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Optimizing the Efficacy of Epitope-Directed DNA Vaccination

Monika C. Wolkers,* Mireille Toebes,* Masaru Okabe,* John B. A. G. Haanen,*† and Ton N. M. Schumacher2*†

An increasing number of clinical trials has been initiated to test the potential of prophylactic or curative vaccination with tumor Ag-encoding DNA vaccines. However, in the past years it has become apparent that for many Ags and in particular for tumor Ags the intracellular processing and presentation are suboptimal. To improve epitope-directed DNA vaccines we have developed a murine model system in which epitope-specific, DNA vaccine-induced T cell immunity can be followed by MHC tetramer technology directly ex vivo. We have used this well-defined model to dissect the parameters that are crucial for the induction of strong cytotoxic T cell immunity using two independent model Ags. These experiments have led to a set of five guidelines for the design of epitope-directed DNA vaccines, indicating that carboxyl-terminal fusion of the epitope to a carrier protein of foreign origin is the most favorable strategy. DNA vaccines that are based on these guidelines induce high-magnitude CD8+ T cell responses in >95% of vaccinated animals. Moreover, T cell immunity induced by this type of optimized DNA vaccine provides long-term protection against otherwise lethal tumor challenges. The Journal of Immunology, 2002, 168: 4998–5004.

Since its discovery, DNA vaccination has proven itself as an effective strategy for the induction of T cell immunity (reviewed in Ref. 1). DNA vaccines have been shown to induce long-lasting immunity that results in protection from microbial infections and from tumor outgrowth in animal model systems (2–4). In addition, encouraging results have been obtained with the induction of parasite-specific T cell immunity in human DNA vaccination trials (5). To further increase the efficacy of DNA vaccines, several approaches have been developed in the past years. These include the provision of genes encoding cytokines such as IL-12 and IL-2, or costimulatory molecules such as B7-1 and B7-2 (6–9). A conceptually different approach for the optimization of DNA vaccines is to maximize the generation of T cell epitopes from DNA vaccine-encoded gene products. The dissection of the MHC class I Ag processing pathway over the past decades has defined rules that determine the efficiency of Ag processing. Both the proteasomal degradation system and the TAP transport system have been shown to restrict the repertoire of peptides that is available for MHC class I binding (10–14). Importantly, due to this restriction, the presentation of both viral and in particular tumor Ag-derived T cell epitopes is often less than maximal, and inefficient processing has been shown to adversely affect the magnitude of T cell responses (15–17). Early attempts to increase vaccination efficiency by optimizing epitope generation have focused on the use of minigene-encoded T cell epitopes. However, vaccination with minigene DNA vaccines did not improve and may however reduce the efficiency of T cell induction in comparison to whole gene vaccines (Ref. 18 and Results and Discussion).

In this study, we dissected the requirements for optimal induction of CD8+ T cell immunity by comparison of a panel of epitope-directed DNA vaccines. Our results indicate that the immunogenicity of these model Ags is optimal when fused to the carboxyl terminus of a gene of foreign origin. Furthermore, pre-existing T cell immunity to the carrier protein only marginally affects the efficiency of this strategy. Using this optimized strategy, >95% of vaccinated mice contain significant numbers of Ag-specific CD8+ T cells that can be monitored directly ex vivo. Epitope-specific T cell memory induced by these vaccines is detectable for >3 mo and protects mice from outgrowth of Ag-expressing tumors.

Materials and Methods

Animals

C57BL/6 mice, C57BL/10 mice, and MHC class II-deficient mice (19) crossed to the C57BL/6 background were obtained from the experimental animal department of The Netherlands Cancer Institute (Amsterdam, The Netherlands). Green fluorescent protein (GFP)-transgenic mice (20) were kindly provided by Dr. R. Torensma (Department of Tumor Immunology, Nijmegen University Medical Center, Nijmegen, The Netherlands). All mice were handled in accordance with institutional guidelines.

