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Alternative roles for interferon-gamma in the immune response to DNA vaccines encoding related melanosomal antigens

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Abstract

Tyrosinase-related proteins-1 and -2 (gp75/TRP-1 and TRP-2) are melanosomal membrane glycoproteins recognized by antibodies and T-cells from patients with melanoma. Xenogeneic DNA immunization against gp75/TRP-1 generates antibody-dependent tumor immunity and autoimmune depigmentation. In contrast xenogeneic *TRP-2* DNA immunization induces immunity mediated by CD8+ T-cells. The role of IFN-gamma in the generation of tumor immunity and autoimmune depigmentation in these two models was investigated. No tumor protection and minimal depigmentation was observed after immunization with human *TRP-2* DNA in mice deficient in IFN-gamma ligand. Repletion with recombinant murine IFN-gamma restored tumor immunity. Experiments using IL4 deficient mice demonstrated that tumor immunity was unaffected but that autoimmune depigmentation was potentially accelerated, consistent with down-modulation of autoimmunity against TRP-2 by IL4. In contrast, IFN-gamma was not required for the generation of immunity to gp75/TRP-1. In fact, exogenous IFN-gamma ablated autoantibody responses against gp75/TRP-1 after xenogeneic DNA immunization, consistent with a down-regulatory effect of IFN-gamma. These results show that immunity to TRP-2 following DNA immunization uses an IFN-gamma-dependent Th1 pathway, but immunity to gp75/TRP-1 is down-regulated by IFN-gamma.

Introduction

Vaccination with plasmid DNA can induce both humoral and T-cell responses to a variety of antigens, including otherwise poorly immunogenic self antigens. We have previously shown that immunization of mice against tissue-specific antigens with DNA encoding xenogeneic (human) gp75/tyrosinase-related protein-1 (hgp75/TRP-1) and hTRP-2 results in tumor immunity and autoimmune depigmentation (1,2). Although gp75/TRP-1 and TRP-2 are homologous glycoproteins expressed in the same cellular compartment, they elicit qualitatively distinct immune responses. Tumor immunity and autoimmunity in mice immunized with DNA encoding human gp75/TRP-1 involves a mechanism that requires Th2-type antibodies, which is independent of CD8+ T-cells. On the other hand, immunity following immunization with *hTRP-2* DNA reveals a strict requirement for CD8+ T-cells and is unaffected by the absence of B-cells. The above results are consistent with a Th2 response required for the generation of tumor immunity and autoimmunity to xenogeneic *gp75/TRP-1* DNA immunization but a Th1 response for immunity to TRP-2.

IFN-gamma is a pleiotropic cytokine considered central to Th1 responses (3). Targeted deletions of both the IFN-gamma ligand and its receptor have been performed in mice (4,5,6,7,8,9,10). Such animals are essentially normal in a "clean" environment but display reduced macrophage function in response to pathogens and inability

to control mycobacteria and leishmania infections (10,11). The latter observation is significant because it implies a deficient Th1 response considered crucial for the control of leishmania. Interestingly, the cytotoxic T-cell response to influenza virus is normal in IFN-gamma receptor deficient mice, showing that inability to mount an effective Th1 response is dependent on the particular pathogen (6).

The importance of endogenous IFN-gamma in tumor immunity has been demonstrated by several groups using B16 mouse melanoma vaccines secreting GM-CSF (12,13). In addition, recent studies with IFN-gamma receptor deficient animals support a role for IFN-gamma in endogenous tumor surveillance mechanisms, as these animals have a heightened sensitivity to chemically-induced sarcomas (14) as well as spontaneous epithelial cancers (15).

The *IFN-gamma* gene has been shown to be an effective genetic adjuvant for DNA vaccines eliciting Th1 responses; however, the role of endogenous IFN-gamma in the mechanism of DNA immunization remains unknown (16,17). We have addressed the question of the role of endogenous IFN-gamma in the response to DNA immunization with human *gp75/TRP-1* and *hTRP-2* using mice deficient in IFN-gamma ligand. Our results show that IFN-gamma is required for both tumor immunity and autoimmunity resulting from immunization with *hTRP-2* DNA. Moreover, repletion studies using recombinant murine IFN-gamma suggest that the cytokine must be present during the priming phase of immunization against TRP-2 in order for tumor immunity to develop.

