

# Targeted Interleukin 2 Therapy Enhances Protective Immunity Induced by an Autologous Oral DNA Vaccine against Murine Melanoma<sup>1</sup>

Andreas G. Niethammer,<sup>2</sup> Rong Xiang, J. Michael Ruehlmann,<sup>3</sup> Holger N. Lode, Carrie S. Dolman, Stephen D. Gillies, and Ralph A. Reisfeld<sup>4</sup>

Department of Immunology, The Scripps Research Institute, La Jolla, California 92037 [A. G. N., R. X., J. M. R., H. N. L., C. S. D., R. A. R.]; Lexigen Pharmaceuticals Corp., Lexington, Massachusetts 02173 [S. D. G.]; and Charité Children's Hospital, 13353 Berlin, Germany [H. N. L.]

## ABSTRACT

We demonstrate that a mouse-human chimeric anti-ganglioside GD2-interleukin (IL)-2 fusion protein (ch14.18-IL2) substantially amplifies tumor-protective immunity against murine melanoma induced by an autologous oral DNA vaccine containing the murine ubiquitin gene fused to murine melanoma peptide epitopes gp100<sub>25–35</sub> and TRP-2<sub>181–188</sub>. This combination therapy led to the complete rejection of a lethal challenge with B78D14 murine melanoma cells in six of eight mice and a marked suppression of s.c. tumor growth in the two remaining animals. The tumor-protective immunity was mediated by MHC class I antigen-restricted CD8<sup>+</sup> T cells together with CD4<sup>+</sup> T cell help, which was required only for tumor cell killing in the effector phase of the immune response. A single oral vaccination with the DNA vaccine, which was carried by attenuated *Salmonella typhimurium*, was equally as effective as three such vaccinations applied at 2-week intervals. The immunological mechanisms involved in this antitumor effect were suggested by a decisively increased secretion of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and IFN- $\gamma$  from CD4<sup>+</sup> and CD8<sup>+</sup> T cells and a markedly up-regulated expression on CD8<sup>+</sup> T cells of high-affinity IL-2 receptor  $\alpha$  chain (CD25), costimulatory molecule CD28, and adhesion molecule lymphocyte function-associated antigen-2 (LFA-2/CD2). Additionally, the combination therapy induced increased expression of costimulatory molecules B7.1 and CD48 on murine antigen-presenting cells. Taken together, our results suggest that IL-2 targeted to the tumor microenvironment by a specific antibody-IL-2 fusion protein is a potent enhancer of tumor-protective immunity induced by an oral DNA vaccine that may ultimately enhance the chances of success in its clinical application.

## INTRODUCTION

The application of DNA vaccines encoding tumor rejection antigens for cancer therapy was prompted by the cloning and identification of a number of melanoma self-antigens that are recognized by mouse and human T lymphocytes (1, 2). Such autoreactive T cells can escape deletion in the thymus and reach the periphery, where they may be activated to induce effective antitumor immune responses (3, 4). Among the melanoma-associated antigens recognized by T cells are gp100 and tyrosinase-related proteins TRP-1 and TRP-2, which are lineage-specific differentiation antigens expressed on melanocytes and melanoma cells of both mice and man (5–9). Requirements for breaking peripheral T-cell tolerance to these antigens were established when CTLs against gp100 were elicited by immunization of mice with a recombinant vaccinia virus encoding human gp100. Although a fortuitous heteroclitic immune response was generated against the corresponding murine gp100 epitope that substantially suppressed established pulmonary murine melanoma metastases in syngeneic mice, an autologous vaccine encoding murine gp100 failed to generate this tumor-protective immune response (5). More recently, however,

we have demonstrated that peripheral T-cell tolerance toward murine gp100 and TRP-2 antigens could indeed be broken by an autologous vaccine. This was accomplished with a DNA vaccine containing the murine ubiquitin gene fused to minigenes encoding murine peptide epitopes gp100<sub>25–35</sub> and TRP-2<sub>181–188</sub> that were delivered by oral gavage using an attenuated strain of *Salmonella typhimurium* as carrier. These peptide epitopes did contain dominant anchor residues for MHC class I antigen alleles H-2Db and H-2Kb, respectively. Importantly, tumor-protective immunity was mediated by MHC class I antigen-restricted CD8<sup>+</sup> T cells that secreted the proinflammatory IFN- $\gamma$  and induced tumor growth suppression after a lethal challenge with murine melanoma cells (10). The use of *Salmonella* as carrier for a vaccine and their potential to transport plasmid DNA through the gastrointestinal tract into the Peyer's patches and thus to induce T-cell responses have been described previously (11, 12). Also, the live attenuated *S. typhimurium* may actually provide a "danger signal" and stimulate the innate immune system (13, 14).

Three lines of prior evidence provided the rationale for our attempt to improve the efficacy of our DNA vaccine by combining it with small boosts of a recombinant antibody-IL-2<sup>5</sup> fusion protein, ch14.18-IL2. First, we demonstrated previously that such immunocytokines target to the tumor microenvironment, where they effectively activate and expand CD8<sup>+</sup> T cells. This, combined with CD4<sup>+</sup> T-cell help, induced effective antitumor immune responses and eradicated established metastases of murine melanoma (15) and colon carcinoma (16). Second, we could show that a humanized KS1/4-IL-2 fusion protein (huKS1/4-IL2) elicited a long-lived cellular memory immune response against murine colon carcinoma cells that was substantially amplified by additional boosts with noncurative doses of this same immunocytokine targeted to the tumor microenvironment (16). Third, the fact that IL-2 is also of key importance for boosting the efficacy of a tumor vaccine in the clinic was clearly demonstrated by other investigators (4, 17–20). In addition, modified gp100<sub>209–217</sub> peptide-induced T-cell responses resulted in a striking objective clinical response rate of 42% among melanoma patients only when these individuals received concurrent boosts with high doses of IL-2 (21). In fact, IL-2 may be a *sine qua non* in breaking peripheral T-cell tolerance against melanoma.

Here, we describe the effect of a combination immunotherapy using a single vaccination with our oral DNA vaccine to induce a tumor-protective immune response against murine melanoma that was rendered decisively more effective by boosts with noncurative doses of the mouse-human chimeric version of an anti-ganglioside GD2-IL-2 fusion protein (ch14.18-IL2). We further demonstrate that the induction of CD8<sup>+</sup> T effector cells together with CD4<sup>+</sup> T-cell help and the up-regulation of key adhesion and/or costimulatory molecules on both T cells and APCs provide a mechanism for an effective protective tumor immunity induced by this combination immunotherapy.