DNA constructs

DNA vaccines were generated by the introduction of target genes or gene fragments into the vector pcDNA3.1. A truncated form of nucleoprotein (NP) derived from influenza A/NT/60/68 (aa: 1, 2, and 328–498), which contains the H-2D^d-restricted epitope NP366 (aa: ASNENMDAM), was used for vaccination (NP). The minigene NP366 was generated with the primer set NP366 top (5'-GATCTGATCCACATGGTGGCTTAG-3') and NP366 bottom (5'GGGCTGGTGTTTGAGAACAGATCCTGAAACCCATGTTGCTTATG-3'). To ensure amino-terminal processing comparable to the parental protein, the four naturally flanking amino acid residues of NP366 (aa: GVQI) were included in the minigene. The DNA vaccine encoding the fusion protein NP366-GFP, including the flanking amino acid residues GVQI, was generated with the primer set NP366-GFP top (5'-GGGGGATCCTAAGCCACCATGGGTGTTCAGATCGCTCCTCAGGAAAACATGGACGCTATGGAAGCT-3') and NP366-GFP bottom (5'GGGGGATCCTAAGCCACCATGGGTGTTCAGATCGCTCCTCAGGAAAACATGGACGCTATGGAAGCT-3'). Generation of gene constructs encoding a carboxyl-terminal fusion of either NP366 or the H-2D^d-restricted epitope E7 49 from human papilloma virus

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18 U.S.C. Section 1734 solely to indicate this fact.

Abbreviations used in this paper: GFP, green fluorescent protein; NP, nucleoprotein; HPV, human papilloma virus; IRES, internal ribosome entry site.
(HPV)16 E7 (aa: RAHYIVNTF) to GFP, together with the NP-derived flanking amino acid residues (see above; GFP-NP<sub>366</sub> and GFP-E7<sub>49</sub>, respectively), was performed as previously described (21). A DNA vaccine encoding a carboxy-terminal fusion of NP<sub>366</sub> to the male-specific minor histocompatibility Ag Dby (Dby-NP<sub>366</sub>) was generated with the primer set Dby top (5′-GGGAATTCG CACCATGATGCAATGGCAGGCG-3′) and Dby-NP<sub>366</sub> bottom (5′-GTTGA CTGTGGGGCAATGTGTGAGTCTTCACACGAAAACATGGAC GCTATGTAAGGCGCAGAAAGG-3′).

Genes were cloned into the BamHI and NotI sites of the plasmid pcDNA3.1 to generate pcDNA.NP<sub>366</sub>, pcDNA.NP<sub>366</sub>-GFP, pcDNA.GFP-NP<sub>366</sub>, pcDNA.GFP-E7<sub>49</sub>, and pcDNA.NP<sub>366</sub>-GFP. For the generation of pcDNA.NP<sub>366</sub>-IRES-GFP, internal ribosome entry site (IRES)-GFP was inserted downstream of the NP<sub>366</sub> minigene into the cloning sites NotI and SalI. Dby-NP<sub>366</sub> was cloned into the EcoRI and NotI sites of pcDNA3.1 to generate pcDNA.Dby-NP<sub>366</sub>. Sequences were confirmed by sequence analysis. All DNA batches were purified using EndoFree Plasmid kit (Qiagen, Hilden, Germany). Analysis of DNA purity in this manner revealed endotoxin contents of <0.25 EU/ml.

**Immunizations and ex vivo analysis of Ag-specific CD8+ T cells**

Mice were injected i.m. in the hind leg with 100 μg of DNA in 50 μl HBSS (Life Technologies, Paisley, U.K.) three times at 14-day intervals. At day 8 postimmunization, ~50 μl of peripheral blood was drawn for analysis of T cell responses. Erythrocytes were removed by incubation in erylysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA (pH 7.4)) on ice for 15 min. Cells were washed twice with PBA (1× PBS, 0.5% BSA and 0.02% sodium azide) and stained with FITC- or PE-conjugated anti-CD8β (BD PharMingen, San Diego, CA) together with PE- or allophycocyanin-conjugated NP<sub>366</sub> or E7<sub>49</sub> tetramers (22, 23) at room temperature for 15 min in PBA. Cells were washed twice and analyzed by flow cytometry. Live cells were selected based on propidium iodide exclusion. Statistical analysis was performed with the Student t test after logarithmic transformation.