In contrast to the requirement for IFN-gamma in the response to TRP-2, IFN-gamma appears to play a suppressive role in the response to gp75/TRP-1. Immunization of C57BL/6 mice with human *gp75/TRP-1* DNA characteristically results in the induction of a Th2 antibody response capable of producing both tumor immunity and autoimmune depigmentation. Addition of the *IFN-gamma* gene as an adjuvant to human *gp75/TRP-1* DNA vaccination ablates the antibody response. In addition, immunization of IFN-gamma receptor knockout mice with human *gp75/TRP-1* DNA results in higher antibody titers than those found in wild type controls. Taken together, these results show that IFN-gamma is required for immunity induced in response to some DNA vaccines, but it suppresses response to others. These observations of antigen-specific conditions for optimal immunization are important to the development of effective cancer vaccination strategies.

Results

IFN-gamma, but not IL4, is required for tumor protection induced by immunization with *hTRP-2* DNA

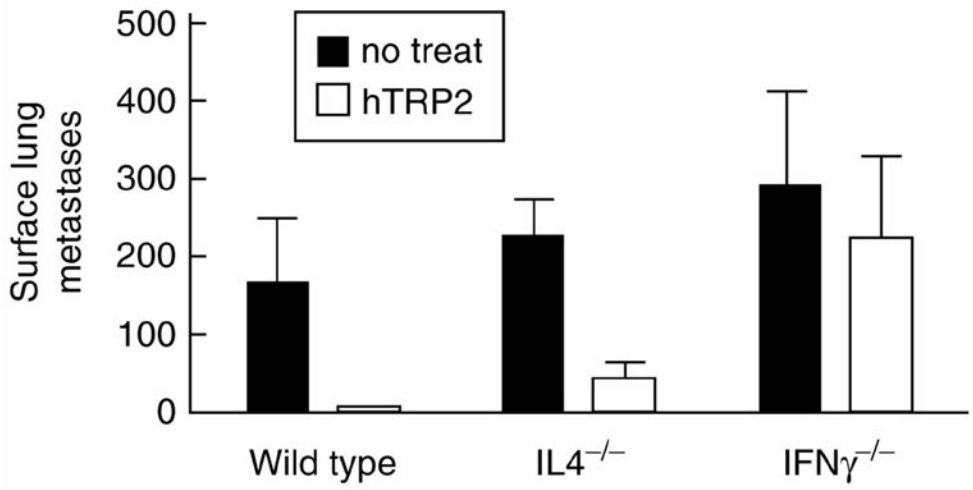

Figure 1. Protection of mice immunized with *hTRP-2* from syngeneic tumor challenge.
Groups of 3-5 C57BL/6 mice (wild type) and mice of the same background deficient in IL4 (IL4$^{-/-}$) or IFN-gamma (IFN-gamma$^{-/-}$) were immunized weekly with *hTRP-2*. Intravenous tumor challenge was performed 7 days after the last immunization and lung metastases were counted 14 days after challenge. Error bars represent standard error of the mean.

We have previously shown that immunization of C57BL/6 mice with *hTRP-2* DNA results in protection from syngeneic tumor challenge with B16 melanoma cells. Tumor immunity required both CD4+ and CD8+ T-cells, implying a Th1 type response. We therefore sought to confirm the Th1 bias of this response by studying the roles of prototypical Th1 (IFN-gamma) and Th2 (IL4) cytokines. C57BL/6, IL4$^{-/-}$ and IFN-gamma$^{-/-}$ mice, all of C57BL/6 genetic background, were immunized with *hTRP-2* cDNA using a gene gun at weekly intervals for 5 weeks, followed by intravenous tumor challenge with B16F10LM3 cells. As expected, immunization of wild-type mice resulted in a marked reduction in the number of surface lung metastases (Fig. 1). Similarly, significant protection was also observed in IL4$^{-/-}$ mice immunized with *hTRP-2*. However, mice lacking the *IFN-gamma* gene were not protected, showing a requirement for this cytokine in tumor immunity induced by *hTRP-2* DNA.

Autoimmune depigmentation induced by hTRP-2 is dependent on IFN-gamma

In our prior experiments with *hTRP-2* DNA immunization, extensive coat depigmentation occurred in almost all immunized animals within 42 days. Therefore, we investigated whether IL4$^{-/-}$ and IFN-gamma$^{-/-}$ mice would develop autoimmune depigmentation. All mice that were immunized with the *hTRP-2* gene were observed for autoimmune depigmentation for at least 70 days after starting immunization (Fig. 2A). Both wild type and IL4$^{-/-}$ mice began demonstrating evidence of depigmentation as soon as hair re-grew in previously depilated areas, between days 35-42. The rate of depigmentation was more rapid in the IL4$^{-/-}$ mice, compared with wild-type mice (Fig. 2B). IFN-gamma$^{-/-}$ mice did not show any significant evidence of autoimmune depigmentation, implying that IFN-gamma is required for induction of autoimmunity as well as tumor immunity.

