

The Roles of MHC Class II, CD40, and B7 Costimulation in CTL Induction by Plasmid DNA¹

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DNA-based vaccines generate potent CTL responses. The mechanism of T cell stimulation has been attributed to plasmid-transfected dendritic cells. These cells have also been shown to express plasmid-encoded proteins and to become activated by surface marker up-regulation. However, the increased surface expression of CD40 and B7 on these dendritic cells is insufficient to overcome the need for MHC class II-restricted CD4⁺ T cell help in the priming of a CTL response. In this study, MHC class II^{-/-} mice were unable to generate a CTL response following DNA immunization. This deficit in CTL stimulation by MHC class II-deficient mice was only modestly restored with CD40-activating Ab, suggesting that there were other elements provided by MHC class II-restricted T cell help for CTL induction. CTL activity was also augmented by coinjection with a vector encoding the costimulatory ligand B7.1, but not B7.2. These data indicate that dendritic cells in plasmid DNA-injected mice require conditioning signals from MHC class II-restricted T cells that are both CD40 dependent and independent and that there are different roles for costimulatory molecules that may be involved in inducing optimal CTL activity. *The Journal of Immunology*, 2001, 166: 3061–3066.

Cytotoxic T lymphocytes play a key role in the adaptive immune system by eliminating cells infected with intracellular pathogens or expressing neoantigens, such as tumor markers. The differentiation of naive CD8⁺ CTL into effector cells is often dependent on the activity of CD4⁺ Th cells. This Th activity engages the APC, which then becomes competent at providing the necessary signals to prime the naive CD8⁺ cell. The cognate interaction between APCs and CD8⁺ T cells involves peptides primarily from endogenously expressed proteins. However, alternative pathways exist for the acquisition, processing, and loading of exogenously derived proteins (1–4). The stimulation of naive CTL by peptides from exogenous proteins has been referred to as cross-priming.

In vivo priming of CTL by DNA injection predominantly occurs by such an Ag transfer to an APC (5). The injection of naked plasmid DNA into the skin and muscle of mice results in the uptake of DNA into neighboring cells. These nonlymphoid tissues express the plasmid-encoded protein. The antigenic peptide is then presented to T cells in the context of the MHC-encoded class I molecules of bone marrow-derived cells and not by injected myocytes (6, 7). The bulk of the immune response is dependent on the Ag expressed by nonlymphoid tissues and transferred to bone mar-

row-derived APCs with the Ag expressed by directly transfected APCs playing a minor role (5).

Although directly transfected dendritic cells have been isolated following plasmid DNA inoculation, they do not appear to function similarly to virally infected cells. Virally infected dendritic cells can generate CTL in the absence of T cell help, but dendritic cells that acquire exogenous Ag require signals by MHC class II-restricted Th cells (8, 9). This conditioning effect has been attributed to signaling through CD40 (8, 10, 11). Injection of naked plasmid DNA that does not encode Ag can nonspecifically augment the expression of CD40, B7.1, and B7.2 on dendritic cells (12). This enhanced surface expression of costimulators does not circumvent the need for T cell help in generating CTL in plasmid DNA-injected mice (5, 13, 14).

In this report, the elements required for CTL priming by plasmid DNA vaccination are investigated. There is an absolute dependence on MHC class II-restricted Ag presentation for generating MHC class I-restricted CTL. However, in CD4 deficient mice, there was diminished but detectable CTL, suggesting that not all of the MHC class II-restricted T cells bear CD4 coreceptors. The function of MHC class II-restricted help could not be fully restored by nonspecifically ligating CD40 with an activating Ab. Interestingly, the addition of a B7.1-, but not a B7.2-expressing plasmid, could also generate CTL in IA^b-deficient mice, suggesting a separation of function for these CD28 ligands. Finally, the combination of nonspecific CD40 ligation and the addition of a B7.1-expressing plasmid synergistically augmented CTL responses in MHC II^{-/-} mice.

Materials and Methods

Mice

MHC class II^{-/-}, CD4^{-/-}, CD40 ligand-deficient (CD40L^{-/-}),³ and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were bred and maintained under standard conditions in the

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³ Abbreviations used in this paper: CD40L, CD40 ligand; ELISPOT, enzyme-linked immunospot.