**In vitro restimulation of splenocytes**

LPS blasts were generated from C57BL/10 splenocytes by incubation with 25 μg/ml LPS from Salmonella typhosa (Sigma-Aldrich, St. Louis, MO) and 7 μg/ml dextran-sulfate in IMDM (Life Technologies) supplemented with complete medium (5% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5 × 10<sup>-5</sup> M 2-ME) for 3 days at 37°C. LPS blasts were washed twice with serum-free IMDM (Life Technologies). A total of 30 × 10<sup>6</sup> cells/ml were incubated with 50 μg/ml NP<sub>366</sub> peptide (aa: ASNENMDAM) in serum-free medium for 1 h at room temperature. Peptide-loaded blasts were irradiated with 30 Gy and subsequently washed twice with complete medium.

For in vitro restimulations, spleen cells were isolated from C57BL/10 mice and MHC class II-deficient mice that had been immunized with the indicated DNA vaccine. A total of 5 × 10<sup>6</sup> cells were restimulated with 0.5 × 10<sup>6</sup> NP<sub>366</sub>-peptide loaded LPS blasts for 7 days in complete medium. At day 3 of culture, 10 CU/ml human rIL-2 was added. Cells were harvested, purified over a Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) gradient, and analyzed for Ag-specific CD8<sup>+</sup> T cells by flow cytometry, or used for further functional analysis as described below.

**Intracellular cytokine staining**

A total of 1 × 10<sup>6</sup> spleen cells were cultured for 4 h in complete medium supplemented with 50 U/ml human rIL-2 and 1 μl/ml brefeldin A (Golgistopect; BD PharMingen) in the presence of 0.1 μg/ml NP<sub>366</sub> peptide or E7<sub>49</sub> control peptide. Cells were stained with anti-CD8β-PE, washed twice, and subsequently intracellular cytokine stains were conducted using a Cytofix/Cytoperm kit (BD PharMingen) according to the manufacturer’s protocol. Intracellular staining was performed with FITC-conjugated anti-IFN-γ (clone XMG 1.2).

**Cytotoxicity analysis**

In vitro restimulated spleen cells were prepared as described above and tested in a chromium release assay. Splenocytes were serially diluted in triplicate in 96-well U-bottom tissue culture plates (Costar, Corning, NY). Labeled target cells were incubated with 10 μM NP<sub>366</sub> or E7<sub>49</sub> peptide for 20 min at room temperature. A total of 1 × 10<sup>3</sup> peptide-loaded cells were added to the effector cells. Chromium release was measured in supernatants (25 μl) harvested after a 4-h incubation at 37°C. Percentage of lysis was
calculated from the following formula: 100 × [(cpm experimental release − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release)].

**Virus infection and tumor challenge**

Purified influenza A/NT/60/68 virus was kindly provided by Dr. R. Con- 
salves (National Institute for Medical Research, London, U.K.). Virus was 
grown and titrated in the Department of Virology, Erasmus University 
(Rotterdam, The Netherlands). Mice were infected intranasally with 25 
hemagglutinating units of virus.

For tumor challenge experiments, mice were immunized three times i.m. 
with a 14-day interval with the indicated DNA vaccine. Induction of Ag-
specific T cell immunity was confirmed by MHC tetramer staining of pe-
ripheral blood cells by flow cytometry. Thirty-five days after the third 
vaccination mice were challenged i.c. with 2.5 × 10^6 TC-1 cells (24). 
Every 3–4 days after tumor cell inoculation, tumor size was measured in 
two dimensions. Mice were sacrificed when tumors became necrotic or 
reached a diameter of >15 mm.

**Results and Discussion**

**Carboxyl-terminal fusion of T cell epitopes to GFP enhances induction of T cell immunity**

We and others have previously documented that pronounced in-
fluenza A NP-specific CD8+ T cell responses can be monitored 
directly ex vivo by MHC tetramer technology in influenza A-in-
fected mice (22, 25). To establish the potential of DNA vaccines in 
the induction of CD8+ T cell immunity, we immunized naive mice 
i.m. with a DNA vaccine encoding a large fragment of influenza A 
NP. Although up to 4% of NP366-specific CD8+ T cells can be 
detected in some mice, T cell immunity induced by this vaccine 
appears highly variable, and measurable T cell responses are ob-
erved in only 20% of the vaccinated mice (Fig. 1, A and B). It has 
previously been argued that suboptimal T cell induction may be 
the result of inefficient Ag processing. Therefore, to bypass the 
requirement for epitope liberation from the parental protein, mice 
were vaccinated with a minigene DNA construct encoding NP366 
as a short peptide fragment. Comparison of the vaccination effi-
ciency of the minigene DNA vaccine and the NP gene DNA vac-
cine reveals a nonsignificant increase in the number of mice with 
measurable NP366-specific T cell responses (25 vs 20%), albeit 
with a reduction in the magnitude of T cell immunity (Fig. 1C).