Received 12/8/00; accepted 6/19/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This is The Scripps Research Institute's manuscript number 13672-IMM.

<sup>2</sup> A. G. N. is a fellow of the Deutsche Krebshilfe.

<sup>3</sup> J. M. R. is a fellow of the Deutsche Forschungsgemeinschaft.

<sup>4</sup> To whom requests for reprints should be addressed.

<sup>5</sup> The abbreviations used are: IL, interleukin; TNF, tumor necrosis factor; KO, knockout; mAb, monoclonal antibody; PE, phycoerythrin; APC, antigen-presenting cell.

## MATERIALS AND METHODS

**Animals, Cell Lines, and Bacterial Strains.** C57BL/6J mice and C57BL/6J-Cd8a tm1 Mak (CD8 KO; stock number 002665) and C57BL/6J-Cd4 tm1 Mak (CD4 KO; stock number 002663) mice, 6–8 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) and from the breeding colony at The Scripps Research Institute (La Jolla, CA). These animals were housed under specific pathogen-free conditions. The B78D14 murine melanoma cell line has been described previously (22). This cell line was derived from B16 melanoma cells by transfection with genes encoding for  $\beta$ -1,4-*N*-acetylgalactosaminyl transferase and  $\alpha$ -2,8-sialyl transferase. These transfections induced a constitutive expression of gangliosides GD2 and GD3. This cell line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum in the presence of 400  $\mu$ g/ml G418 and 50  $\mu$ g/ml hygromycin B. The attenuated *S. typhimurium* AroA– strain SL7207 was kindly provided by Dr. B. A. D. Stocker (Stanford University, Stanford, CA). Bacteria were routinely grown at 37°C in Luria-Bertani broth or on agar plates (Sigma Chemical Co., St. Louis, MO) and supplemented, if required, with 100  $\mu$ g/ml ampicillin. The ability of the bacterial strain used to transfer the DNA to phagocytes *in vitro* has been examined and described by us previously (16).

**Vaccine Vectors.** The construction and amplification of the vectors pUb-M and pUb-V were reported previously (10). Briefly, pUb-M contains the murine ubiquitin gene fused to minigenes encoding murine melanoma peptide epitopes gp100<sub>25–35</sub> (EGSRNQDWL) and TRP-2<sub>181–188</sub> (VYDFVWL) with dominant H-2Db 5(N), 9(L), and H-2Kb 5(F), 8(L) anchor residues, respectively. pUb-V is an empty vector control that contains only the murine ubiquitin gene.

**Oral Immunization and Tumor Challenge.** Groups of eight female C57BL/6J mice were immunized by oral gavage of each animal with 100  $\mu$ l of PBS containing  $2 \times 10^6$  *Salmonella* harboring one of the plasmids, pUb-M or pUb-V, respectively. Mice were immunized either once or three times at 2-week intervals, respectively, and then challenged 1 week after the last immunization by s.c. injection of  $5 \times 10^5$  B78D14 murine melanoma cells into the left frontal flank. Animals were examined every other day, and tumor diameters were measured with microcalipers in two dimensions. Tumor volumes were calculated according to the following formula:  $\frac{1}{2} \times \text{length} \times \text{width}^2$ . Twenty days after tumor cell challenge, mice were sacrificed and analyzed for tumor growth.

**Boost with Targeted IL-2.** The construction of the mouse-human chimeric anti-GD2 antibody-IL-2 fusion protein (ch14.18-IL2) has been described previously (23). Groups of eight C57BL/6J mice immunized with pUb-M and pUb-V, respectively, received noncurative doses of ch14.18-IL2 (5  $\mu$ g) or 100  $\mu$ l of PBS for 4 consecutive days by injection into the lateral tail vein, starting 1 day after tumor cell challenge. Controls included mice that received only vaccination with pUb-M, PBS, or a combination therapy with pUb-V and ch14.18-IL2 at the same dose level used for boosting pUb-M. Animals were examined daily until the tumor became palpable, and tumor diameters were measured in two dimensions every other day. Mice were sacrificed 20 days after tumor cell challenge.

**Immune Response in CD4 and CD8 T-cell-deficient Mice.** To evaluate the role of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in mediating the immune responses elicited by our vaccine/boost protocol, groups of six C57BL/6J-CD4 KO and C57BL/6J-CD8 KO mice were treated and challenged as described above. The vaccine was applied once by oral gavage, 1 week before tumor cell challenge. C57BL/6J mice ( $n = 6$ ) were then treated 1 day later with dosages of ch14.18-IL2 (5  $\mu$ g) or PBS (100  $\mu$ l) for 4 consecutive days. Tumor volumes were compared at day 20 after tumor cell challenge and expressed as a percentage of the growth observed in the untreated group of mice.

**Depletion of CD4<sup>+</sup> T Cells.** Depletion of CD4<sup>+</sup> T cells was accomplished by weekly i.p. injection of 500  $\mu$ g of rat antimouse anti-CD4 mAb (GK1.5). We have described the effect of these antibodies previously (15, 24). Additional experimental groups included mice depleted only in the prechallenge immunization phase, mice depleted additionally through boosting, and mice depleted only after completion of the ch14.18-IL2 fusion protein boost.

**Activation of Splenocytes following Different Treatment Regimens.** The extent of systemic activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as dendritic cells was evaluated in mice that had received either pUb-M plus ch14.18-IL2, pUb-M alone, or empty vector pUb-V plus ch14.18-IL2 or PBS. One week after challenge, the spleens were harvested, collected in RPMI 1640, and kept on ice before analysis for intracellular IFN- $\gamma$  and TNF- $\alpha$  by flow cytometry as described below.

**Intracellular Cytokine Staining of IFN- $\gamma$  and TNF- $\alpha$  in CD4<sup>+</sup> and CD8<sup>+</sup> T Cells.** The following mAbs were used for intracellular cytokine staining: (a) anti-CD4 conjugated with FITC, 01064A; (b) anti-CD8 conjugated with FITC, 01044D; (c) anti-IFN- $\gamma$  conjugated with PE, 18114A (PharMingen, La Jolla, CA); (d) anti-CD3e PerCP-Cy5.5, PC1658; and (e) anti-TNF- $\alpha$  APC, PC1652 (BD Biosciences, San Jose, CA). All these reagents were purified rat antimouse mAbs. All antibodies and protocols for this assay were generously provided by Dr. Ken Davis (BD Biosciences). Cell suspensions were prepared by gently pushing tissue through a wire screen into a culture dish with RPMI 1640, followed by transfer into 15-ml polypropylene tubes. Erythrocytes were removed by adding lysis buffer (0.84% NH<sub>4</sub>Cl in H<sub>2</sub>O) for 2 min at room temperature. Cells were then washed in RPMI 1640 and diluted to a final concentration of  $1\text{--}3 \times 10^7$  cells/ml.