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Plasmids

The construction of pACB and pCMVint-based vectors have been previously described (15). The pACB-Z plasmid expresses the *Escherichia coli* Lac Z cDNA. The ACB-OVA and ACB-TPASIIINFEKL plasmids encode full-length OVA and the H-2K^b-restricted epitope SIINFEKL, respectively. The nCMV-B7.1 and nCMV-B7.2 constructs encode the cDNAs for CD80 and CD86, which were kindly provided by Dr. G. Freeman (Dana-Farber Cancer Institute, Boston, MA) (16, 17). Plasmid DNA for injection was prepared as previously described (6).

Antibodies

CD40-activating Ab FGK45 (18) (prepared from hybridoma supernatants) and control rat IgG (Sigma, St. Louis, MO) were injected in PBS as described in the figure legends.

CTL assay

Cytolytic T cell assays were performed as described (6). Briefly, splenocytes were restimulated in culture with peptide and rIL-2 for 5 days. The dead cells were eliminated with gradient centrifugation, and the remaining cells were tested for their ability to lyse peptide-pulsed EL4 cells in a 4-h culture. The peptides used include the H-2K^b-restricted epitope from OVA, SIINFEKL (257–264) (19), the H-2K^b-restricted epitope from β -galactosidase, DAPIYTNV (96–103) (20), and from influenza nucleoprotein the H-2D^b-restricted peptide, ASNENMETM (366–374) (21) (Molecular Research Laboratories, Durham, NC). Background controls with an irrelevant peptide for nonspecific target and effector cell lysis were included on each plate. Lysis was detected using the CytoTox 96 assay kit (Promega, Madison, WI) per the manufacturer's instructions. After background subtraction, lysis was calculated by $100 \times [(test\ release - spontaneous\ release) / (maximum - spontaneous\ release)]$.

Enzyme-linked immunospot (ELISPOT) assay for single-cell IFN- γ secretion

At the time of sacrifice, splenocytes were titrated starting at 2.0×10^6 in 96-well plates in duplicate with and without peptide. The cells were transferred in $100\ \mu\text{l}$ to 96-well nitrocellulose plates (Millipore, Bedford, MA) previously coated overnight at 4°C with rat anti-mouse IFN- γ (R46A2; BD PharMingen, San Diego, CA) Abs in PBS and blocked with RPMI 1640 supplemented with 10% FBS, 1% penicillin and streptomycin, 2 mM L-glutamine, and 50 μM 2-ME. Cells were incubated 16–20 h at 37°C. After culture, wells were washed thoroughly with balanced salt solution and dH₂O, and incubated for 2 h with biotinylated anti-mouse IFN- γ (XMG1.2; BD PharMingen) Ab in PBS containing 1% BSA, washed again, and then incubated for 1 h with HRP-streptavidin (Zymed, South San Francisco, CA). Plates were developed using tetramethylbenzidine membrane substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and counted. Only large spots that were well defined with fuzzy edges were recorded.

Results

Induction of CTL from plasmid injection requires MHC class II-restricted T cell help

Mice injected with plasmid-encoding minigenes for MHC class I-restricted T cell epitopes generate poor if any CTL responses unless an additional signal is provided (5, 22). To evaluate the role of Th cells in providing these signals, MHC class II and CD4 gene-disrupted mice were injected with plasmid DNA encoding β -galactosidase (Fig. 1). Six weeks following the initial injection, the mice were sacrificed and their splenocytes were assessed for their ability to lyse cells pulsed with the H-2K^b-restricted epitope from β -galactosidase, DAPIYTNV (20). The CD4^{-/-} mice were able to mount a diminished CTL response compared with wild-type mice. However, the MHC class II^{-/-} mice were not able to generate a measurable response. The residual CTL response in the CD4^{-/-} mice suggested that there were MHC class II-restricted T cells that were capable of providing T cell help for the generation of CTL, which did not bear the CD4 coreceptor. Hence, CTL activity from plasmid DNA injection is more dependent on MHC