Similarly, Whitton et al. (18) have shown that, despite circumvent-
ning the requirement for Ag processing, the use of minigene DNA 
encoding epitope-liberated Ags can be a limiting step in the generation of 
epitope-specific T cell responses that could be visualized directly ex vivo (Fig. 
2). Together, these results show that fusion of a minigene to GFP renders 
DNA vaccination highly efficient. The power of this strategy is empha-
sized by the fact that epitope-specific T cell immunity is not only 
detected after in vitro restimulation but can be visual-
ized directly ex vivo in 97% of the vaccinated mice.

We next established whether amino-terminal location of the 
NP366 epitope within the fusion protein would result in equally 
pronounced T cell induction. Vaccination with DNA encoding an 
aminoterminal fusion of NP366 to GFP also resulted in Ag-spe-
cific T cell responses that could be visualized directly ex vivo (Fig. 
3). However, both the frequency of successful vaccination and the 
magnitude of the vaccination-induced T cell response are clearly 
reduced when compared with carboxyl-terminal fusion of NP366 
(p < 0.001), indicating that carboxyl-terminal fusion of the 
epitope is favored to amino-terminal fusion for efficient induction 
of T cell immunity.

To examine whether direct linkage of the T cell epitope to GFP 
is required for the increased effectiveness of epitope-directed DNA 
vaccination or whether coadministration of GFP is sufficient, we

**Remarkably, when this fusion gene is used as a DNA vaccine, 
pronounced NP366-specific CD8+ T cell responses can be moni-
tored directly ex vivo in 38 of 40 (95%) vaccinated mice (Fig. 1, 
A and C; all mice that harbor >0.3% Ag-specific CD8+ T cells 
were scored as positive). Mice vaccinated with the GFP-NP366 
fusion gene display epitope-specific T cell immunity of in average 
of 2.5% of the CD8+ T cell population present in peripheral blood 
(Fig. 1D). Vaccination with a carboxyl-terminal fusion of the 
HPV16-derived Ag E749 to GFP also induced epitope-specific T 
cell immunity in 20 of 20 mice (Fig. 1, C and D), indicating that 
the potency of this strategy is not restricted to the NP366 epitope. 
Immunization with these fusion gene vaccines induces functional 
epitope-specific T cell immunity as judged by the ability to pro-
duce IFN-γ and to kill target cells upon in vitro stimulation (Fig. 
2). Together, these results show that fusion of a minigene to GFP 
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![Diagram](http://www.jimmunol.org/)

**FIGURE 2.** DNA vaccination results in functional epitope-specific T cell immunity. C57BL/10 mice were immunized with GFP-NP366 (n = 3), and induction of epitope-specific T cells was confirmed by analysis of peripheral blood cells (data not shown). Seventeen days post-third vacci-
nation, splenocytes were restimulated in vitro with NP366-loaded LPS 
blasts. A, The amount of NP366-specific spleen cells was determined by 
MHC class I tetramer staining (left panel). The percentage of IFN-γ-pro- 
ducing CD8+ T cells was determined upon stimulation with NP366 (middle 
panel) or the control peptide E7α49 (right panel). B, Spleen cells were tested 
in a 51Cr release assay against EL4 target cells pulsed with NP366 (●) or the 
control peptide E7α49 (■). The mean percentage of specific lysis per E:T 
ratio is shown. A total of 3.5% of effectors are NP366-specific CD8+ T 
cells as determined by MHC class I tetramer staining.
Peripheral blood was drawn at day 8 post-third vaccination and analyzed from a single dicistronic mRNA (NP366-IRES-GFP). In none of the mice immunized with the NP366-IRES-GFP DNA vaccine, and the average is depicted as a line. Compare the GFP-NP366 vaccine with a DNA construct that expresses the NP366 peptide and GFP as separate translation products from a single dicistronic mRNA (NP366-IRES-GFP). In none of the eight mice immunized with the NP366-IRES-GFP DNA vaccine an epitope-specific CD8+ T cell response is detectable (Fig. 3, p < 0.001). In contrast, the fusion gene GFP-NP366 induces an NP366-specific CD8+ T cell response in all vaccinated mice in this experiment (Fig. 3).