For intracellular cytokine staining, cell suspensions (100 ml/well) were transferred into 96-well tissue culture plates. To each well, 0.2  $\mu$ g of brefeldin A (B7651; Sigma Chemical Co.) was added for 1 h to allow cytokines to accumulate within the cell by blocking protein transport into post-Golgi compartments. Fc receptors were blocked by adding Fc blocking reagent (01241D; PharMingen). Aliquots of cells were washed and then subjected to staining for CD3e, CD4, or CD8. Cells were then permeabilized by Perm/wash buffer (2091KZ; PharMingen) and stained for intracellular IFN- $\gamma$  and TNF- $\alpha$ . Lymphocytes were analyzed on a FACScalibur with Cell Quest Software (BD Biosciences).

**Flow Cytometry Analysis.** Two-color flow cytometric analyses were performed with single-cell suspensions prepared from lymphatic tissues. Anti-CD8 (01044) and anti-CD-4 (01844) were used in FITC-conjugated form in combination with PE-conjugated rat antimouse mAbs CD2 (01175), CD25 (01105A), CD28 (01675), CD48 (09175), and CD80 (09605). Lymphocytes were incubated for 1 h at 4°C with FITC- and PE-labeled antibodies, washed, and analyzed immediately with a Becton Dickinson FACScan. A total of 10,000 labeled cells per sample were analyzed. All labeled antibodies were purchased from BD-PharMingen (La Jolla, CA).

**Statistics.** The statistical significance of differential findings between experimental groups of animals was determined by Student's *t* test. Findings were regarded as significant if two-tailed *P*s were  $< 0.05$ .

## RESULTS

**Comparison of Tumor-protective Immunity Achieved by One versus Three Oral Immunizations with DNA Vaccine.** To assess whether three vaccinations are actually required to achieve protective immunity, mice were immunized by oral gavage either once or three times with attenuated *S. typhimurium* harboring vector pUb-M. One week after the last immunization, these animals were challenged with  $5 \times 10^5$  B78D14 murine melanoma cells. As shown in Fig. 1, there was essentially no difference in the effectiveness of the vaccine, irrespective of whether the mice were vaccinated one or three times. In each case, mean tumor growth was reduced about 7-fold compared with that seen in the control groups that treated with either PBS or the empty vector, pUb-V. Two of eight mice rejected the tumor challenge completely when immunized either once or three times with pUb-M. The mice vaccinated with the empty vector containing only ubiquitin (pUb-V) showed no tumor protection, as did the other control group that received only PBS. Mean tumor growth was 1143 mm<sup>3</sup> in the control group receiving empty vector pUb-V and 1141 mm<sup>3</sup> in the untreated group receiving only PBS. Differences in tumor growth between treatment and nontreatment groups were statistically significant ( $P < 0.05$ ).

**Tumor Protection Induced by the DNA Vaccine Is Markedly Increased by Boosts with ch14.18-IL2 Fusion Protein.** Based on our hypothesis that vaccination-induced antitumor activity can be further enhanced by targeting IL-2 to the tumor microenvironment, groups of eight C57BL/6J mice each were either immunized once with the DNA vaccine (pUb-M) alone or additionally injected i.v. with four daily noncurative doses of the ch14.18-IL2 fusion protein (5  $\mu$ g each), starting 24 h after tumor cell challenge. Controls also

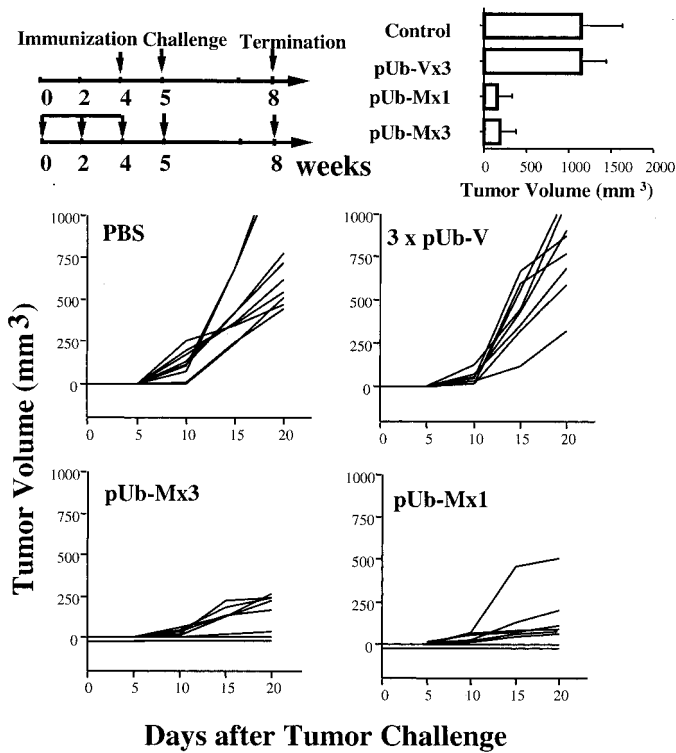


Fig. 1. Equal tumor-protective immunity is induced by one immunization versus three immunizations with DNA vaccine. Groups of eight C57BL/6J mice were immunized by oral gavage either once or three times every other week with  $10^6$  attenuated *S. typhimurium* carrying the pUb-M plasmid. One week after the last vaccination, mice were challenged s.c. with a lethal dose of  $5 \times 10^5$  B78D14 murine melanoma cells. Control groups included eight mice each treated with either PBS or immunized with attenuated *S. typhimurium* harboring only the empty vaccine vector pUb-V. Animals were examined daily until the tumor became palpable, after which its diameter was measured in two dimensions every other day. Lines represent tumor growth in individual mice. The bars represent mean tumor growth and SD 25 days after challenge.

included mice treated only with PBS or, importantly, mice that received the empty vector, pUb-V, plus the ch14.18-IL2 fusion protein at the same dose level used for boosting the pUb-M vaccine. When compared with controls receiving only PBS, the ch14.18-IL2 fusion protein and empty vector pUb-V combined showed no antitumor effect at day 20 after tumor challenge (Fig. 2). Significantly, however, boosts of the pUb-M vaccine with ch14.18-IL2 markedly enhanced tumor-protective immunity when compared with treatment with the vaccine alone ( $P < 0.01$ ; Fig. 2). In fact, in this case, mean tumor growth was reduced almost 10-fold to  $28 \text{ mm}^3$ , and six of eight mice rejected the tumor cell challenge completely. In contrast, in the group of mice that received pUb-M vaccine without the fusion protein boost, only two of eight mice completely rejected the tumor cell challenge. The experiment had to be terminated on day 20 after tumor challenge because of excessive tumor growth in control groups; however, mice that had completely rejected the tumor ( $n = 6$ ) were examined until day 30 and still revealed no tumor growth at this time point.

