

DNA Vaccines, Combining Form of Antigen and Method of Delivery to Raise a Spectrum of IFN- γ and IL-4-Producing CD4⁺ and CD8⁺ T Cells¹

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DNA-based immunizations have been used to determine the patterns of type 1 and type 2 cytokines that can be induced in vivo for Ag-specific CD4⁺ and CD8⁺ T cells. IL-4 was used as a signature cytokine for a type 2 T cell response and IFN- γ as the signature cytokine for a type 1 response. Gene gun deliveries of secreted Ags were used to bias responses toward type 2 and saline injections of cell-associated Ags to bias responses toward type 1. The studies revealed that gene gun bombardments of DNAs expressing secreted Ags strongly biased responses toward type 2, inducing IL-4-producing CD8⁺ as well as CD4⁺ T cells. Saline injections of DNAs expressing cell-associated Ags strongly biased responses toward type 1, inducing IFN- γ -producing CD8⁺ and CD4⁺ cells. A mixed type 1/type 2 response of IFN- γ -producing CD8⁺ T cells and IL-4-producing CD4⁺ T cells was found for gene gun deliveries of cell-associated Ags. Saline injections of secreted Ags raised a weakly type 1-biased response characterized by only slightly higher frequencies of IFN- γ - than IL-4-producing CD4⁺ and CD8⁺ T cells. Studies in B cell knockout and hen egg lysozyme Ig transgenic mice revealed that B cells were required for the generation of IL-4-producing CD8⁺ T cells. *The Journal of Immunology*, 2003, 171: 1999–2005.

In this study, we use DNA-based immunizations to ask whether immunizations that affect the cytokine patterns of Ag-induced CD4⁺ T cells also affect the pattern of cytokines expressed by Ag-induced CD8⁺ T cells. Injections of DNA in saline tend to elicit CD4⁺ T cells that produce IFN- γ , the signature cytokine for type 1 T cell help (Th1 cells). In contrast, gene gun deliveries of DNA coated onto gold beads to the epidermis tend to elicit CD4⁺ T cells that produce IL-4, the signature cytokine for type 2 T cell help (Th2 cells) (1, 2). In murine models, these signature cytokines are accompanied by different predominant isotypes of Ag-specific Ab. IFN- γ -producing CD4⁺ T cells support rearrangements of Ig H chain to IgG2a whereas IL-4-producing CD4⁺ T cells are associated with rearrangements to the IgG1 or IgE isotypes (3). In general, DNAs that express secreted Ags establish stronger IL-4- and IgG1-biased responses than DNAs that express cell-associated Ags (as reviewed in Ref. 4). Thus, a continuum in the biases of responses toward type 1 or type 2 T cell help can be found in DNA-based immunizations with gene gun delivery of secreted Ags eliciting the strongest Th2-biased responses and saline injections of cell-associated Ags initiating the strongest Th1-biased responses.

Classically, effector CD8⁺ T cells have been thought of as cytolytic cells (Tc) that produce high levels of IFN- γ (5, 6). However, when CD8⁺ T cells are cultured in the presence of IL-4, IL-2,

and blocking Ab to IFN- γ , IL-4-producing CD8⁺ T cells are established (7). These IL-4-producing CD8⁺ T cells are called Tc2 cells to distinguish them from IFN- γ -producing CD8⁺ T cells, which are referred to as Tc1. Tc2 cells have been shown to have lytic function equivalent to Tc1 cells in vitro but when infused into mice, Tc2 have exhibited differences in their ability to traffic to sites of viral infection or metastases (8, 9).

Below we report the induction of IL-4-producing Ag-specific CD8⁺ T cells by the subset of DNA-based immunizations that are the most strongly biased toward IL-4-producing CD4⁺ T cells: gene gun delivery of DNAs expressing secreted Ags. Gene gun immunizations with DNAs expressing cell-associated Ags raised predominantly IL-4-producing CD4⁺ T cells but IFN- γ -producing CD8⁺ T cells. We also demonstrate that the ability of gene gun-delivered secreted Ags to raise IL-4-producing CD8⁺ T cells is dependent on the presence of B cells and suggest that the IL-4 bias of Tc2 cells reflects the secreted Ag undergoing uptake and presentation by B cells.

Materials and Methods

Cell lines, animals

Human embryonic kidney cells (293T; American Type Culture Collection, Manassas, VA) were used for transient transfections of DNA expression vectors. Female 6- to 8-wk-old BALB/c and C57BL/6 mice (Charles River Breeding Laboratories, Wilmington, MA), C57BL/6 μ MT mice (10) (The Jackson Laboratory, Bar Harbor, ME), and B cell transgenics for hen egg lysozyme (HEL)³ (11) (a kind gift from Dr. R. Mittler, Emory Vaccine Research Center) were used for immunizations.