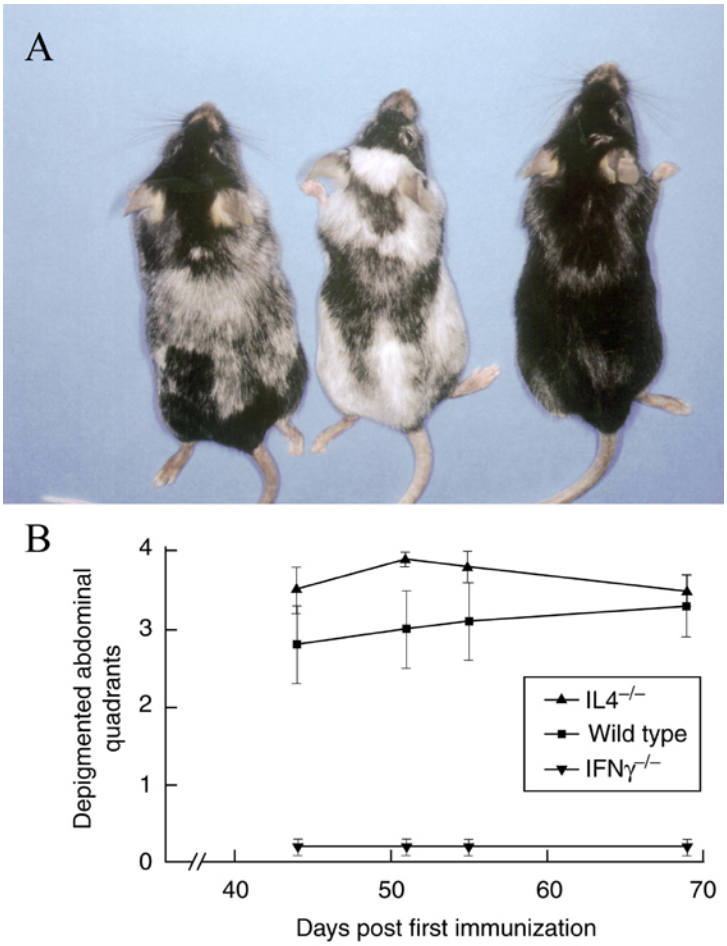

Figure 2. Coat depigmentation in mice immunized with *hTRP-2.* Groups of 12-19 C57BL/6 mice (wild type) and mice of the same background deficient in IL4 (IL4$^{-/-}$) or IFN-gamma (IFN-gamma$^{-/-}$) were immunized with *hTRP-2* and observed for the appearance of white hair. (A) The appearance of representative mice observed for 70 days after immunization- *left to right*: wild type, IL4$^{-/-}$, IFN-gamma$^{-/-}$. (B) The number of depigmented abdominal quadrants measured over time. IL4$^{-/-}$ mice depigmented most quickly, but wild type animals eventually achieved the same degree of depigmentation.

IFN-gamma is required during the priming phase of immunization

To further elucidate the role of IFN-gamma in tumor immunity induced by *hTRP-2* DNA, we repleted IFN-gamma$^{-/-}$ mice with recombinant murine IFN-gamma during the entire experiment or only during the effector (tumor challenge) phase and assessed tumor protection (Fig. 3). Again, we observed that wild type C57BL/6 mice immunized with *hTRP-2* DNA were well protected. IFN-gamma$^{-/-}$ mice that were immunized with *hTRP-2* DNA but not repleted with IFN-gamma were not protected. However, in immunized mice that were repleted with IFN-gamma during both the priming and effector phase (56 days), the number of surface lung metastases was significantly and substantially reduced compared to the knockout mice that were not repleted ($P < 0.0002$). In contrast, IFN-gamma$^{-/-}$ mice immunized with *hTRP-2* DNA and repleted only during the effector phase, immediately prior to tumor cell injection, did not demonstrate significant protection from tumor challenge. Therefore the presence of IFN-gamma$^{-/-}$ during the effector phase alone is not sufficient to mediate significant tumor immunity.