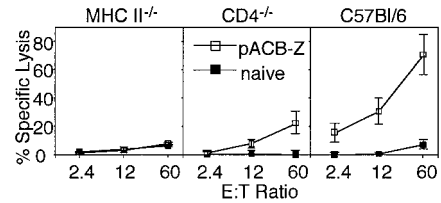


FIGURE 1. CTL induction is dependent on MHC class II molecules. Groups of four adult MHC class II^{-/-}, CD4^{-/-}, and C57BL/6 mice were immunized with 50 μg pACB-Z intradermally on days 0 and 7 or left untreated. After 6 wk, the mice were sacrificed and their splenocytes were assayed for their ability to lyse peptide-pulsed EL4 cells after 5 days of in vitro restimulation. Values plotted are average peptide-specific background lysis \pm SEM. The maximum lysis seen with the negative control peptide (ASNENMETM) was 3.9%. This experiment was performed four times.

class II-restricted T cell help than additional stimulus through the CD4 coreceptor.

Nonspecific T cell help augments CTL from plasmid vaccination

Previously, cross-priming was reported to require Ag-specific T cell help to elicit CTL (9). More recently, the activation of CD40 by a cross-linking Ab has been shown to substitute for T cell help and restore CTL generated by cross-priming in MHC class II-deficient mice (8, 10, 11). As CD40 activation is a nonspecific signal, the priming of CTL by plasmid DNA injection may only need non-Ag-specific T cell help. To evaluate whether the signals from MHC class II-restricted T cells were Ag specific, a minigene plasmid was coinjected with a plasmid expressing an irrelevant Ag (β -galactosidase) in wild-type mice (Fig. 2). This minigene plasmid encoded the H-2K^b-restricted epitope from OVA residues 257–264 (SIINFEKL) fused to a leader sequence to facilitate transport into the endoplasmic reticulum (22). Mice coinjected with minigene and vector alone did not mount a substantial CTL response. In contrast, the mice that received the minigene and vector encoding β -galactosidase had a markedly greater CTL response. The augmentation in the CTL response suggested that plasmid-expressed β -galactosidase contained epitopes that were able to generate bystander T cell help that was not specific for OVA. Indeed, the splenocytes of the mice injected with β -galactosidase-expressing plasmid secreted IFN- γ to in vitro stimulation with β -galactosidase but not OVA and vice versa with the splenocytes

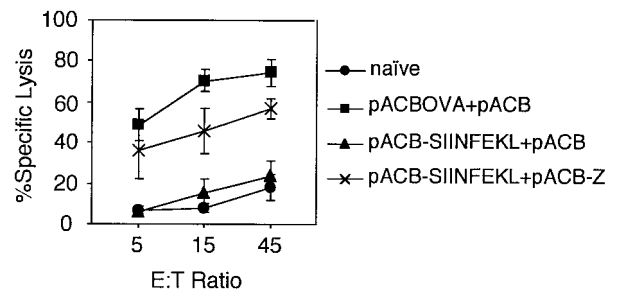


FIGURE 2. Nonspecific T cell help augments CTL from plasmid vaccination. Four adult C57BL/6 mice per group were injected with 50 μg of each of the indicated plasmids intradermally on days 0 and 7 or left untreated. The pACB backbone vector was used to equalize the total amount of DNA each mouse received. After 2 wk, the mice were sacrificed and their splenocytes were assayed for their ability to lyse SIINFEKL peptide-pulsed EL4 cells after 5 days of in vitro restimulation. Values plotted are average peptide-specific background lysis \pm SEM. The maximum lysis seen with the negative control peptide (ASNENMETM) was 3.5%. This experiment was performed twice.

from the mice inoculated with the OVA-encoding plasmid (data not shown).

CTL induction by plasmid DNA inoculation is dependent on CD40L signaling

As the signals required to provide T cell help did not appear to require strict Ag specificity, the role of CD40L was evaluated as an avenue of Ag-independent dendritic cell activation. Mice that were targeted mutants in CD40L expression were injected with pACB-Z and compared with wild-type mice (Fig. 3). The CD40L-deficient mice were unable to generate any CTL response, suggesting that the CD40/CD40L pathway is also critical in generating this response from plasmid DNA injection. In addition to their CTL activity, the splenocytes were assayed for their ability to secrete IFN- γ , IL-4, IL-5, or IL-10 in response to *in vitro* restimulation (data not shown). The splenocytes from wild-type control mice produced IFN- γ and IL-10, but the splenocytes from CD40L^{-/-} mice did not make measurable amounts of cytokine. The lack of cytokine secretion suggests that there was an inadequate MHC class II-restricted response, which may have indirectly resulted in a defect in CTL response.