Prior studies have indicated that protein translation from IRES is slightly less efficient compared with translation from conventional start sites (E. Hooijberg, personal communication). To directly examine the relative levels of expression in both configurations, the two constructs were cotransfected into COS-7 cells and protein levels were determined by Western blot analysis. This reveals that levels of GFP are ~3-fold higher for the fusion protein as compared with expression from the dicistronic vector (data not shown).

Consequently, the requirement for genetic fusion of the target Ag and carrier protein may reflect an intrinsic property of the fusion protein, such as protection from cytosolic proteases, or may be a consequence of a reduced production of a putative GFP-derived Th epitope from the dicistronic vector (see next section). Regardless of the underlying mechanism, covalent linkage of the Ag and carrier protein is clearly preferable when compared with current dicistronic vector systems.

**Efficiency of the GFP carrier protein depends on nonself recognition**

We next studied the mechanisms that may underlie the dramatic improvement of T cell induction upon vaccination with the fusion gene used. GFP may serve as a carrier protein that facilitates the entry of the linked epitope into the Ag processing machinery and/or prevents epitope degradation by cytosolic proteases. In addition, GFP may enhance Ag-specific CD8+ T cell immunity by the provision of T cell help. To dissect whether T cell help is required for the induction of CD8+ T cell responses by DNA vaccination, we immunized MHC class II-deficient mice with the GFP-NP366 fusion gene. In these mice devoid of CD4+ T cells no detectable Ag-specific T cell response is induced, whereas 8 of 10 wild-type mice were successfully vaccinated (Fig. 4A, p < 0.005). In vitro restimulation of splenocytes from vaccinated MHC class II-deficient mice did not result in the outgrowth of Ag-specific T cells, whereas splenocyte cultures from wild-type mice contained high numbers of NP366-specific CD8+ T cells, mounting up to 40% of the CD8+ T cell population (Fig. 4B). These findings indicate that induction of CD8+ T cell immunity by DNA vaccination is critically dependent on CD4+ T cell help, in line with previous observations by Levy et al. (31). This CD4+ T cell dependency is a property of the DNA vaccination strategy and not of the GFP-NP366 Ag, as shown by the fact that it is also observed for the DNA vaccine encoding the NP protein (data not shown), and that NP366-specific T cell induction in mice harboring tumors expressing the identical fusion gene is independent of CD4+ T cells (21).

The CD4+ T cell help that is required for successful vaccination may be induced by a GFP-encoded helper epitope. Alternatively,

**FIGURE 3.** Optimal induction of CD8+ T cell immunity by carboxyl-terminal fusion of the Ag to GFP. C57BL/10 mice were immunized with a DNA vaccine encoding a carboxyl-terminal fusion (GFP-NP366, n = 13) or amino-terminal fusion of the Ag to GFP (NP366-GFP, n = 10). Alternatively, mice were vaccinated with a minigene DNA vaccine (NP366, n = 8) or with the dicistronic vaccine encoding NP366 and GFP as two separate translation products from a single mRNA (NP366-IRES-GFP, n = 8). Peripheral blood was drawn at day 8 post-third vaccination and analyzed for NP366-specific CD8+ T cells. Each dot represents an individual mouse, and the average is depicted as a line.