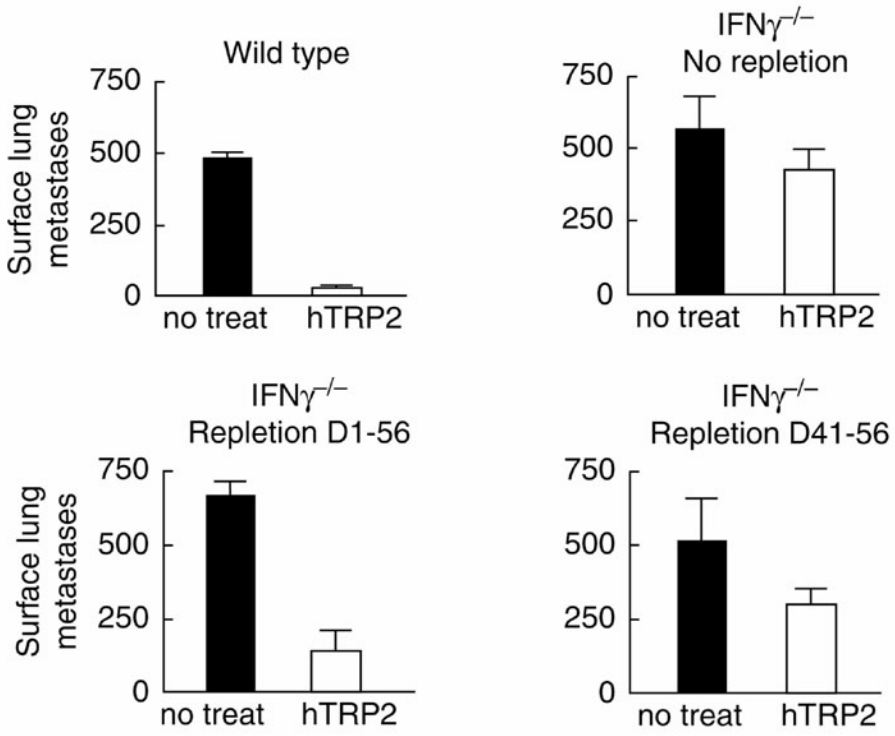

Figure 3. Effect of repletion with recombinant murine IFN-gamma on tumor immunity in IFN-gamma$^{-/-}$ animals. Groups of 5-7 wild type and IFN-gamma$^{-/-}$ mice were immunized with *hTRP-2*. The IFN-gamma$^{-/-}$ mice were divided into three groups: One group received no repletion, one group was repleted with recombinant murine IFN-gamma from the first day of immunization through the end of the tumor challenge (days 1-56), and one group was repleted with recombinant murine IFN-gamma during the effector phase only (days 41-56). Syngeneic tumor challenge with intravenous B16 melanoma was performed and lung metastases were counted twelve days after tumor injection.

Intracellular cytokine staining

We have previously identified a novel human TRP-2 class II MHC epitope that is recognized by CD4+ T cells from C57BL/6 mice immunized with *hTRP-2* DNA (18). We investigated the cytokine profiles of CD4+ T cells from wild type, IFN-gamma$^{-/-}$ and IL4$^{-/-}$ mice. Consistent with our prior experience, CD4+ T cell lines from wild type C57BL/6 mice immunized with *hTRP-2* DNA produced both IFN-gamma and IL4 in response to the specific TRP-2 peptide (Fig. 4). CD4+ T cells from IL4$^{-/-}$ mice produced levels of IFN-gamma comparable to those seen in wild type mice and no IL4, as would be expected based on the disruption of the IL4 gene. When CD4+ T cell lines were generated from IFN-gamma$^{-/-}$ mice immunized with *hTRP-2* DNA, as expected there is no IFN-gamma production but there was almost a 9-fold decrease in IL4 production compared to wild type mice. The significant

decrease in IL4 production suggests that the lack of IFN-gamma results in a loss of ability to prime CD4+ T cells in response to *hTRP-2* DNA.