CD40 ligation restores modest CTL activity in MHC class II^{-/-} mice immunized with plasmid DNA

Nonspecific CD40 activation by Ab ligation has been shown to substitute entirely for T cell help in generating CTL via cross-priming (8, 10, 11). A similar strategy was used by injecting plasmid DNA encoding the Ag and a CD40-activating Ab, FGK45, into IA^b-deficient mice (Fig. 4). The CD40-activating Ab was only able to restore a partial CTL response compared with wild-type mice. These data suggest that although CD40/CD40L interaction is critical for generating a CTL response, it is not sufficient for the magnitude of the entire response. Hence plasmid DNA injection likely uses both CD40-dependent and -independent pathways of T cell help in the induction of a CTL response.

Plasmid DNA encoding B7.1 enhances CTL independent of CD40

Other signaling components involved in naive T cell activation include CD28 and B7 ligands. To further investigate the minimal requirements necessary to prime a MHC class I-restricted CTL response, MHC II^{-/-} mice were injected with combinations of plasmids encoding Ag and the costimulatory ligands B7.1 and B7.2 (Fig. 5). The B7.1-coinjected mice were capable of generat-

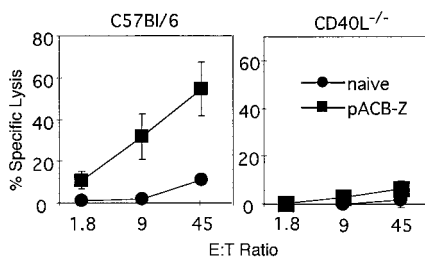


FIGURE 3. CTL induction by pDNA inoculation is dependent on CD40L signaling. Four B6/129 F₂ and CD40L^{-/-} mice per group were injected intradermally at the tail base on days 0, 7, and 14 with 50 μ g of plasmid-encoding β -galactosidase. After 6 wk, the mice were sacrificed and their splenocytes were assayed for their ability to lyse DAPIYTNV peptide-pulsed EL4 cells after 5 days of *in vitro* restimulation. Values plotted are average peptide-specific background lysis \pm SEM. The maximum lysis seen with the negative control peptide (ASNENMETM) was 3.3%. This experiment was performed twice.

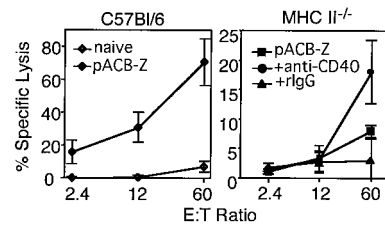


FIGURE 4. CD40 ligation restores modest CTL activity in MHC class II^{-/-} mice immunized with plasmid DNA. Four adult C57Bl/6 or MHC class II^{-/-} mice per group were injected with 50 μ g pACB-Z intradermally on days 0, 7, and 14. Additionally, some groups concurrently received 100 μ g of FGK45 (CD40-activating Ab) or rat IgG control Ab *i.p.* After 6 wk, the mice were sacrificed and their splenocytes were assayed for their ability to lyse EL4 cells pulsed with DAPIYTNV peptide. Values plotted are average peptide-specific background lysis \pm SEM. The maximum lysis seen with the negative control peptide (ASNENMETM) was 2.7%. This experiment was performed twice.

ing a measurable CTL response, whereas the B7.2-coinjected mice were not able to mount a response. The frequency of cells that secreted IFN- γ in response to *in vitro* peptide stimulation was also augmented in the B7.1-coinjected group. Previous experiments showed that coexpression of B7.1 in the vicinity of a minimal MHC class I-restricted peptide was sufficient to prime a CTL response in the absence of CD4⁺ T cell help (22). In other experiments using wild-type mice, the B7.2 plasmid was shown to augment Ab and responses associated with the presence of MHC class II epitopes (data not shown and Ref. 22).