**FIGURE 4.** Optimal vaccination efficiency depends on fusion with a nonself carrier protein and CD4+ T cell help. A, C57BL/6J (n = 10), GFP-transgenic (n = 5), and MHC class II-deficient mice (n = 5) were vaccinated with the GFP-NP366 DNA vaccine. At day 8 post-third vaccination, peripheral blood was drawn and monitored for NP366-specific CD8+ T cells. B, Five weeks after the third vaccination, splenocytes were restimulated in vitro for 7 days with NP366-loaded LPS blasts and 20 CU/ml human rIL-2. The percentage of NP366-specific CD8+ T cells was determined by flow cytometry.
cryptic open reading frames encoded in the plasmid used may contain MHC class II-restricted T cell epitopes as previously shown for an MHC class I-restricted epitope (32). To establish whether T cell recognition of GFP-derived fragments contributes to the induction of NP366-specific CD8⁺ T cell immunity, we vaccinated GFP-transgenic mice that express GFP in all nucleated cells (20). In two of five mice no T cell response was detectable, and in the remaining three animals the T cell response was significantly reduced in magnitude as compared with wild-type animals (0.54 vs 3.7%, Fig. 4, A, p < 0.05). Thus, efficient DNA vaccination requires CD4⁺ T cell help and depends on nonself recognition of the vaccine-encoded carrier protein. Collectively, these data suggest that one important element of the success of the GFP fusion strategy is the provision of CD4⁺ T cell help through recognition of a GFP-encoded CD4⁺ T cell epitope.

Preexisting T cell immunity to GFP does not affect application of fusion gene DNA vaccines

Our current data show that carboxyl-terminal fusion of CD8⁺ T cell epitopes to a foreign carrier protein dramatically increases the efficiency of epitope-specific CD8⁺ T cell induction by DNA vaccination. During clinical application, preexisting CD4⁺ and/or CD8⁺ T cell immunity to the carrier protein may influence the efficiency of this vaccination strategy. For instance, tetanus toxin has been used as a source of CD4⁺ T cell help in human DNA vaccination trials (33) but is also included in childhood vaccination programs. Preexisting CD4⁺ T cell immunity to a carrier protein could conceivably promote the efficiency of epitope-directed DNA vaccination by providing increased CD4⁺ T cell help. Conversely, preexisting CD8⁺ T cell immunity to the carrier protein could possibly reduce the efficiency of DNA vaccination, either by competition for or by inactivation of APCs (34–36). Based on the results in CD4-deficient and GFP-transgenic mice, GFP appears to contain a Th epitope. To address whether preexisting T cell immunity to GFP affects the induction of T cell responses to the Ag of interest, mice were first immunized three times with the GFP-NP366 vaccine, and 5 wk later with GFP-E749. Equal amounts of E749-specific CD8⁺ T cells were detected in pretreated and in nontreated mice (Fig. 5A), indicating that preexisting T cell immunity to GFP did not further increase the induction of CD8⁺ T cells.

We next studied how preexisting CD8⁺ T cell immunity to an epitope contained within the carrier protein influenced the induction of the desired epitope-directed T cell immunity. To this purpose, we vaccinated mice that had undergone an infection with an influenza A strain (A/NT/60/68) that contains the NP366 epitope. Five weeks post-influenza A infection mice have developed NP366-specific CD8⁺ T cell memory (Fig. 5B), and 8 days after infection the mice contain large populations of NP366-specific effector T cells (referred to as “activated”) (22, 25). Vaccination of these mice was performed with a DNA vaccine that contains the NP366 epitope at the amino terminus and the HPV16 E749 epitope at the carboxyl terminus (NP366-GFP-E749), and the CD8⁺ T cell response against the E749 epitope was monitored. Although vaccination with the amino-terminal fusion of NP366 is less efficient as compared with carboxyl-terminal fusion (see above), the Ag is sufficiently liberated from this precursor gene to be well recognized by NP366-specific T cells and can therefore serve well to induce recall responses (N. Brouwenstijn and T. Schumacher, unpublished observations).

In mice undergoing an acute influenza A infection, the magnitude of the E749-specific T cell response is significantly reduced compared with naive mice (Fig. 5C, p < 0.01). However, preexisting T cell memory to the fusion partner only slightly affects the magnitude of T cell immunity compared with nontreated mice. Together, these data indicate that, whereas effector type CD8⁺ T cell immunity to a carrier protein may hinder the induction of epitope-specific T cell immunity, CD8⁺ T cell memory to the fusion partner does not significantly affect T cell induction by DNA vaccination.
DNA vaccination protects against lethal tumor challenge