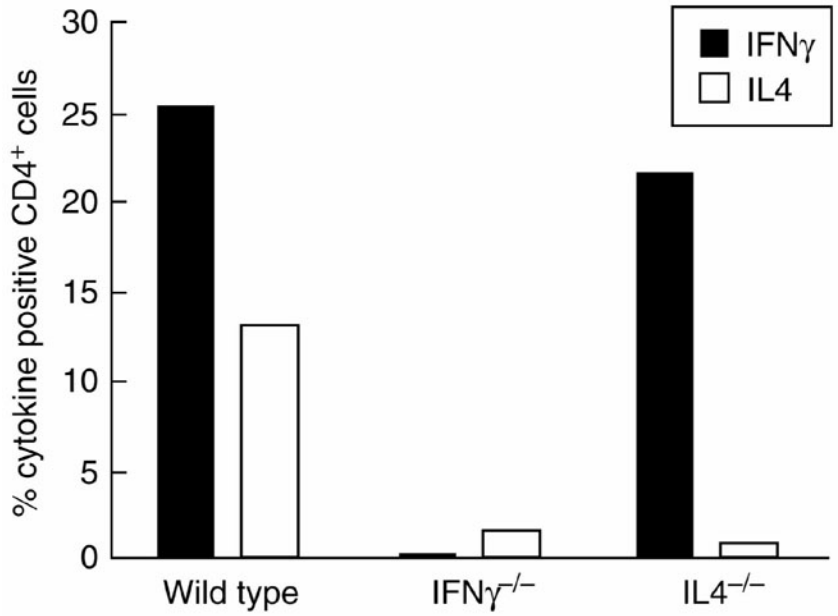

Figure 4. Cytokine release by CD4+ cell lines generated from C57BL/6 mice immunized with *hTRP-2* DNA. Five days after the last immunization, CD4+ T lymphocytes were isolated from the spleen and draining inguinal lymph nodes by magnetic bead cell sorting (MACS) and co-cultured with naive irradiated splenocytes with the addition of hTRP-2 237-256 peptide. Following three weekly rounds of *in vitro* stimulation, cells were stimulated with plate-bound anti-CD3 and anti-CD28 for 6 hours, in the presence of Brefeldin A for the last 2 hours. Flow cytometry was then performed on the cells in order to analyze intracellular cytokines.

IFN-gamma suppresses the antibody response to immunization with *hgp75* DNA

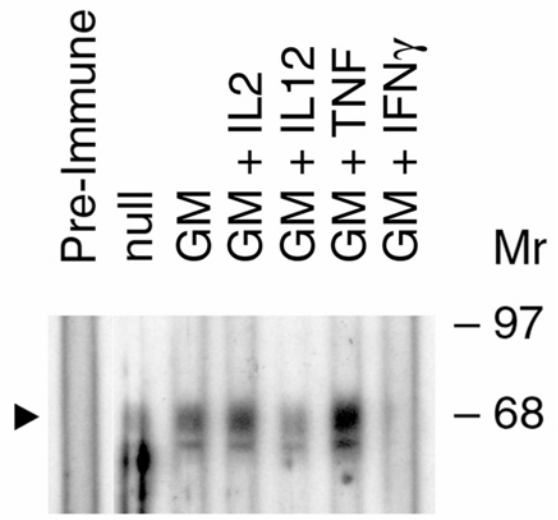

Figure 5. Effect of cytokines on the antibody response to hgp75/TRP-1. Groups of three C57BL/6 mice were immunized five times with *hgp75/TRP-1* preceded by treatment with various murine cytokine DNA constructs or control (null) plasmid. Sera were analyzed for the presence of antibodies detecting hgp75/TRP-1 in an immunoprecipitation-Western blot assay. Results are representative of all three mice per group (GM = GM-CSF).

Prior studies from our group have documented the ability of *hgp75/TRP-1* DNA to induce a Th2-type antibody response in C57BL/6 mice and the additive effect of the gene encoding GM-CSF on this response (19). We were interested in investigating the effect of combinations of cytokines on the antibody response to hgp75/TRP-1. The results (Fig. 5) indicate that combining *IL2*, *TNF-alpha* or *IL12* with *GM-CSF* did not significantly enhance the antibody response to *hgp75/TRP-1* DNA. Interestingly, the addition of *IFN-gamma* completely ablated the antibody response to hgp75/TRP-1 in 3/3 mice tested. To follow up on this observation, we immunized IFN-gamma-receptor deficient animals with *hgp75/TRP-1* DNA and assessed gp75/TRP-1 autoantibody titers against mouse gp75/TRP-1. The results (Fig. 6) show that IFN-gamma receptor deficient animals produced higher titer antibodies than controls ($P = 0.003$). These results were confirmed in both immunoprecipitation-Western blot assays with serial dilutions of serum, as well as in a B16 cell-based ELISA (data not shown).