**Optimal Induction of Tumor-protective Immunity Requires CD4<sup>+</sup> T-cell Help.** The specific roles of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the induction of a tumor-protective immunity following the DNA vaccine treatment and targeted IL-2 boosts 24 h after tumor cell challenge were delineated by experiments performed in CD8 KO and CD4 KO C57BL/6J mice, respectively. Thus, as shown in Fig. 3, the DNA vaccine plus ch14.18-IL2 fusion protein boosts proved completely ineffective in CD8 KO mice, which revealed the same extent of tumor growth as C57BL/6J mice treated only with PBS. Importantly, however, experiments performed in CD4 KO mice clearly indicated that CD4<sup>+</sup> T-cell help is required to achieve optimal tumor-

protective immunity because CD8<sup>+</sup> T cells in the absence of CD4<sup>+</sup> T cells were only partially effective in reducing tumor growth when compared with the decisive reduction in tumor growth observed in fully immunocompetent mice treated with pUb-M and boosts of ch14.18-IL2 fusion protein ( $P < 0.01$ ). However, in the absence of CD4<sup>+</sup> T cells, tumor growth was still effectively suppressed when compared with naïve mice receiving only PBS ( $P < 0.01$ ).

**CD4<sup>+</sup> T-cell Help Is Required in the Effector Phase.** To further evaluate the role of CD4<sup>+</sup> T cells in generating a full antitumor response, CD4<sup>+</sup> T cells were immunologically depleted over different time periods. The results presented in Fig. 4 reveal that CD4<sup>+</sup> T cells

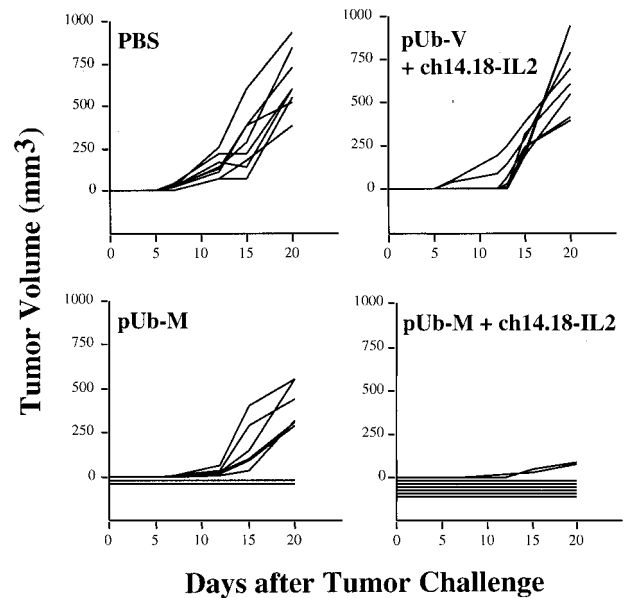


Fig. 2. Tumor-protective immunity induced by an oral DNA vaccine is amplified by boosts with ch14.18-IL2 fusion protein. C57BL/6J mice vaccinated once by oral gavage with attenuated *S. typhimurium* harboring either pUb-M plasmid or control plasmid pUb-V were challenged 1 week later by s.c. injection of  $5 \times 10^5$  B78D14 murine melanoma cells. This was followed 24 h later by  $5 \mu\text{g}$  of ch14.18-IL2 fusion protein injected i.v. for 4 consecutive days. Other control groups were mice treated by oral vaccination with pUb-M alone or PBS. Lines represent tumor growth in individual mice ( $n = 8$  mice/group).

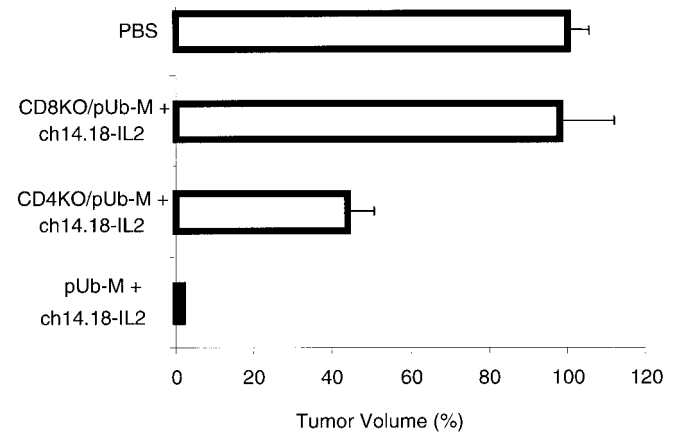
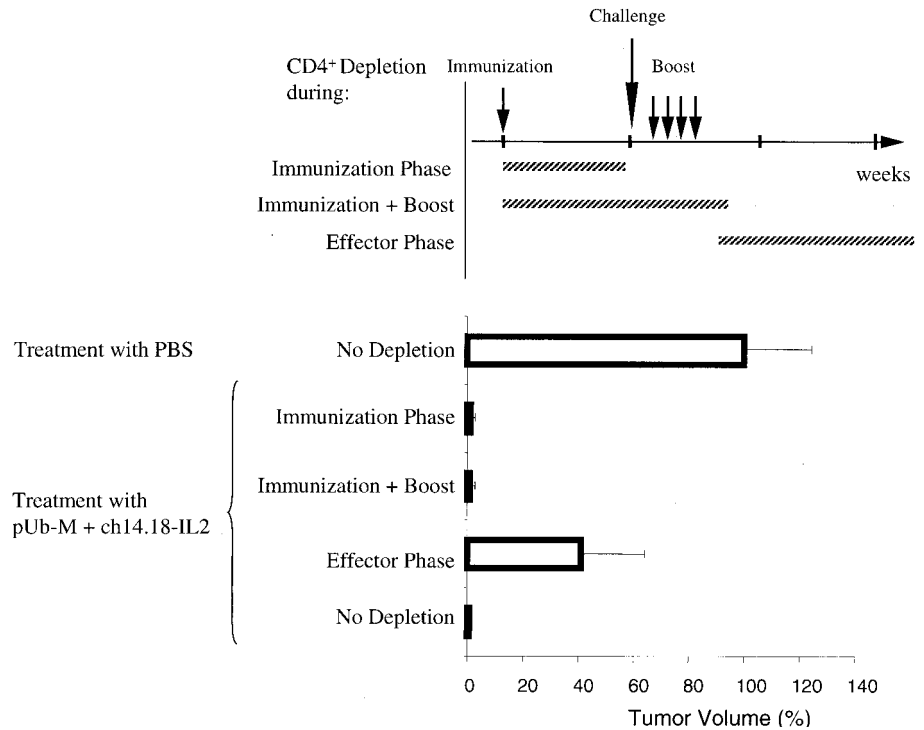