Expanding on the use of plasmid-expressed costimulatory ligands, the B7.1- and B7.2-encoding plasmids were also tested in CD40L-deficient mice. Again, coadministration of the B7.1-expressing plasmid was able to generate CTL (Fig. 5). The priming of peptide-specific precursor cells with this combination was confirmed by ELISPOT. This stimulation suggests that the administration of a B7.1 transgene can work independently of CD40 signaling. The effect of B7.1 costimulation and CD40 ligation are not limited to β -galactosidase, as similar results were obtained using OVA as the Ag (Fig. 6). In side-by-side comparisons, the B7.1 and anti-CD40 protocols appeared to be roughly equivalent in priming functional CTL responses in MHC class II-deficient mice (Figs. 6 and 7).

Coadministration of B7.1-expressing plasmid and concurrent CD40 ligation synergistically restores CTL activity in MHC class II^{-/-} mice

As both B7.1 and CD40 appeared to be influential in the generation of CTL following plasmid DNA injection, they were tested in conjunction with one another (Fig. 7). In the experiment presented, the groups that received either CD40-activating Ab or the B7.1 plasmid had a small but detectable CTL response. However, there was an additive effect seen in the group that received the combination of costimulatory signals. The MHC class II^{-/-} mice that received both CD40-activating Ab and plasmid-encoding B7.1 mounted a CTL response similar to the wild-type animals in the control group. A variety of potential mechanisms are suggested by these results. The CD40-activating Ab may be acting in a short temporal window, whereas Ag-bearing B7.1-expressing cells may be continually present to stimulate T cells. This hypothesis suggests that B7.1-transfected cells are activating a separate set of T cells increasing the total number of precursor cells. Alternatively, the B7.1-transfected cells may have been providing additional signals through CD28, enhancing the stimulus to the same cohort of T

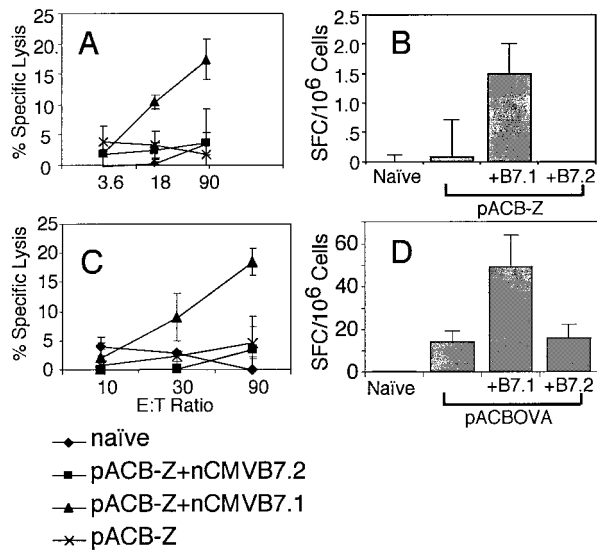


FIGURE 5. Plasmid DNA encoding B7.1 enhances CTL in CD40L^{-/-} and MHC class II^{-/-} mice. **A**, Four adult CD40L^{-/-} mice per group were injected with 50 μ g pACB-Z intradermally and the indicated plasmid on days 0, 7, and 14 or left untreated. After 6 wk, the mice were sacrificed and their splenocytes were assayed for their ability to lyse EL4 cells pulsed with DAPIYTNV peptide. **B**, The splenocytes were plated in graded amounts with and without DAPIYTNV peptide overnight. The number of peptide-specific IFN- γ -secreting spot-forming cells (SFC) were assayed in parallel. **C**, Similarly, four adult MHC class II^{-/-} mice per group were injected with 50 μ g pACB-Z intradermally and the indicated plasmid on days 0 and 7 or left untreated. After 2 wk, the mice were sacrificed and their splenocytes were assayed for their ability to lyse EL4 cells pulsed with DAPIYTNV peptide. Values plotted are average peptide-specific background lysis \pm SEM. The maximum lysis seen with the negative control peptide (ASNENMETM) was 3.6%. This experiment was performed three times. **D**, Four adult MHC class II^{-/-} mice per group were injected with 50 μ g pACB-OVA intradermally and the indicated plasmid on days 0, 7, and 14 or left untreated. After 4 wk, the mice were sacrificed. The splenocytes were plated in graded amounts with and without SIINFEKL peptide overnight. The number of peptide specific SFCs were assayed by ELISPOT. Shown are the averages for four mice per group + SEM.

cells. Thus, CD40 activation may result in suboptimal B7.1 expression, but augments other surface ligands, and the addition of B7.1-expressing plasmid further compensates for the lack of T cell help. These results indicate that the signals from Th cells to condition the APC are optimal when they are continually present while APCs acquire Ag and are sufficiently potent to activate cells despite low levels of Ag.