Plasmid DNA

DNA vaccines were constructed in pGA vectors that use the CMV immediate early promoter, including intron A and the bovine growth hormone polyadenylation sequence for transcriptional control, and include a Kozak sequence to promote ribosome entry onto mRNAs (12). The pGA expression vectors for influenza A/PR/8/34 tMHA, sHA, and sHA(C3d)₃ have

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³ Abbreviations used in this paper: HEL, hen egg lysozyme; HA, hemagglutinin; NP, nucleoprotein; TLR9, Toll-like receptor 9.

been previously described (13); a pGA vector encoding influenza nucleoprotein (NP) was generated from the NP gene from A/PR/8/34 in pCMV/NP using standard techniques (14). ptmHA-OVA expressed the complete A/PR/8/34 H1 hemagglutinin (HA) fused to a C-terminal H-2^b-restricted OVA class I (SIINFEKL), class II (ISQAVHAHAHAINEAGR), and FLAG (DYKDDDDK; Stratagene, La Jolla, CA) epitopes. ptmHA-NP expressed HA fused to C-terminal H-2^b-restricted NP class I (ASNENMDTM) and class II (ARSALILRGSAHKSCLPACVYGP) epitopes. psHA-OVA and psHA-NP expressed these same epitopes fused to a secreted form of HA that was truncated at nt 1633, immediately upstream of its transmembrane and cytoplasmic regions. These plasmids were constructed using oligonucleotides and standard molecular biology techniques. All DNA used in vaccination studies was grown in DH5 α *Escherichia coli* and purified using Endotoxin-free Maxiprep or Gigaprep kits (Qiagen, Valencia, CA).

DNA immunization

For saline-based immunizations, mice were anesthetized with ketamine: xylazine (10:1) and injected with 50 μ l of a 1 μ g/ μ l solution of DNA dissolved in sterile PBS into each quadriceps (100 μ g of total DNA/mouse). Gene gun-vaccinated mice were anesthetized and the abdomens shaved and bombarded with two shots of a 2 μ g of DNA/mg of gold powder (1 mg of gold/mouse). DNA-loaded gold beads were generated as previously described (14).

ELISA for Abs

Sera were assayed by ELISAs to measure influenza-specific IgG1 and IgG2a as described (15).

ELISPOT analyses

Splenocytes were harvested at 2 wk following booster immunizations and processed as described elsewhere (16). Briefly, multiscreen 96-well plates (Millipore, Billerica, MA) were coated overnight at 4°C with 4 μ g/ml anti-mouse IFN- γ or IL4 Ab (R4-6A2 or BVD4-1D11; BD PharMingen, San Diego, CA) and then washed three times with 0.1% Tween 20 in PBS. Peptides were dissolved in DMSO in a 0.01 M stock solution and stored at -20°C. Stimulations of splenocyte preparations were performed in RPMI 1640 containing 10% FBS, 10 mM L-glutamine, nonessential amino acids, HEPES buffer, and penicillin/streptomycin (complete medium). Peptides were diluted in complete medium and used at a final concentration of 10 μ M. The choice of peptides to be used in these assays was determined by previous reports (17–21). For the HA immunogen, H-2^d-restricted HA class I peptide (LYEKVKSQL) and a pool of five class II peptides (SFERFEIFPKE, HNTNGVTAACSH, CPKYVRSALRM, KLKNSYVNKKGK, and NAYVSVVTSNYNRRF) were used. For the NP immunogen, H-2^d-restricted NP class I peptide (TYQRTRALV), H-2^b-restricted class I peptide (ASNENMDTM), a pool of three H-2^d-restricted class II peptides (FWRGENGRKTRSA YERM CNILKKGK, RLIQNSLTIERMVLSAFDE RNK, and AVKGVGTVMELIRMIKRGINDRN), and one H-2^b-restricted class II peptide (ARSALILRGSAHKSCLPACVYGP) were similarly used. For OVA responses, H-2^b-restricted OVA class I peptide (SIINFEKL) and class II peptide (ISQAVHAHAHAINEAGR) were used. An irrelevant peptide derived from HIV envelope protein (IGPGRAFYAR) (22) was used as a negative control for T cell responses in BALB/c mice, whereas either H-2^b-restricted OVA or NP peptides were used as negative controls in C57BL/6 mice not vaccinated with vectors encoding these peptides. PMA plus ionomycin (50 ng/ml PMA and 500 ng/ml ionomycin) were used as positive controls for the induction of a cytokine response. One million or 500,000 cells were incubated in duplicate wells in the presence of the optimal concentration of peptide and 1 μ g/ml costimulatory anti-CD28 and anti-CD49d Abs (37.51 and R1-2, respectively; BD PharMingen) (23) for 36 to 40 h at 37°C in a humidified atmosphere containing 5% CO₂. The plates were washed five times with 0.1% Tween 20 in PBS and incubated with 0.5 μ g/ml biotin-conjugated anti-mouse IFN- γ or IL-4 Abs (XMGI.2 or BVD6-24G2; BD PharMingen) overnight at 4°C. Plates were subsequently washed five times and treated with streptavidin-HRP (Vector Laboratories, Burlingame, CA) followed by development with stable diaminobenzidine (Research Genetics, Huntsville, AL). Spot-forming units were counted using an Immunospot cell counting device (Cellular Technologies, Cleveland, OH) and normalized for 10⁶ splenocytes. All counts were adjusted for background (stimulation with an irrelevant peptide) before averaging.

Cell depletion and flow cytometry

Total splenocytes were depleted of either CD4 or CD8 cells by magnetic beads (Dyna, Delaware, NY). The purity of the depleted populations was

verified by the use of fluorescent-labeled anti-mouse CD4 and CD8 Abs (L3T4 and Ly-2; BD PharMingen).