Previously, it has been described that, irrespective of the original magnitude of a CD8+ T cell response, 90–95% of Ag-specific CD8+ T cells die following pathogen clearance, and the remaining 5–10% of the T cell population enter the memory pool (reviewed in Refs. 37 and 38). DNA vaccination results on average in 2.5% of epitope-specific CD8+ T cells in the second week after vaccination. With a reduction of 90%, T cell memory may be expected to drop to undetectable levels in the peripheral blood very rapidly. However, 37 and 84 days after DNA vaccination, Ag-specific CD8+ T cells can still be monitored (37 days post-vaccination: 86% of the original magnitude; 84 days post-vaccination: 36%). To determine whether the Ag-specific T cell memory induced by this optimized vaccination strategy can confer protection from subsequent tumor challenge, mice vaccinated with GFP-E7 49 were challenged with the HPV E6/E7 transformed TC-1 tumor cell line 5 wk after the third vaccination (24). Only three of eight mice that were vaccinated with a control DNA vaccine GFP-NP 366 were protected from tumor challenge (Fig. 6A). In contrast, seven of eight mice vaccinated with GFP-E7 49 were protected from tumor growth and remained tumor-free for >70 days, indicating that epitope-directed DNA vaccination provides effective epitope-specific protection from tumor challenge. Whether the reduced incidence of tumor outgrowth in mice vaccinated with the control DNA vaccine as compared with naive mice (Fig. 6B) is due to nonspecific immune activation or involves recognition of the cryptic T cell epitope that is shared between the DNA vaccine and the TC-1 cell line (32) remains to be established. In conclusion, these data indicate that DNA vaccines encoding T cell epitopes fused to the carboxyl terminus of a nonself carrier protein not only induce pronounced, long-lived T cell immunity but also provide long-term protection from tumor outgrowth.

To determine whether efficient induction of epitope-directed T cell responses by carboxyl-terminal fusion to a carrier protein of foreign origin is a more general principle, we used the murine male-specific minor histocompatibility Ag Dby as fusion partner. Because Dby is located on the Y chromosome, it is foreign to female mice but (ubiquitously) expressed in male mice. Vaccination of female mice with Dby-NP 366 vaccine resulted in epitope-specific T cell responses in six of seven mice (Fig. 7A), and these cells are functional as determined by IFN-γ staining upon in vitro restimulation (Fig. 7C). In contrast, only one of eight male mice had detectable levels of NP 366-specific CD8+ T cells ex vivo (<0.05). Furthermore, this difference in the magnitude of epitope-directed T cell immunity between male and female mice is maintained after in vitro restimulation (Fig. 7B, p < 0.005). These results confirm that fusion vaccines can be highly efficient and that at least a part of this efficiency can be attributed to nonself recognition of the carrier protein. The observation that Dby contains an immunodominant CD4+ T cell epitope but no known CD8+ T cell epitopes (39) supports the notion that this nonself recognition serves to provide CD4+ T cell help. These data are consistent with prior observations of Rice et al. (40) that indicate that carboxyl-terminal fusion of a CD8+ T cell epitope to a tetanus toxin fragment containing a CD4+ T cell epitope results in a highly efficient DNA vaccine.

In this study, we have determined five guidelines for the design of epitope-specific DNA vaccines. 1) Genetic fusion of T cell epitopes to a carrier protein can dramatically improve DNA vaccination compared both to epitope minigenes as well as to the parental protein. Twenty of 20 mice (100%) vaccinated with this type of DNA vaccine encoding a HPV-derived T cell epitope displayed pronounced T cell immunity directly ex vivo. Likewise, 38 of 40 mice (95%) immunized with an influenza A NP-containing DNA vaccine were efficiently vaccinated. Furthermore, one of the two mice that scored negative in ex vivo MHC tetramer analysis was shown to contain Ag-specific T cells after one round of in vitro restimulation (1.4% NP 366-specific CD8+ T cells, Fig. 4),
suggesting that the screening method used may still underestimate the percentage of successfully immunized mice. 2) Fusion of the epitope to the carboxyl terminus of a carrier protein is superior to amino-terminal fusion or coexpression of the two genes. 3) Successful vaccination is critically dependent on CD4+ T cell help. 4) Successful vaccination is significantly reduced when the carrier protein is a self-protein. 5) Preexisting CD8+ T cell memory against other MHC class I-restricted epitopes contained within the vaccine does not prevent the development of effective immunity against the vaccine-encoded target Ag. Collectively, the guidelines defined in this study should form a useful starting point for the development of epitope-directed DNA vaccines, in particular those encoding human tumor-associated Ags.

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