Discussion

DNA vaccines are being evaluated as an attractive alternative to conventional protein vaccines as they can induce potent CTL responses. The cellular mechanisms involved in establishing these responses are being investigated. Clearly, MHC class II presentation plays a decisive role in the induction of CTL following plasmid DNA vaccination (13, 22). However, there appear to be MHC class II-restricted T cells that are capable of providing support for a weak CTL response in CD4-deficient mice. This dependence on CD4⁺ T cell help for the generation of CTL suggests cells presenting Ag do not encounter adequate amounts of DNA to be sufficiently activated to prime CTL.

The cells that have been described to express the encoded Ag are predominantly found at the site of plasmid inoculation (23). However, studies using bone marrow chimeras showed that the

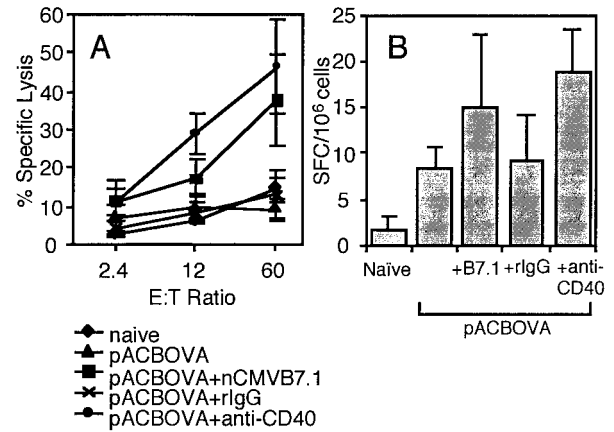


FIGURE 6. Plasmid DNA encoding B7.1 and anti-CD40 ligand enhance CTL similarly. Four adult MHC class II^{-/-} mice per group were injected with 50 μ g pACB-OVA intradermally and the indicated plasmid or Ab on days 0, 7, and 14 or left untreated. After 4 wk, the mice were sacrificed and their splenocytes were assayed for their ability to lyse EL4 cells pulsed with SIINFEKL peptide. Values plotted are average peptide-specific background lysis \pm SEM (**A**). The splenocytes were also titrated with and without SIINFEKL peptide overnight. The number of peptide-specific IFN- γ -secreting cells were assayed. ELISPOTS were performed in duplicate for each dilution, and shown are the average number of peptide-specific spot-forming cells (SFC) per million for four mice per group + SEM (**B**).

antigenic peptide involved in priming a CTL response is presented in the context of MHC class I molecules on bone marrow-derived cells and not by injected myocytes (6, 7, 24). Thus, immune responses are initiated by Ag that is acquired by APCs. These APCs may either become directly transfected themselves or process extracellular proteins. A role for directly transfected Langerhans cells was suggested by the appearance of Ag-expressing dendritic cells in draining lymph nodes following biolistic immunization (25, 26). However, in our studies the MHC class II^{-/-} mice were unable to mount a CTL response consistent with a cross-priming mechanism rather than endogenous expression of the Ag by dendritic cells similar to viral infections (8).

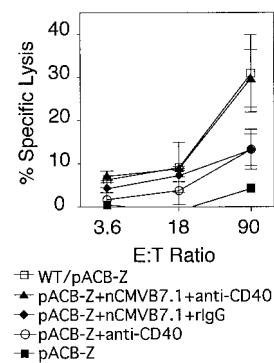


FIGURE 7. Coadministration of B7.1-expressing plasmid and concurrent CD40 ligation synergistically restores CTL activity in MHC class II^{-/-} mice. Four adult wild-type (WT) and MHC class II^{-/-} mice per group were injected with 50 μ g pACB-Z intradermally and the indicated plasmid on days 0, 7, and 14 or left untreated. Additionally, some groups concurrently received 200 μ g of FGK45 (CD40-activating Ab) or rat IgG control Ab i.p. After 6 wk, the mice were sacrificed and their splenocytes were assayed for their ability to lyse EL4 cells pulsed with DAPIYTNV peptide. Values plotted are average peptide-specific background lysis \pm SEM. The maximum lysis seen with the negative control peptide (ASNENMETM) was 3.7%. This experiment was performed twice.

