta_2m^{-/-}$ target cells, but the lysis of $\beta_2m^{-/-}$ targets was 50 times less than that of $\beta_2m^{+/+}$ targets (47). Furthermore, the frequency of alloantigen-specific CD8⁺ CTL precursors has been estimated to be 20–200 times higher than that of precursors against viral Ags (50). To our knowledge, no studies have been reported on the induction of CD8⁺ CTL in $\beta_2m^{-/-}$ and $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ mice during viral infection (23, 51). Accordingly, our $\beta_2m^{-/-}$ or $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ animals immunized with vMN462 or pCEnv did not generate Ag-specific CD8⁺ CTLs. Although many investigators using the β_2m knockout mice have reported MHC class II-restricted CD4⁺ CTL in their experiments (23, 51), such analysis was irrelevant in our experiment using EL4 cells as target cells since they do not express MHC class II molecules. These results established the use of $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ or $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ chimeric animals as an appropriate model to examine the CD8⁺ antiviral CTL responses restricted by the MHC class I molecules of the APCs or muscle cells.

Since $\beta_2m^{-/-}$ professional APCs could not be involved in the induction of CD8⁺ CTL in $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ mice, the high level

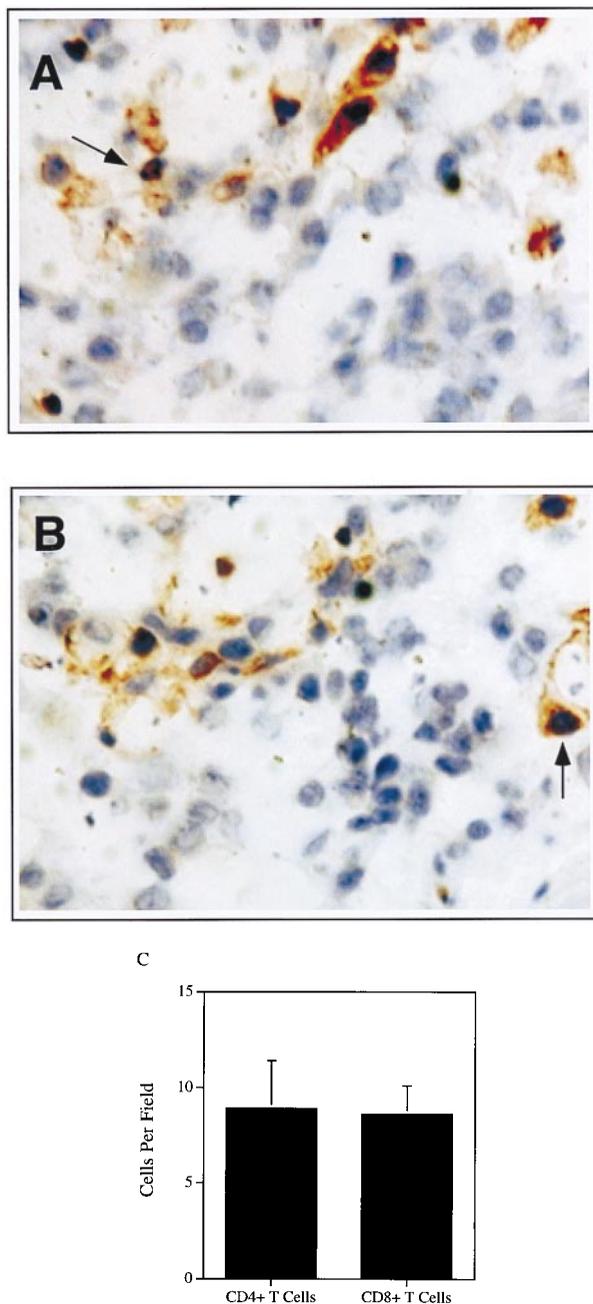


FIGURE 9. Presence of CD4⁺ and CD8⁺ T cells in muscle. The infiltrating cells in muscle following coimmunization with pCEnv + pCD86 were further stained with anti-CD4 (A) or anti-CD8 (B) Abs. Representative examples of CD4⁺ and CD8⁺ T cells are highlighted with black arrows. A and B are shown at $\times 20$ magnification. C, CD4⁺ and CD8⁺ T cells were counted from the stained slides.

of CTL responses generated by immunization of these animals with pCEnv + pCD86 supports our hypothesis that nonhemopoietic cells such as muscle cells can be engineered to function as APCs. In contrast, pCEnv + pCD80 immunization did not result in high levels of CTL responses (Figs. 5 and 6). These results were consistent with previous results obtained in normal mice, and they again support the critical role of CD86 expression presentation of Ag. However, these studies shed little light on the function of CD80 in this process. CD80 and CD86 have limited homology (28%), but both have been shown to efficiently costimulate proliferation of T cells and to induce cytokine production in vitro (2, 52, 53). Although more work may be needed to fully elucidate the

differential roles of CD80 and CD86, we speculate that CD86 may be able to activate CD8⁺ T cells better than CD80 because of a higher affinity for CD28, which is involved in T cell activation, and we could speculate that CD80 could have higher avidity for CTLA-4, which is involved in suppression of T cells (54–57).