Statistical analysis

ELISPOTs were performed in duplicate wells per stimulation condition for a given mouse. The duplicate well counts were averaged and these averages were averaged and SDs calculated for the three to five mice in each group. A one-way ANOVA was used to test for differences in the ratios of IL-4 and IFN- γ ELISPOTs among groups. A value of $p < 0.05$ was considered significant. For two of the measures, the data distributions were strongly skewed and, therefore, did not meet the requirements for ANOVA. In these two cases, we used the nonparametric Wilcoxon test for independent samples. A two-sided $p < 0.05$ was considered statistically significant.

Results

Optimizing the conditions for detection of cytokine-producing cells

Initially, experiments were conducted to optimize the detection of IL-4 as well as IFN- γ -producing T cells. The highest frequencies of IL-4- and IFN- γ -producing CD8⁺ T cells were seen when Abs to CD28 and CD49d were added to peptide stimulations (Fig. 1). The frequencies of responding T cells were measured using known class I and class II peptides for influenza A/PR/8/34 HA and NP in an ELISPOT assay (23). Total splenocytes from BALB/c mice, infected at least 4 wk earlier with a sublethal dose of influenza A/PR/8/34, were harvested, processed, and stimulated in the presence of varying concentrations of peptide. The cultures were supplemented with or without 1 μ g/ml Abs that recognize murine CD28 and CD49d. The choice of these Abs was based on a study demonstrating improved Ag-specific CD4⁺ T cell responses in human peripheral blood stimulated in the presence of Ag and Ab to human CD28 and CD49d (24). CD28 is a surface-expressed protein with a well-characterized costimulatory role in T cell activation (for review, see Ref. 25). CD49d is an integrin molecule involved in cell-cell adhesion (for review, see Ref. 26). Addition of these Abs increased the frequencies of both class I and class II

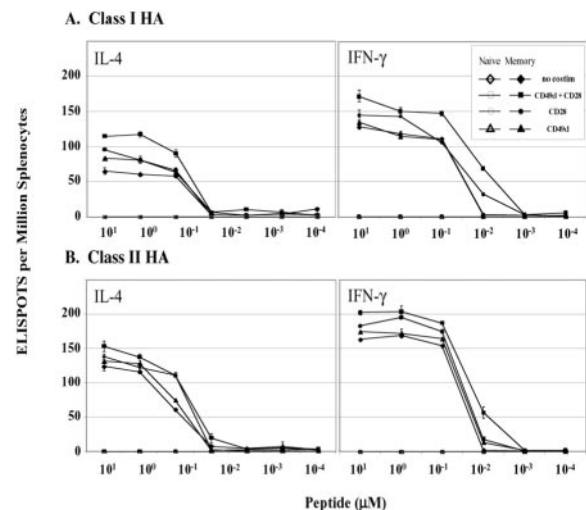


FIGURE 1. Enhancement of the detection of IL-4- and IFN- γ -producing ELISPOTs by Ab to CD28 and CD49d. Frequencies of IL-4 and IFN- γ ELISPOTs in response to stimulation with class I (A) or class II (B) HA peptides. BALB/c splenocytes pooled from three mice immunized intranasally with a sublethal dose of A/PR/8/34 influenza virus >4 wk earlier were used for stimulations. The graphs are plotted based on the average ELISPOTs for replicate wells. Peptides were serially diluted in stimulation media containing 1 μ g/ml of the indicated costimulatory Ab. Increased responses in the presence of the costimulatory Ab were seen in three independent experiments.

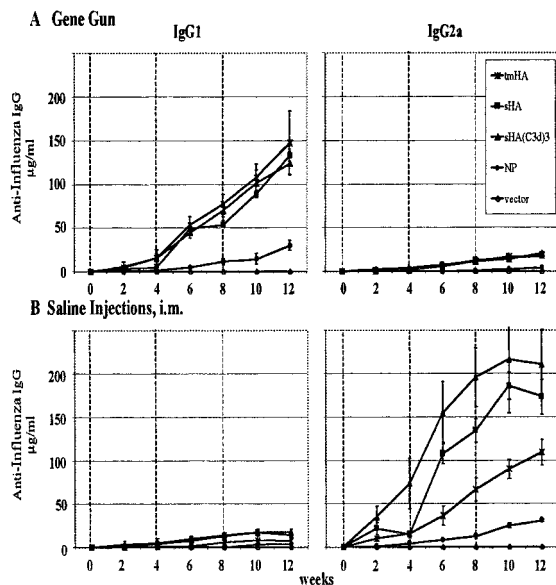


FIGURE 2. Temporal levels of anti-influenza IgG1 and IgG2a in DNA-vaccinated mice. Anti-influenza IgG1 and IgG2a in BALB/c mice receiving gene gun injections of 2 µg (A) or i.m. saline injections of 100 µg (B) of DNA encoding sHA, sHA(C3d)₃, tmHA, NP, or empty vector. DNA was delivered at 0, 4, and 8 wk and sera were tested for influenza-specific Ab every 2 wk. Each point represents average isotype levels for three to five mice with SDs. Vertical dashed lines indicate DNA deliveries.