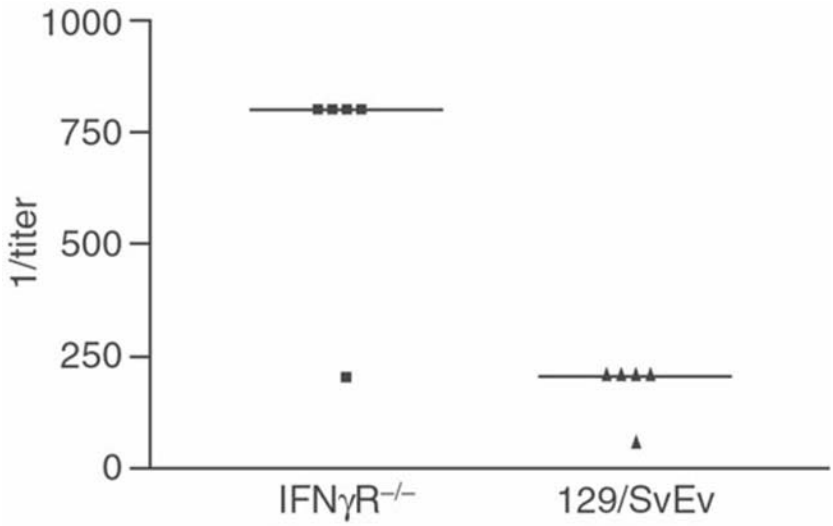

Figure 6. Anti-hgp75/TRP-1 antibody titers in wild type (129/SvEv) or IFN-gamma receptor deficient (IFN-gammaR-/-) mice. Groups of five mice were immunized with *hgp75/TRP-1* weekly for five weeks. Sera were collected one week following the final immunization and used in a B16 cell-based ELISA at serial dilutions to determine titer (last dilution at which absorbance was above background). The horizontal lines represent median titer. Results were confirmed using immunoprecipitation-Western blot assays with serial dilutions of sera.

Discussion

Immunization with plasmid DNA has emerged as a simple and potent means for generating both antibody and T-cell responses to many types of antigens. The mechanism(s) underlying the success of DNA immunization are still being investigated; however, much interest has been focused on the role of cytokines in the generation of immune responses to antigens encoded by plasmid DNA. Endogenous cytokines are induced in response to unmethylated CpG immunostimulatory motifs within the plasmid DNA and are thought to contribute to the inherent immunogenicity of DNA vaccines (20,21). We and others have shown that genes encoding cytokines such as GM-CSF can serve as immunologic adjuvants, allowing for responses to otherwise non-immunogenic peptides and augmenting the responses to DNA vaccines (19). In this report, we have shown that IFN-gamma is required for the response to TRP-2, a melanocytic differentiation antigen, but it represses the response to gp75/TRP-1, a related molecule given by the same route.

The requirement for IFN-gamma for the induction of a Th1 T-cell response, such as to hTRP-2, is not surprising given the pleiotropic functions of this cytokine in the processes of antigen presentation, T-cell and macrophage activation and cellular cytotoxicity (3). The availability of mice with targeted deletions of either the IFN-gamma ligand or its receptor has aided in the understanding of the specific roles for this cytokine in an otherwise redundant immune system. IFN-gamma$^{-/-}$ mice are unable to mount a protective response against *Leishmania major* and are unusually sensitive to *Mycobacterium bovis*, yet have a normal CD8+ T-cell response to influenza

infection (7,8,9,10,11). This implies some degree of antigen-specificity to the defects. Similarly, mice lacking the IFN-gamma receptor share sensitivity to intracellular pathogens while demonstrating normal T-cell responses to viral infection (5,6).

Our finding that IFN-gamma is required for the response to hTRP-2, yet inhibits the response to hgp75/TRP-1, also points to an antigen-specific role for IFN-gamma in the response to DNA vaccines. Although no tumor immunity or autoimmunity occurred in IFN-gamma$^{-/-}$ mice vaccinated with *hTRP-2*, IFN-gammaR$^{-/-}$ mice immunized with *hgp75/TRP-1* had a higher-titer antibody response than wild type controls (Figure 6). This demonstrates the suppressive effects of small amounts of endogenous IFN-gamma on the Th2 antibody response to hgp75/TRP-1. In addition, when the *IFN-gamma* gene was included as an adjuvant with hgp75/TRP-1 and GM-CSF, the antibody response was abrogated completely. The type of immune response induced by an antigen (Th1 vs Th2) appears to be pre-determined, and attempts to 're-polarize' this response may, in fact, have a detrimental effect.