Recently, the need for CD4⁺ T cell help was described for generating CTL via cross-priming mechanisms (9). This CD4⁺ T cell help was originally described to be Ag specific (9); however, a nonspecific stimulus through CD40 was later shown to restore CTL by cross-priming in MHC class II^{-/-} mice (8, 10, 11). Bystander T cell help from coadministration of a plasmid expressing β -galactosidase was proficient in generating CTL against the OVA-derived SIINFEKL epitope encoded by a minigene. This result suggested that the signals required for priming CTL from plasmid injection could be Ag independent or nonspecific. Plasmid DNA encoding MHC class I-restricted epitopes has been used to selectively induce CTL without generating an Ab response (22, 27). The addition of a well-chosen signal sequence to facilitate transport of the minigene-encoded peptide into the endoplasmic reticulum could additionally enhance the generation of CTL by creating MHC class II-restricted neopeptides that stimulate T cell help.

To further evaluate the costimulation necessary for the generation of CTL in MHC class II^{-/-} mice, nonspecific activation through CD40 cross-linking was attempted. The CTL response was only modestly restored following CD40 ligation. In contrast, protein either coadministered or directly conjugated to CpG-containing oligonucleotides induces CTL in the absence of MHC class II or CD40L (28). There is an adjuvant effect of the CpG dinucleotides motifs in unmethylated DNA that results in activation of the innate immune system and stimulation of a predominantly Th1 response (29). The difference between plasmid injection and oligonucleotide and protein coadministration may be due to the relative strength of the stimulus from the unmethylated CpG of phosphothioate oligonucleotides compared with the phosphodiester nucleotides in plasmid DNA. Alternatively, the amount of antigenic material may dictate the relative dependence on costimulatory signals (30).

Previously, coinoculation of plasmids encoding Ag and the costimulator molecules B7.1 and B7.2 enhanced CTL production by plasmid DNA vaccination (22, 31–33). The two costimulatory ligands showed a divergence of functions where B7.2 preferentially augmented responses when MHC class II epitopes were present, and B7.1 was able to generate a CTL response to a MHC class I epitope in the absence of MHC class II epitopes (22). Similar results were seen using MHC class II^{-/-} mice in this report. In the presence of costimulation by B7.1, MHC class II-restricted Th cells were not necessary for CTL induction. This activation of naive CD8⁺ T cells by B7.1 costimulation was similar to reports in several tumor models (34–37).

Ligation of CD40 has been shown to result in up-regulation of B7.1 and B7.2 on APCs (38). However, there is ~100-fold increase in the surface expression of B7.2 and only 10-fold induction of B7.1 (39). The lower level of B7.1 may be related to the relatively modest induction of CTL in MHC class II^{-/-} mice following CD40 activation with plasmid DNA injection. Plasmid DNA vaccination probably uses CD40-dependent and -independent signaling pathways of dendritic cell conditioning by MHC class II-restricted T cells (40). This lends itself to a model whereby CD40 signaling leads to the up-regulation of costimulatory molecules, but additional signals probably augment the expression of B7.1 on APCs (41) to prime a more potent CTL response.

The generation of a strong CTL response would be advantageous for vaccines directed against tumors and infectious agents. A long-lived cellular immunity can be generated in murine models with plasmid and other DNA-based vaccines. The strategies to optimize these vaccines should include a MHC class II-restricted epitope as well as the candidate MHC class I-restricted epitopes (42–44). The addition of B7.1 continuously expressed at a site

distal to the infection or tumor may provide perpetual CTL activation that could overcome some of the immunosuppressive factors seen in these diseases. The simplicity of gene vaccination with a combination of factors that would allow selective CTL generation warrants future studies.

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