Ag-specific IL-4- and IFN-γ-producing T cells by up to 2-fold (Fig. 1). Although most of the increase was seen with anti-CD28 alone, responses were consistently the highest when both of the Abs were added to cultures. Unvaccinated mice, or mice vaccinated with empty vector, gave no peptide-specific ELISPOTs in the presence or absence of the costimulatory Abs (Fig. 1 and data not shown). Moreover, the costimulatory Abs did not induce the production of IL-4 or IFN-γ without the addition of the cognate peptide at the appropriate dosages, suggesting that TCR-mediated signaling was required to induce cytokine production (Fig. 1). Henceforth, all peptide-specific stimulations were supplemented with anti-CD28 and anti-CD49d Abs.

Ig and CD4⁺ T cell biases elicited by gene gun and saline injections of vaccine DNAs

The establishment of the expected IgG1-biased immune responses by gene gun delivery of DNA and IgG2a-biased responses by i.m. saline injections of DNA was verified using plasmids expressing two secreted and two cell-associated Ags. The secreted Ags were a truncated form of HA (sHA) that had been deleted for its trans-membrane and cytoplasmic regions, and sHA fused to three copies of the complement component C3d (sHA(C3d)₃) (13). The cell-associated Ags included the membrane-bound form of HA (tmHA) and the influenza virus NP. As previously reported (1), both the secreted and cell-associated Ags primed predominantly IgG1 responses when delivered by gene gun, but predominantly IgG2a when delivered by i.m. saline injections (Fig. 2). A positive control for each experiment included serum from mice infected with A/PR/8/34 influenza virus which elicited a predominantly IgG2a response (data not shown). The Ig-isotype biases were stable over time and did not change with the number of DNA inoculations (Fig. 2).

Consistent with the Ig bias of the Ab responses, the gene gun immunizations raised predominantly IL-4-producing CD4⁺ T cells (Th2-biased responses), whereas i.m. injections raised predominantly IFN-γ-producing CD4⁺ T cells (Th1-biased responses) (Fig. 3). Splenocytes harvested at 2 wk after the second DNA immunization were stimulated with class II peptides for HA and NP for 36 h and scored in ELISPOT assays for IL-4- and IFN-γ-producing cells. All expression vectors delivered via gene gun raised 1.5 to 4 times more Ag-specific IL-4 than IFN-γ-producing CD4⁺ T cells (Fig. 3A). In contrast, i.m. injections raised from 1.5 to 3 times more IFN-γ than IL-4-producing CD4⁺ T cells (Fig. 3B). A sublethal influenza virus infection primed three to four times more IFN-γ than IL-4-producing cells (Fig. 3). In all experiments, the Ig isotype profiles were in accordance with the type of Th response, consistent with the observation that Ig rearrangement is influenced by the cytokine profiles of the Ag-specific Th cells (27).

CD8 T cell biases elicited by gene gun and saline injections of vaccine DNAs

Splenocytes from the same mice analyzed for CD4⁺ T cell responses in Fig. 3 were tested for the frequencies and cytokine patterns of CD8⁺ T cells by stimulation with class I peptides for

FIGURE 3. Th2-biased responses in gene gun-vaccinated mice and Th1-biased responses in i.m. vaccinated mice. Frequencies of class II-specific HA and NP responses in mice receiving gene gun bombardments of 2 µg of DNA (A) or saline injections of 100 µg of DNA (B). BALB/c mice received vaccinations at 0 and 4 wk and splenocytes were analyzed at 6 wk for IFN-γ or IL-4 class II peptide-stimulated ELISPOTs. Data are averages and SDs for four to five mice per group and are representative of three independent experiments.