Fig. 3. Tumor-protective immunity induced by DNA vaccine is mediated by CD8<sup>+</sup> T cells and requires CD4<sup>+</sup> T-cell help. Two groups each of six C57BL/6J CD8 KO and CD4 KO mice, respectively, were treated once by oral gavage with *S. typhimurium* harboring the pUb-M plasmid and challenged 1 week later with tumor cells. This was followed 24 h later by i.v. injections of  $5 \mu\text{g}$  of ch14.18-IL2 fusion protein on 4 consecutive days. Control groups included six C57BL/6J mice treated in a manner identical to the KO mice and C57BL/6J mice that received only PBS. The mean tumor growth of the PBS control mice 20 days after tumor challenge was considered as 100%. Bars, SDs.



Fig. 4. CD4<sup>+</sup> T-cell help is only required in the effector phase. CD4<sup>+</sup> T cells were depleted in C57BL/6J mice over different time periods. Depletion was accomplished in groups of six mice each by weekly i.p. injection of 500 μg of rat antimouse anti-CD4 mAb (GK1.5). All experimental groups were immunized with the pUb-M vaccine and boosted with ch14.18-IL2 fusion protein. These mice were either depleted of CD4<sup>+</sup> T cells throughout the immunization phase; the immunization, challenge, and boost phases combined; or only throughout the effector phase. Control groups included nondepleted C57BL/6J mice treated identically and an additional group of mice that received only PBS. The mean tumor growth of the PBS control mice 20 days after tumor challenge was considered as 100%. Bars, SDs.

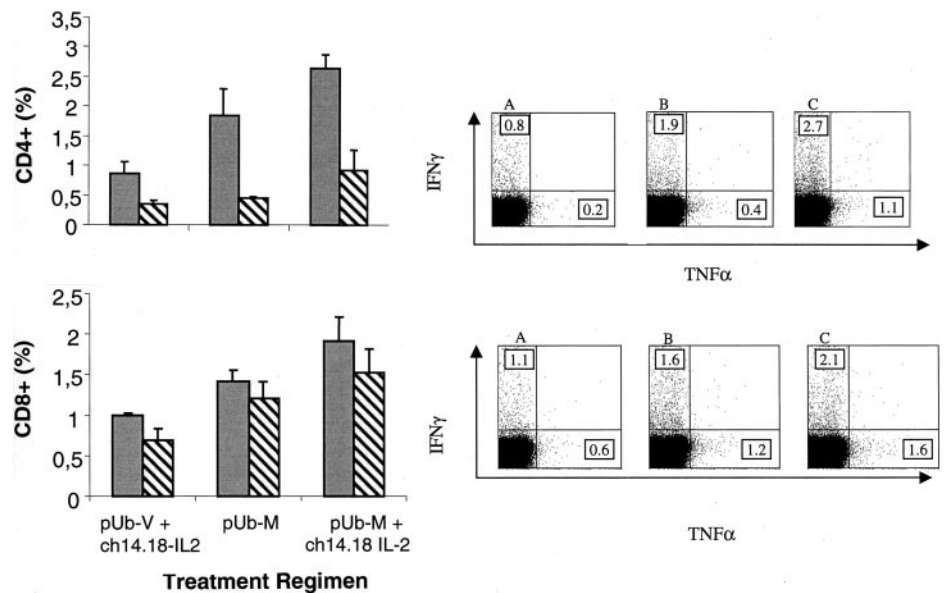


are necessary in the effector phase starting 1 day after completion of the boost. Thus, tumor protection in this group was significantly impaired when compared with undepleted mice ( $P < 0.01$ ). When compared with naïve mice receiving only PBS, suppression of tumor growth was still significant ( $P < 0.01$ ). In contrast, depletion of CD4<sup>+</sup> T cells throughout the immunization phase and ch14.18-IL2 boost had no effect on the extent of tumor protection when compared with that of undepleted mice.

**Combination of DNA Vaccine and Targeted IL-2 Therapy Increases Secretion of IFN- $\gamma$  and TNF- $\alpha$  from CD4<sup>+</sup> and CD8<sup>+</sup> T Cells.** The percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing IFN- $\gamma$  and/or TNF- $\alpha$  could be significantly increased depending on the treatment regimen. Specifically, Fig. 5 demonstrates average IFN- $\gamma$  and TNF- $\alpha$  expressions as representative dot plots. Thus, the average percentage of CD4<sup>+</sup> T cells expressing intracellular IFN- $\gamma$  more than

tripled from 0.86% to 2.6% after treatment with pUb-M vaccine plus ch14.18-IL2 boosts ( $P < 0.01$ ). In addition, the number of CD4<sup>+</sup> T cells expressing intracellular TNF- $\alpha$  more than doubled in mice treated with pUb-M plus the ch14.18-IL2 boosts as compared with mice treated with only the empty vector plus fusion protein or pUb-M alone, respectively ( $P < 0.05$ ). The percentage of CD8<sup>+</sup> T cells that expressed IFN- $\gamma$  comprised, on average, 1.9% of all CD8<sup>+</sup> T cells, as compared with 0.9% and 1.4% of all CD8<sup>+</sup> T cells when treated with pUb-V or pUb-M alone ( $P < 0.05$ ), whereas the number of TNF- $\alpha$ -positive CD8<sup>+</sup> T cells doubled from 0.7% to 1.4% ( $P < 0.05$ ). These data plus mean values and SEs are depicted in Fig. 5. Taken together, these results underline the potential of the boost with targeted IL-2 to amplify T-cell response and also emphasize the importance of CD4<sup>+</sup> T-cell help in achieving an optimal immune response elicited by the DNA vaccine.