The requirement for IFN-gamma in the response to hTRP-2 also contrasts with the role of IL4 in the same response. Although no tumor immunity or autoimmune depigmentation occurs in animals lacking IFN-gamma, these responses are intact (or enhanced) in animals deficient in IL4. The more rapid depigmentation observed in IL4$^{-/-}$ mice supports the idea that Th2 cytokines, such as IL4, can temper Th1 responses, and that the absence of such opposing cytokines allows the intended response to occur more rapidly.

The experiments described in this paper raise the question of exactly when in the course of the immune response to DNA vaccines cytokines are required. IFN-gamma is released from CD8+ CTL and could therefore be partially responsible for tumor cell killing. However, repletion of IFN-gamma$^{-/-}$ mice with IFN-gamma protein only appears to be effective if it is done starting from the initiation of vaccination, and not solely during the effector phase, consistent with a role for this cytokine in events other than just tumor cell lysis. IFN-gamma may be needed for effective priming of naive Th1 cells to hTRP-2 or it may contribute to the processing and presentation of the antigen. Support for such an 'early' function of IFN-gamma in vaccine-induced tumor immunity can be found in the work of Winter *et al*. (12). This group showed that although IFN-gamma$^{-/-}$ mice are not protected from B16 tumor challenge after active immunization with irradiated B16 cells, adoptive transfer of T cells from immunized animals can mediate tumor regression. The absence of IFN-gamma results in deficiencies of other cytokines, such as IL12 and TNF-alpha, in a *Plasmodium chabaudi* system (22). This can further impair the response to antigens normally inducing a Th1 response.

The results from the experiments described in this report have implications for the design of optimal cancer vaccination strategies. First, it is now clear that cytokines used as adjuvants to enhance the response to one antigen may inhibit the response to other (related) antigens. Therefore, experiments to identify which type of cytokine (Th1 vs Th2) should be considered, so as to determine appropriate pairing of antigen with cytokine. Second, multi-epitope vaccines may be best administered at separate injection sites. The local cytokine milieu induced by one component of a multi-epitope vaccine may not be best for the other component(s). As more is learned about the roles of specific cytokines in antigen presentation and induction of immune responses to vaccines, more refined vaccination programs combining specific antigens with cytokines can be developed.

Abbreviations

gp75/TRP-1, tyrosinase-related protein-1; TRP-2, tyrosinase-related protein-2

Acknowledgements

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Materials and methods

Mice

C57BL/6 mice (females 6-8 weeks old) were acquired from the National Cancer Institute (Bethesda, Maryland) breeding program. Homozygotic mice genetically deficient for IFN-gamma (IFN-gamma$^{-/-}$) and IL4 (IL4$^{-/-}$), on a C57BL/6 background, were obtained from the Jackson Laboratories (Bar Harbor, ME). IFN-gamma receptor deficient animals were kindly supplied by Dr. Jan Vilcek, New York University Medical Center (New York, NY) (6). These mice were bred and kept in a pathogen-free Memorial Sloan-Kettering Cancer Center vivarium according to National Institutes of Health Animal Care guidelines. All mice entered the study between 7 and 10 weeks of age.

Cell lines and tissue culture

B16F10/LM3 is a pigmented mouse melanoma cell line of C57BL/6 origin, derived from the B16F10 line, provided by Dr. Isaiah Fidler (M.D. Anderson Cancer Center, Houston, TX) (23). This and other tumor cell lines were cultured as described (23).

Plasmid constructs

The human TRP-2 expression vector *hTRP-2* (original construct kindly provided by Drs. S.A. Rosenberg and J.C. Yang, National Cancer Institute, Bethesda, MD) has been previously described (24). The human gp75/TRP-1 vector *hgp75* has also been described previously (1). Vectors containing murine cytokine genes were kindly provided by Dr. J. Fuller, Powderject Vaccines (Madison, WI).

DNA immunization

The method of DNA immunization has been reported (1,25). In brief, *hTRP-2* or *hgp75* DNA was coated onto 1.0 µm diameter gold bullets (Bio-Rad Laboratories). Animals were immunized by delivering gold-DNA complexes using a helium-driven gun (Accell; PowderJect Vaccines Inc.) into each abdominal quadrant (1 µg plasmid DNA/quadrant) for a total of four injections. Mice were immunized weekly for a total of 5 weeks. Cytokine genes (*GM-CSF*, *IL2*, *TNF-alpha*, *IL12*, *IFN-gamma*) were delivered weekly for 5 weeks using the same method four to seven days before each vaccination with antigen.