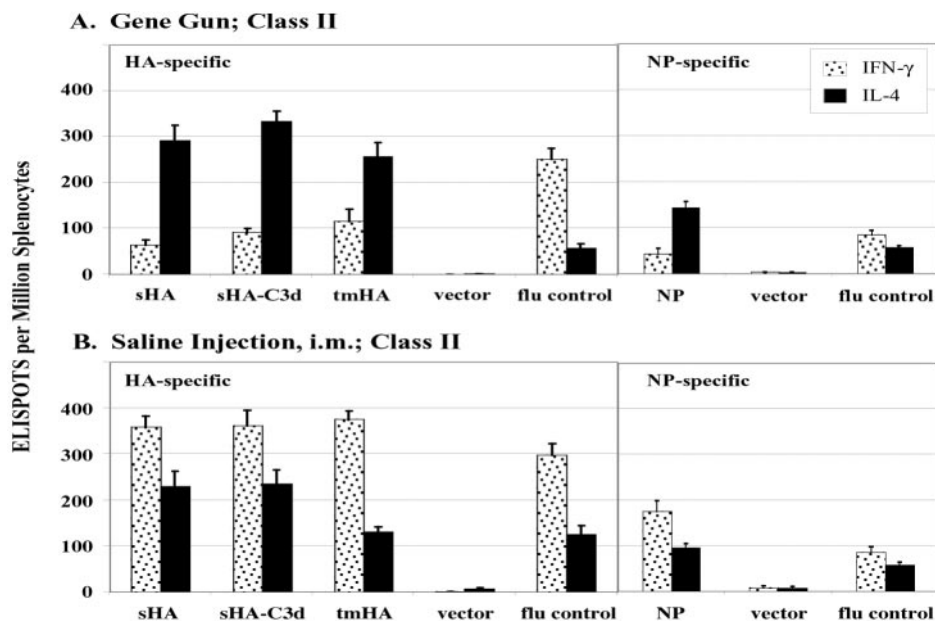
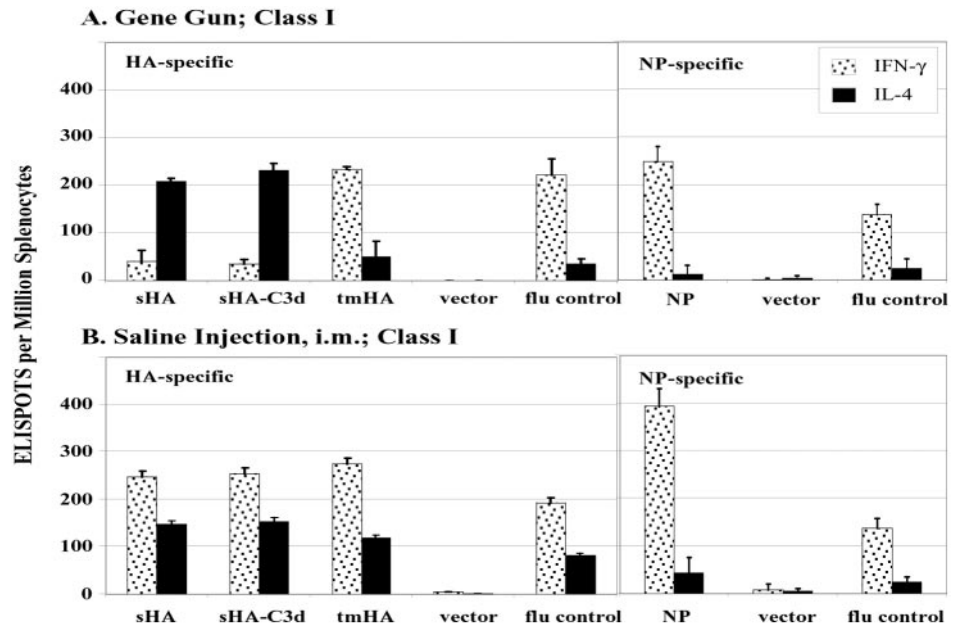


FIGURE 4. Lymphokine biases of CD8 cells raised by various immunizations. Frequency of class I-specific HA and NP responses in mice receiving gene gun inoculations of 2 μ g of DNA (A) or saline injections of 100 μ g of DNA (B). BALB/c mice were vaccinated at weeks 0 and 4 and splenocytes were analyzed at week 6 for class I peptide-stimulated IFN- γ and IL-4 ELISPOTs. Data are averages and SDs for four to five mice per group and are representative of three independent experiments.



either HA or NP (Fig. 4). Gene gun deliveries of sHA and sHA(C3d)₃, but not cell-associated tmHA or NP, primed four to five times more IL-4 than IFN- γ -producing CD8⁺ T cells (Fig. 4A). For gene gun deliveries of tmHA and NP, despite the IL-4 bias of the raised CD4⁺ T cells (Fig. 3A), most CD8⁺ T cells were IFN- γ producing (four to nine times more IFN- γ - than IL-4-producing CD8⁺ T cells). As expected, i.m. deliveries of the cell-associated Ags mounted 2.5 to 8 times more IFN- γ than IL-4-producing CD8⁺ T cells and influenza virus infections 2 to 4 times more IFN- γ than IL-4-producing cells (Fig. 4B). Intramuscular saline injections of secreted Ags raised a less strongly IFN- γ -biased profile with only 1.5 times more IFN- γ - than IL-4-producing CD8 T cells. Similar profiles occurred in independent experiments and were maintained for up to 6 mo after boost (data not shown).

Depletions of CD4⁺ and CD8⁺ T cells before ELISPOT analyses confirmed that the class II and class I peptides were stimulating CD4⁺ and CD8⁺ T cells, respectively (Fig. 5). Cells were depleted using magnetic beads coated with anti-CD4 or anti-CD8 Abs. FACS analyses of the depleted populations revealed that depletions were at least 97% effective (data not shown). As can be seen in Fig. 5, depletions of CD4⁺ cells removed IL-4-producing CD4⁺ T cells, whereas depletions of CD8⁺ cells, removed IL-4-producing CD8⁺ T cells. These results confirm that the IL-4-producing class I peptide-stimulated cells were Ag-specific CD8⁺ T cells.

Similar results in C57BL/6 mice

To allow studies in C57BL/6 mice, DNAs expressing HA molecules fused to known H-2^b class I and class II epitopes for either OVA (sHA-OVA, sHA(C3d)-OVA, and tmHA-OVA) (20, 21) or influenza NP (sHA-NP and tmHA-NP) (17, 19) were constructed using standard recombinant DNA technology. Protein expression was confirmed using transient transfections and Western blotting (data not shown). These constructs allowed assessment of whether the phenomena seen in the BALB/c mouse, an H-2^d haplotype, occurred in C57BL/6 mice, an H-2^b haplotype. Moreover, the availability of transgenic and gene knockout mice on the C57BL/6 background allowed for further mechanistic studies.