Fig. 5. Boosts of targeted IL-2 therapy increase intracellular expression of IFN- $\gamma$  and TNF- $\alpha$  in CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Groups of C57BL/6J mice ( $n = 4$ ) were treated with various regimens of the DNA vaccine, and their splenocytes were analyzed. Increases in the mean percentage of IFN- $\gamma$ -positive (▣) and TNF- $\alpha$ -positive (▨) CD4<sup>+</sup> and CD8<sup>+</sup> T cells after vaccination with pUb-M plus boosts with ch14.18-IL2 fusion protein are compared with vaccination with empty vector plus boosts with ch14.18-IL2 pUb-V or pUb-M vaccine alone ( $P < 0.01$ ). Also shown are representative dot plots for the respective treatment groups. A, pUb-M boosted with ch14.18-IL2; B, pUb-M alone; C, control vector pUb-V boosted with ch14.18-IL2. All mice were challenged with tumor cells as described in "Materials and Methods."



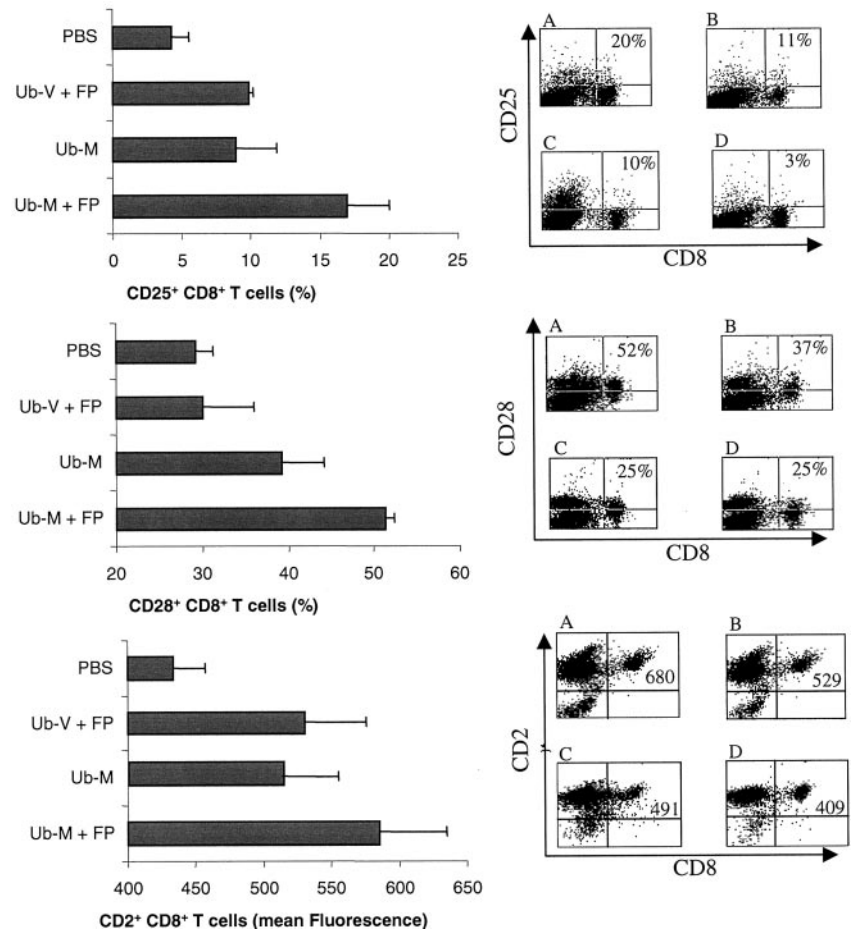


Fig. 6. Up-regulation of T-cell activation markers on CD8<sup>+</sup> T cells induced by DNA vaccine and boosts with ch14.18-IL2. C57BL/6J mice were immunized, challenged with melanoma cells, and boosted with ch14.18-IL2 fusion protein as described in "Materials and Methods." Controls included mice treated with the pUb-M vaccine alone, mice treated with empty vector pUb-V plus fusion protein, and mice treated with PBS. Fluorescence-activated cell-sorting analyses were performed with splenocytes obtained from mice 1 week after tumor cell challenge. Anti-CD2, -CD25, and -CD28 antibodies were used in PE-conjugated form in combination with FITC-conjugated antimouse mAb CD8. Each bar represents the mean and SE for three mice. Also shown are representative dot plots for the respective treatment groups and two color stainings. A, pUb-M boosted with ch14.18-IL2; B, pUb-M alone; C, control vector pUb-V boosted with ch14.18-IL2; D, treatment with PBS. All mice were challenged with tumor cells as described in "Materials and Methods."

**Expression of Activation Markers and Costimulatory Molecules on CD8<sup>+</sup> T Cells Is Induced by the DNA Vaccine and Enhanced by Boosts with the Antibody-IL-2 Fusion Protein.** We further analyzed the expression of well-recognized markers of T-cell activation on CD8<sup>+</sup> T cells, based on our finding that CD8<sup>+</sup> T cells are mainly responsible for the antitumor effect of our DNA vaccine. These markers included CD25, the high-affinity IL-2 receptor  $\alpha$  chain, and CD28, a member of the immunoglobulin superfamily expressed on T cells whose ligation with the costimulatory molecule B7.1 expressed on APCs will costimulate growth of naïve T cells. We also demonstrated up-regulation of LFA-2 (CD2) on CD8<sup>+</sup> T cells, which serves to enhance their adhesion to APCs via CD48 and thus costimulates T-cell activation. Fig. 6 depicts flow cytometry data that indicate up-regulation of the high-affinity IL-2 receptor  $\alpha$  chain (CD25) and the costimulatory molecule CD28 induced by vaccination with pUb-M. This effect was further enhanced by boosts with the ch14.18-IL2 fusion protein producing an 88% increase in CD25-expressing cells and a 31% increase in CD28 ( $P < 0.05$ )-expressing cells. Treatment with the empty vector pUb-V plus ch14.18-IL2 fusion protein had an effect similar to that of pUb-M vaccination alone in the case of CD25 but showed no effect on the expression of CD28. Treatment with pUb-M alone or with pUb-V plus ch14.18-IL2 up-regulated CD2 expression about 20% compared to treatment with PBS ( $P < 0.05$ ), and vaccination with pUb-M plus ch14.18-IL2 boosts produced an additional 14% increase in relative fluorescence.