Depigmentation studies

Depigmentation experiments were performed as described (1,23). In brief, after the final immunization, mice were shaved and depilated over the abdomen and observed for 8 weeks. Scoring of depigmentation was performed by dividing the abdomen into four equal quadrants. Quadrants were recorded as positive when they had an estimated >50% depigmented hairs. Depigmentation was scored 0-4+ according to the number of quadrants that were depigmented in each mouse (e.g., 3+ if three of four quadrants are depigmented >50%).

Antibody responses to gp75/TRP-1 and TRP-2

Antibody responses to syngeneic mouse gp75/TRP-1 and TRP-2 were measured by immunoprecipitation, followed by Western blot assay as described (1). B16F10/LM3 melanoma cells were lysed, followed by immunoprecipitation with mAb TA99 against mouse gp75/TRP-1 or anti-PEP-8, rabbit polyclonal antisera raised against a carboxyl-terminal peptide of TRP-2 (gift of Dr. Vincent Hearing, National Cancer Institute). To quantitate titers, sera were diluted until an endpoint was reached in the immunoprecipitation-Western blot assay. In addition, a B16 cell ELISA was performed as described previously (19). In this assay, serologic reactivity of mouse antibodies against B16 melanoma were assessed using serial dilutions of sera.

Cytokine release assay

CD4+ T cell lines were generated against TRP-2. Five days after the last immunization, CD4+ T lymphocytes were isolated from the spleen and draining inguinal lymph nodes by magnetic bead cell sorting (MACS) (Miltenyi Biotec, Auburn, CA). Briefly, cells were resuspended in PBS supplemented with 0.5% BSA and 2 mM EDTA, and incubated on ice for 15 minutes with anti-CD4 magnetic beads. After washing, the cells were placed on a magnetic column and two additional washes were performed. After removal from the magnet the cells were recovered and 5 x 10^5 cells were co-cultured with naive irradiated (300 cGy) splenocytes (2 x 10^7), in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM beta-mercaptoethanol, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 20 µg/ml hTRP-2 237-256 peptide (sequence NESFALPYWNFATGRNECDV) (18). Cells were restimulated weekly with peptide in media supplemented with 5% supernatant from rat lymphocytes stimulated with concanavalin A.

Intracellular cytokine flow cytometric analyses

Monoclonal antibodies against CD3 (clone 145-2C11) and CD28 (clone 37.51) were used for stimulation for intracellular cytokine staining. Anti-CD4-peridin chlorophyll protein (PerCP) or Cy-Chrome (clone RM4-5), anti-CD44-fluorescein isothiocyanate (FITC) (clone IM7) and anti-CD62L-FITC (clone MEL-14) were used for cell-surface staining. Intracellular staining for cytokines was performed using phycoerythrin (PE) anti-IL4 (clone 11B11) and FITC or PE anti-IFN-gamma (clone XMG1.2). Isotype controls for the intracellular cytokines used the irrelevant rat IgG1 monoclonal R3-34 conjugated to FITC or PE. All antibodies were purchased from PharMingen (San Diego, CA). After two to three rounds of stimulation, cells were stimulated with plate-bound anti-CD3 and 2 µg/ml anti-CD28 for 6 hours, with addition of 10 µg/ml Brefeldin A (Sigma, St. Louis, MO) for the last 2 hours. The cells were then stained for cell-surface markers and intracellular cytokines using the Cytofix/Cytoperm Kit (PharMingen) according to the manufacturers instructions and analyzed on a FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Fluorescence voltages and compensation values were determined using cells single-stained with anti-CD4-FITC, anti-CD4-PE or anti-CD4-PerCP. Acquisition and analysis were performed using CellQuest software (Becton Dickinson Immunocytometry Systems). For each tube, 30000 events were acquired in a live lymphocyte and CD4 double-gate.

Mouse tumor studies

All mice were injected intravenously via the tail vein with 2 x 10^5 B16F10/LM3 melanoma cells. Tumor challenge was performed 7 days after the final immunization. Mice were sacrificed at 14 days after tumor challenge, all lobes of both lungs were dissected, and surface lung metastases were counted under a dissecting microscope. Statistical analysis of tumor growth was performed using the Student's *t* test.

Repletion studies

Mice were repleted with 3000 U of recombinant murine IFN-gamma (Peprotech, Princeton, NJ) by intraperitoneal injection twice per week (26). Different groups of animals received IFN-gamma injections either from the start of immunization until the end of the experiment (priming phase + effector phase) or only after tumor challenge had begun (effector phase).

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