Gene gun delivery of these DNA vectors confirmed that the cytokine biases for secreted and cell-associated Ags observed in

BALB/c mice were the same in C57BL/6 mice (Fig. 6). DNA immunizations at 0 and 4 wk and ELISPOT analyses at 6 wk revealed that both class I and class II responses to sHA-NP (Fig. 6A) and sHA-OVA (Fig. 6B) consisted of predominantly IL-4-producing CD8⁺ T cells and IL-4-producing CD4⁺ T cells. These same NP and OVA peptides stimulated predominantly IFN- γ -producing CD8⁺ T cells but IL-4-producing CD4 cells following gene gun immunizations with tmHA-OVA and tmHA-NP (Fig. 6). Thus, the ability to raise Tc2 in vivo appeared to be strain independent and could be studied in mice with a C57BL/6 genetic background.

Role of B cells in raising IL-4-biased CD8⁺ T cells

In the next series of experiments, gene gun immunizations in B cell-deficient mice revealed that B cells were required for eliciting IL-4-producing CD8⁺ T cells. Cytokine responses to DNA vaccines encoding either sHA-NP or tmHA-NP were compared in μ MT^{-/-} B cell deficient and wild-type C57BL/6 mice (Fig. 7A, left panel). In the absence of B cells, gene gun delivery of sHA-NP elicited ~4.2-fold greater IFN- γ - than IL-4-producing CD8⁺ T cells. In contrast, in wild-type mice, there was a 6.4-fold greater IL-4 ELISPOT count relative to IFN- γ (Fig. 7A, left panel). The

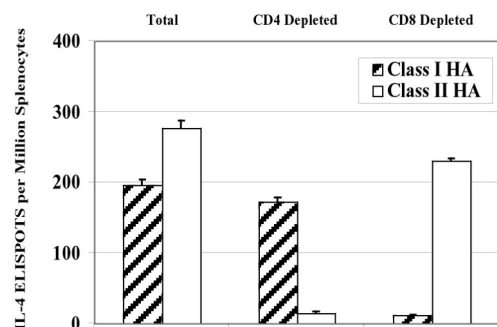
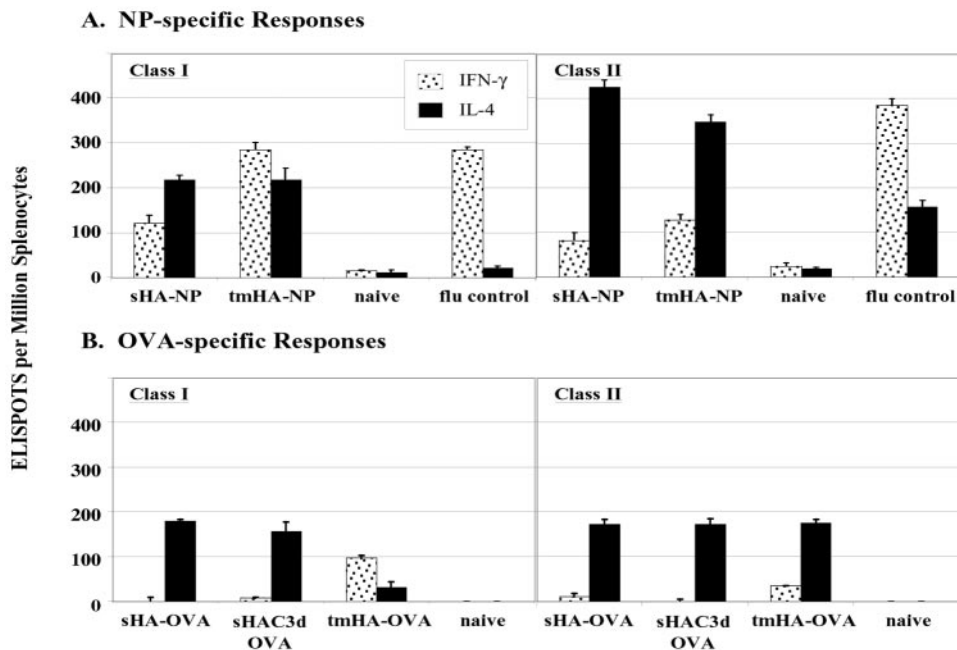


FIGURE 5. Class I peptide-stimulated IL-4 responses are restricted to CD8⁺ cells and class II peptide-stimulated IL-4 responses are restricted to CD4⁺ cells. Total splenocytes from BALB/c mice gene gun vaccinated with 2 μ g of sHA were harvested and aliquots were magnetically depleted of either CD4⁺ or CD8⁺ cells before testing for class I and class II ELISPOTs. Depletions were >95% effective for the target population.

FIGURE 6. IL-4-producing CD8 cells can be raised in the C57BL/6 mouse model. Class I- and class II-restricted IFN- γ and IL-4 ELISPOT responses to NP epitopes (A) and OVA epitopes (B). C57BL/6 mice were gene gun vaccinated at weeks 0 and 4 with 2 μ g of the indicated plasmids and splenocytes were harvested and stimulated in the presence of class I or class II NP or OVA peptides at week 6. Cytokine responses for either IFN- γ or IL-4 were assessed by ELISPOT. Data are the averages and SDs for four to five mice per group and are representative of three independent experiments.



ratios for IFN- γ to IL-4 production were compared between these two groups and found to be significantly different ($p < 0.01$). The absence of B cells also resulted in a shift from an IL-4-bias to approximately equal frequencies of IL-4- and IFN- γ -producing

CD4 $^{+}$ T cells (Fig. 7B, right panel). As expected, the absence of B cells did not affect the 2.5- to 3-fold predominance of IFN- γ ELISPOTS raised by gene gun delivery of the tmHA-NP ($p > 0.4$; Fig. 7A, middle panel).