We have previously reported that the dosage of pCEnv selected for immunization (50 μ g) induces a low level of CTL responses (5–10% specific lysis) in $\beta_2m^{+/+}$ normal mice (8). We utilized this dosage in the current experiment to detect the full CTL enhancement effect of CD86 coimmunization in the reciprocal chimeric mice. As shown in Fig. 5, we observed that immunization of $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeras (which had normal professional APCs) with pCEnv resulted in a low level of CTL responses, similar to the level observed in normal mice. On the other hand, we did not observe the enhancement of CTL responses in $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimeric animals immunized with pCEnv + pCD86. There could be several possible explanations for this finding. First, enhancement of antiviral CTL responses with CD86 coadministration may be restricted to the expression of CD86 in non-APCs, such as muscle cells. This hypothesis is supported by the fact that most professional APCs, such as the dendritic cells and macrophages, already express CD86 constitutively, and the expression is further increased upon activation (5–7). Thus, transfection of these professional APCs with CD86 would not enhance their ability to activate and expand T cells. Moreover, although several studies have identified the importance of directly transfecting APCs, such as the dendritic cells and macrophages, via DNA immunization, the efficiency of such direct in vivo transfection has been low (40, 41). On the other hand, i.m. injection of DNA results in a predominant level of transfection in muscle cells. Therefore, potentiating these nonprofessional APCs to coexpress CD86 may be crucial for the priming and enhancement of CTL expansion. In the case of the $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ mice, whose muscle cells (and other nonhemopoietic cells) lacked functional MHC class I molecules, such CD86-mediated CTL expansion would not be possible. One of the other possible mechanisms of transferring Ag or peptides from peripheral cells to the professional APCs is thought to be a “hand-off” mechanism (38, 39, 58, 59) between transfected non-APC protein factory and the APC. If the “hand-off” hypothesis is correct, then the CTL experiment results from the $\beta_2m^{+/+} \rightarrow \beta_2m^{-/-}$ chimera immunization suggest that such a mechanism would be MHC class I restricted. Finally, it is possible that the animals that responded to vaccinia virus could not generate anti-Env CTLs due to differences in quantity of CD8⁺ T cell precursors specific to the Env protein vs the large number of vaccinia virus Ags.

Recently, another group has reported on the potential of converting muscle cells to APCs. Iwasaki et al. utilized chimeric mice generated with H-2^{bxd} recipients and H-2^b or H-2^d donors and reported on injection of wildtype nucleoprotein immunogen with CD86 (60). Earlier they had reported significant enhancement using plasmid encoding for a mutant form of nucleoprotein and CD86 in normal animals (10). In their subsequent chimera experiments, however, they did not observe any significant enhancement with the addition of CD86 (60). No positive effects were observed in their chimeric animals; therefore, it was difficult to draw extensive conclusions in this study due to a lack of CD86 effect. In our experiments, pCEnv vaccination of $\beta_2m^{-/-} \rightarrow \beta_2m^{+/+}$ chimeric mice did not generate antiviral CTL responses (less than 6% of specific lysis), but immunization with pCEnv and pCD86 induced high anti-HIV CTL activity before (up to 23%) and after (up to 37%) in vitro stimulation (Figs. 5 and 6). The positive results in these experiments allow us to draw additional insight into the role of CD86 and the function of bone marrow-derived APCs in CTL induction. Moreover, our results do not conflict with published

results indicating the bone marrow-derived APCs as the presenter of Ag from DNA immunization (38–41, 60). Rather, we show that nonhemopoietic cells, such as muscle cells, can be engineered to present DNA-encoded Ag by potentiating them to express the key costimulatory molecule CD86. This molecule is normally the sole providence of bone marrow-derived cells.

This interpretation, which is the most direct and simplest, is further supported by the immunohistochemistry results presented in Fig. 8. We observed more infiltration of lymphocytes into the muscle of mice immunized with pCEnv + pCD86 than in the muscle of pCEnv + pCD80-immunized mice. These infiltrating cells included both CD4⁺ and CD8⁺ T cells (Fig. 9). It appears that CD86 expressed in the absence of other cytokines or costimulatory molecules can directly prime T cells that can then destroy the presenting target. Macrophages and dendritic cells would in this sense be analogous to the CD86-displaying muscle cells and must be capable of activating CTLs without suffering their wrath. Protection from inadvertent destruction during the activation process must be mediated by other cell surface molecules, which might include other members of the B7 family, which would be expected to be coordinately regulated. Furthermore, as shown by the higher level of lymphocytic infiltration, transfection of muscle cells with CD86 may enhance their ability to chemoattract T cells. This is especially interesting since it has been recently reported that CD8⁺T cells may expand Ag-specific responses in vivo through the elaboration of specific chemokines at the peripheral site of infection during the effector stage of the immune response (61). Such peripheral restimulation may be crucial in the expansion of T cell responses and bears further investigation.

Our findings clarify that one function of the CD86 molecule on APCs itself is, in the context of MHC class I molecules, to prime and expand T cells. The demonstration that this function segregates with CD86 itself even on non-bone marrow-derived APCs could represent an important step in the pursuit of rationally designed vaccines and immune therapies through the control of MHC class I restriction.

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