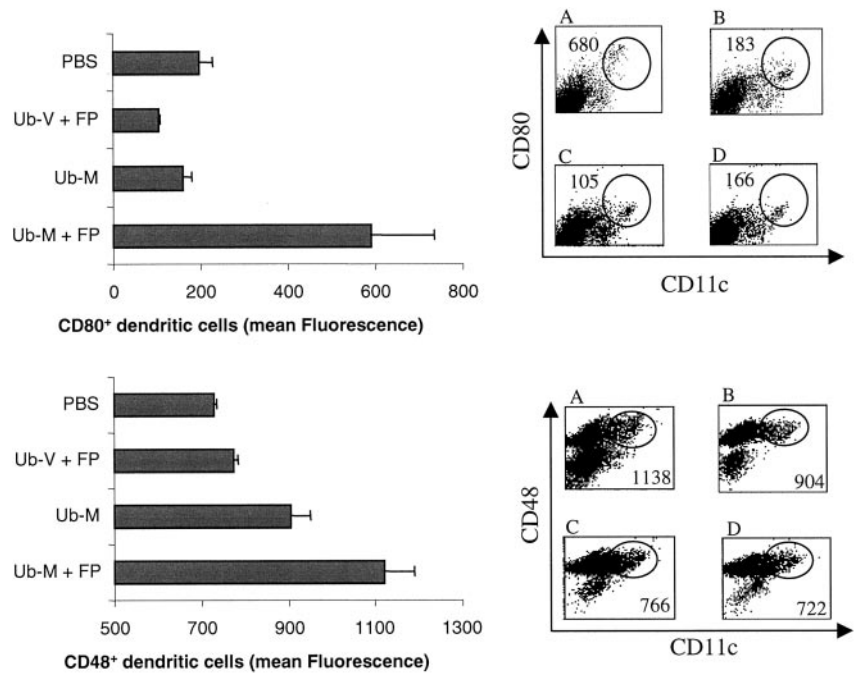
**The Expression of Costimulatory Molecules on Dendritic Cells Is Increased by Immunization with the DNA Vaccine and Further Enhanced by Boosts with Antibody-IL-2 Fusion Protein.** The effective activation of murine T cells requires the binding of the T-cell

receptor to the MHC-peptide complex in the presence of such adhesion and/or costimulatory molecules as B7.1 (CD80), as well as CD48, the major ligand of CD2 and the murine analogue of human lymphocyte function-associated antigen-3 (LFA-3). Consequently, we used two-color flow cytometry to analyze the effect of our DNA vaccine plus ch14.18-IL2 fusion protein boost on the expression of these molecules on dendritic cells isolated from the splenocytes of treated mice. Fig. 7 depicts up-regulation of these markers as indicated by an increase in relative fluorescence within a cell population staining double-positive for CD11c and CD48 or CD80, respectively. Specifically, vaccination with pUb-M alone led to an up-regulation of CD48 but not CD80 on dendritic cells when compared with treatment with PBS ( $P < 0.02$ ). However, boosts with ch14.18-IL2 fusion protein increased the relative fluorescence of CD80 threefold compared with treatment with either PBS, pUb-M alone, or pUb-V plus ch14.18-IL2 fusion protein. Importantly, boosts with ch14.18-IL2 also further increased the expression of CD48 by 24% when compared with treatment with pUb-M alone ( $P < 0.05$ ). In contrast, treatment with the control vector pUb-V plus boosts with ch14.18-IL2 fusion protein did not enhance the expression of either of these two markers when compared with treatment with PBS.

**DISCUSSION**

The breaking of peripheral T-cell tolerance toward poorly immunogenic self-antigens expressed by tumor cells and the subsequent establishment of an effective tumor-protective immune response remain a major challenge for cancer immunotherapy. Here, we demonstrate that this can be achieved in a mouse melanoma tumor model by

Fig. 7. Up-regulation of costimulatory molecules on dendritic cells induced by DNA vaccine boosted with ch14.18-IL2 fusion protein. C57BL/6J mice were immunized, challenged, and boosted with ch14.18-IL2 as described in "Materials and Methods." Experimental groups and controls are the same as those described in the Fig. 6 legend. Two-color fluorescence-activated cell-sorting analyses were performed with splenocytes obtained from mice 1 week after tumor cell challenge. Anti-CD80 and anti-CD48 antibodies were used in PE-conjugated form in combination with FITC-conjugated antimouse mAb CD11c. The increase in relative fluorescence of dendritic cells stained for CD48 or CD80 is indicated. Each bar represents the mean and SE for three mice. Also shown are representative dot plots for the respective treatment groups and two-color stainings. A, pUb-M boosted with ch14.18-IL2; B, pUb-M alone; C, control vector pUb-V boosted with ch14.18-IL2; D, treatment with PBS.



oral delivery of a DNA vaccine carried by attenuated *S. typhimurium* containing the murine ubiquitin gene and genes encoding murine melanoma peptide epitopes gp100<sub>25–35</sub> and TRP-2<sub>181–188</sub> with dominant anchor residues for MHC class I antigen alleles H-2Db and H-2Kb, respectively. Importantly, we could demonstrate in a prophylactic setting that boosts with noncurative doses of an anti-ganglioside GD2 antibody-IL-2 fusion protein (ch14.18-IL2) decisively improved the tumor-protective immunity against murine B78D14 melanoma cells transduced to express ganglioside GD2 as a docking site for the fusion protein. In addition, a single immunization with this DNA vaccine proved equally effective as three immunizations administered at 2-week intervals, attesting to the efficacy of this vaccine.

The rationale for using small noncurative doses of a recombinant antibody-IL-2 fusion protein to improve the efficacy of our DNA vaccine against murine melanoma was based on our previous findings in a CT26 murine colon carcinoma model in BALB/c mice. In this case, the huKS1/4-IL2 fusion protein at a curative dose ( $7 \times 15 \mu\text{g}$ ) elicited a long-lived cellular immune response against murine colon carcinoma cells in only 50% of experimental animals. However, this effect could be substantially amplified by additional boosts with noncurative doses of huKS1/4-IL2, resulting in the complete eradication of experimental lung metastases in all experimental animals. In this particular model, tumor-specific CD8<sup>+</sup> T effector cells were induced, some of which differentiated into long-lived T memory cells (16).