To test whether Ag-specific B cells might be required for raising IL-4-producing CD8 $^{+}$ T cells, transgenic mice for HEL-Ig were gene gun immunized with the sHA-NP construct (Fig. 7B, left

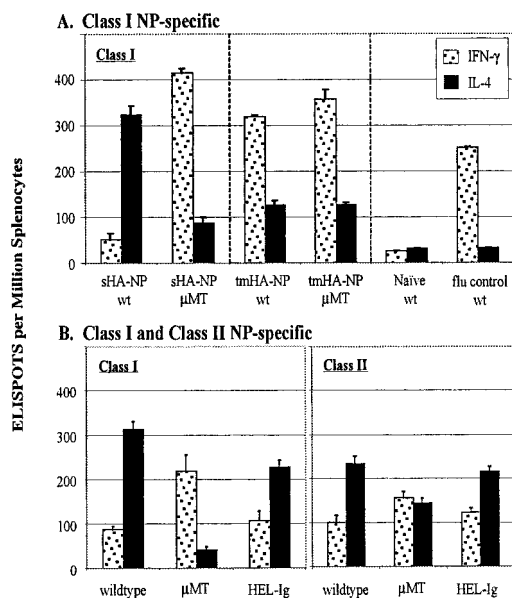


FIGURE 7. B cells are required for eliciting IL-4-producing CD8 cells. A, μ MT $^{-/-}$ as well as wild-type (wt) C57BL/6 mice were gene gun immunized with 2 μ g of the indicated DNAs at weeks 0 and 4 and splenocytes were harvested at week 6. The ratios for IFN- γ :IL-4 ELISPOTS in the wild-type and μ MT $^{-/-}$ mice for sHA are significantly different ($p < 0.01$), whereas the ratios for tmHA are not significantly different ($p > 0.4$). B, Mice transgenic for HEL-Ig, μ MT $^{-/-}$, and wild-type C57BL/6 mice were gene gun immunized with 2 μ g of sHA-NP at 0 and 4 wk and splenocytes were harvested at 6 wk. The ratios for IFN- γ :IL-4 ELISPOTS in the wild-type, μ MT $^{-/-}$, and HEL-Ig Tg mice are different for each group ($p < 0.05$). For both experiments, splenocytes were tested for IL-4- and IFN- γ -producing H-2 ^{b} -restricted class I and class II NP-specific ELISPOTS. Data are the averages and SDs for four to five mice per group and are representative of two independent experiments.

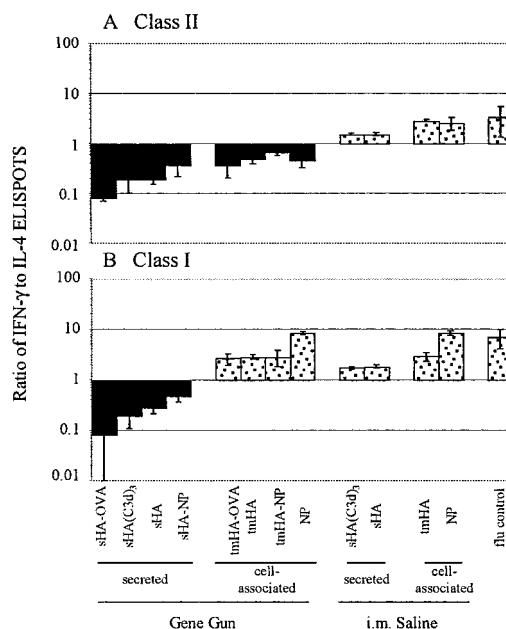


FIGURE 8. Ratio of IFN- γ :IL-4 responses. Data summarize the ratios of IFN- γ to IL-4-producing ELISPOTS for experiments in BALB/c mice. The bars present the means $\pm$ SDs for the ratios of IFN- γ ELISPOTS to IL-4 ELISPOTS from three independent experiments. Values >1 are considered biased toward a type 1 CD4 or CD8 response, whereas values <1 are considered to be biased toward type 2 responses. In instances where responses are close to the axis of 1, our assays do not distinguish whether the response is an equal mixture of IL-4- and IFN- γ -producing T cells or whether cells are Th0 cells that produce both of these cytokines.

panel). Splenocytes harvested from these mice and stimulated with class I peptide revealed a 2- to 3-fold predominance of IL-4-producing CD8⁺ T cells, which was lower than the 3- to 4-fold predominance observed in wild-type mice but different from the 5- to 6-fold predominance of IFN- γ -producing ELISPOTs in the μ MT^{-/-} mice ($p < 0.05$). Thus, the induction of IL-4-producing CD8⁺ T cells in vivo required B cells, but was only partially dependent on Ag-specific B cells.

Discussion

In this study, we use DNA vaccines to demonstrate that IL-4-producing CD8⁺ T cells can be generated in vivo when DNA is delivered by gene gun and the DNA-expressed Ag is secreted (Figs. 3 and 5). This pattern of immune response represented one extreme in the polarization of T cell responses that ranged from IL-4-producing CD4⁺ and CD8⁺ T cells to IFN- γ -producing CD4⁺ and CD8⁺ T cells (Fig. 8). The differences in the lymphokine patterns of raised T cells appeared to be rooted in differences in APC. In particular, we show that B cells influence the generation of IL-4 but not IFN- γ -producing CD8⁺ T cells (Fig. 7).

This study revealed a spectrum of T cell responses that was determined by the form of the Ag as well as the method of vaccination (Fig. 8). Gene gun bombardments of DNA combined with the delivery of secreted Ags initiated predominantly IL-4-producing CD8⁺ as well as IL-4-producing CD4⁺ T cells. In contrast, saline injections of DNAs expressing cell-associated Ags elicited IFN- γ -producing CD8⁺ and CD4⁺ T cells. In between these extremes of polarization, responses in which the CD8⁺ T cells were predominantly IFN- γ -producing and the CD4⁺ T cells were predominantly IL-4-producing occurred after gene gun deliveries of cell-associated Ags. And, overall similar ratios of IFN- γ - and IL-4-producing cells were raised by saline injections of secreted Ags. These similar ratios could have represented similar numbers of IL-4- and IFN- γ -producing cells or type 0 cells that produce both cytokines. As expected, a natural influenza virus infection raised predominantly IFN- γ -producing CD4⁺ and CD8⁺ T cells. Similar patterns of polarization were observed in BALB/c and C57BL/6 mice (Figs. 3 and 5). This spectrum was observed for three different CD8⁺ epitopes (NP, OVA, and HA) carried on the transmembrane or secreted forms of the influenza virus HA.

The requirement for secreted Ag for the induction of IL-4-producing CD8⁺ T cells led us to test whether B cells might be required for this phenomenon. Secreted Ags can undergo Ag processing and presentation following receptor-mediated uptake into B cells, either through Ag-specific Ig (28, 29) or other receptors such as the transferrin receptor (30). B cells, especially germinal center B cells, are strong producers of IL-4, the lymphokine that determines the raising of type 2 CD4⁺ T cell responses (31, 32). Also, it is known that when B cells are ablated, the generation of IFN- γ -producing CD4⁺ T cells is increased (33). In our studies with B cell knockout mice, gene gun delivery of a secreted Ag raised predominantly IFN- γ - instead of IL-4-producing CD8⁺ T cells and the bias of the CD4⁺ T cell response became one equally represented by IL-4 and IFN- γ -producing ELISPOTs. The dependence on B cells for the IL-4-producing CD8⁺ T cell response appeared not to be dependent on an Ag-specific Ig receptor. This was suggested by gene gun deliveries of the secreted HA into transgenics whose B cell populations were almost entirely specific for HEL, raising a type 2-biased response. In this instance, internalization of the HA into the B cell could be by mechanisms other than Ag-specific binding to its cognate Ig. This could occur by HA binding to sialylated protein or lipid receptors and thus undergoing internalization (as reviewed in Refs. 34 and 35).

The molecular mechanisms by which saline injections and gene gun bombardments initiate different biases in T cell responses are complex and not completely understood. Saline injections of DNA deliver large amounts of extracellular vaccine DNA that is then taken up by resident and migrant cells (for review, see Refs. 36 and 37). One popular candidate for the induction of a type 1 response in mice is the Toll-like receptor 9 (TLR9), which can interact with unmethylated cytosine-guanine motifs (CpG) present in bacterial DNAs (38). Injection of CpG DNA s.c. into the footpad of a mouse induces IL-12 and IFN- γ production in the draining lymph nodes (39). In both mouse and human studies, TLR9 is expressed on several cell types including dendritic cells, B cells, macrophages, monocytes, T cells, and NK cells. When stimulated by unmethylated CpG motifs, TLR9 induces the activation of transcription factors, such as NF κ B, that prime predominantly type 1 cytokine responses including type 1 IFNs, IL-12, IFN- γ , and TNF- α (37, 40). However, stimulation of TLR9 does not appear to be the sole mechanism by which a type 1 response is induced because methylation of plasmid DNA does not prevent the induction of a type 1 response following saline injections of DNA (41).

In contrast, gene gun immunizations deliver DNA coated onto gold beads directly into cells, largely bypassing TLR9 and the induction of a type 1 response. Moreover, the gold used in the gene gun inoculations has been shown to promote type 2 responses (42). Although saline injections differ from gene gun deliveries in using much larger amounts of DNA for immunization, for most Ags, these differences in the amounts of DNA have not been key to determining the different biases in the elicited T cell responses (1, 42).

Irrespective of our incomplete understanding of the underlying mechanisms, the ability to produce a spectrum of biases in CD4⁺ and CD8⁺ T cell responses by DNA-based immunizations opens the opportunity for testing how these biases affect the ability of the immune system to respond to infections, modulate autoimmunity, and control allergy. Indeed, studies on in vitro-generated Tc2 cells for the influenza HA have demonstrated that these cells have cytolytic activity, establish memory cells, but have different trafficking patterns and a more limited ability to control infections than the traditional IFN- γ -producing Tc1 cells (8).

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