The mechanism involved in the induction of the tumor-protective immunity induced by the DNA vaccine, boosted by the ch14.18-IL2 fusion protein, clearly involved T lymphocytes. In fact, CD8<sup>+</sup> T cells are absolutely required; experiments performed in CD8 KO mice completely abrogated the antitumor effect of the DNA vaccine because tumor growth was essentially equal to that observed in syngeneic C57BL/6J controls treated only with PBS. The data from intracellular cytokine staining experiments indicate that immunization with the gp100<sub>25–35</sub> and TRP-2<sub>181–188</sub> peptide-based DNA vaccine increased the activation of CD8<sup>+</sup> T cells. Significantly, the number of such cells expressing either IFN- $\gamma$  or TNF- $\alpha$  doubled when compared with that obtained from mice treated with only the empty vector pUb-V, even when combined with ch14.18-IL2. Because vaccination

with only the ubiquitin gene carried by the attenuated strain of *S. typhimurium* was ineffective, it is apparent that neither the plasmid nor the *S. typhimurium per se* induced sufficient activation of T lymphocytes to result in any tumor-protective immunity effective against a lethal challenge of B78D14 murine melanoma cells. It is also evident that the initial CD8<sup>+</sup> T-cell response was directed against the peptides encoded in the DNA vaccine. However, we cannot rule out that this initial peptide-induced CTL attack on the tumor cells does not also lead to the generation of other CD8<sup>+</sup> T cells that recognize additional tumor cell antigens. If this is so, it is clear that these also would be boosted by the ch14.18-IL2 fusion protein. Importantly, it was evident that the DNA vaccine coupled with four boosts ( $5 \mu\text{g}$ ) of noncurative doses of ch14.18-IL2 fusion protein further increased the number of CD8<sup>+</sup> CTLs expressing the proinflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$ . In addition, effective T-cell activation was indicated by the marked up-regulation in expression of such key adhesion and/or costimulatory molecules as CD2, CD28, CD48, and CD80. These findings correlated with a marked increase in T-cell-mediated antitumor immunity observed in our animal model because 75% of mice completely rejected a lethal challenge of B78D14 murine melanoma cells compared with only 25% of such animals treated with the vaccine alone.

CD4<sup>+</sup> T-cell help also played an important role in the DNA vaccine-induced tumor-protective immunity observed in our mouse melanoma tumor model. Thus, experiments performed in CD4 KO mice with the DNA vaccine together with ch14.18-IL2 boosts clearly demonstrated a partial abrogation of the immune response because none of the mice rejected the tumor challenge; in fact, their s.c. tumor growth regressed only partially and was still 40% of that observed in controls treated with PBS. In contrast, this same treatment in fully immunocompetent C57BL/6J mice resulted in complete tumor regression in six of eight mice, whereas the tumor volume observed in the two remaining animals was <10% that of untreated controls. Significantly, immune depletion of CD4<sup>+</sup> T cells during immunization and effector phases indicated that CD4<sup>+</sup> T-cell help was not required for CD8<sup>+</sup> T cell priming but was necessary for tumor cell killing in the effector phase.

Although it was not entirely surprising to find that help provided by



CD4<sup>+</sup> T cells is required for optimal efficacy of our DNA vaccine/IL-2 fusion protein combination therapy, the finding that this help is not required for CD8<sup>+</sup> T-cell priming in the immunization phase but is only needed in the effector phase poses a new finding extending prior results. In this regard, we reported previously that eradication of established hepatic and pulmonary metastases of B78D14 obtained by multiple injections of ch14.18-IL2 fusion protein alone was partially impaired by *in vivo* depletion of CD4<sup>+</sup> T cells (15). Subsequently, we also found that the process resulting in tumor-protective immunity against murine melanoma required CD4<sup>+</sup> T-cell help mediated by the CD40-CD40 ligand interaction and not by endogenous IL-2 production (24). A helper function of CD4<sup>+</sup> T cells in adaptive immunity is well established in the literature and was first described for the B-cell compartment. Subsequent reports also described a role for CD4<sup>+</sup> T cells in the induction of CD8<sup>+</sup> T-cell-mediated immune responses (25).

Data from intracellular cytokine staining experiments correlated with those of our *in vivo* experiments, in which immunization with DNA vaccine doubled the number of inflammatory TH1 CD4<sup>+</sup> T cells expressing IFN- $\gamma$ , whereas boosts with ch14.18-IL2 further increased their number 3-fold compared with controls. Taken together, these data clearly indicate that help by these CD4<sup>+</sup> T cells is required to achieve optimal tumor-protective immunity against a melanoma cell challenge of mice immunized with the DNA vaccine and boosted by the ch14.18-IL2 fusion protein.

Finally, the marked up-regulation in expression of CD48 and B7.1 on dendritic cells and of their respective ligands CD2 and CD28 on CD8<sup>+</sup> T cells as well as the increase in expression of the high-affinity IL-2 receptor  $\alpha$  chain provided further evidence for the effectiveness of the targeted IL-2 boosts in enhancing the immune response evoked by our DNA vaccine. Although vaccination with attenuated *S. typhimurium* carrying the empty vector pUb-V boosted by ch14.18-IL2 fusion protein also up-regulated expression of CD28 and CD2, such treatment did not result in any antitumor effect *in vivo*.

In summary, we demonstrated that boosts with noncurative doses of ch14.18-IL2 fusion protein targeted to the tumor microenvironment are responsible for decisively improving the tumor-protective immunity evoked by our DNA vaccine. This finding correlates with the well-established fact that one of the key biological responses to IL-2 is the activation and clonal expansion of naïve T cells. Our data clearly indicate that an immunotherapy regimen including an autologous oral DNA vaccine boosted with small, noncurative doses of ch14.18-IL2 fusion protein broke peripheral T-cell tolerance toward poorly immunogenic murine melanoma self-antigens because a T-cell-mediated immune response was achieved that induced an effective tumor-protective immunity against a lethal challenge of melanoma cells. It is anticipated that appropriate DNA-based tumor vaccines combined with IL-2 specifically targeted to the tumor microenvironment by antibody-IL-2 fusion proteins might lead to the rational design of treatment regimens that may ultimately prove effective in clinical applications.

## ACKNOWLEDGMENTS

We thank Dr. Ken Davis and Laurel Namura (BD Biosciences) for reagents and assistance with intracellular cytokine staining and Collette Beaton for preparation of the manuscript.

## REFERENCES

- Boon, T., Cerottini, J. C., Van den Eynde, B., van der Bruggen, P., and Van Pel, A. Tumor antigens recognized by T lymphocytes. *Annu. Rev. Immunol.*, *12*: 337–365, 1994.
- Van Pel, A., van der Bruggen, P., Coulie, P. G., Brichard, V. G., Lethe, B., Van den Eynde, B., Uytendaele, C., Renauld, J. C., and Boon, T. Genes coding for tumor antigens recognized by cytolytic T lymphocytes. *Immunol. Rev.*, *145*: 229–250, 1995.
- Sprent, J., and Schaefer, M. Antigen-presenting cells for CD8<sup>+</sup> T cells. *Immunol. Rev.*, *117*: 213–234, 1990.
- Wang, R. F., and Rosenberg, S. A. Human tumor antigens for cancer vaccine development. *Immunol. Rev.*, *170*: 85–100, 1999.
- Overwijk, W. W., Tsung, A., Irvine, K. R., Parkhurst, M. R., Goletz, T. J., Tsung, K., Carroll, M. W., Liu, C., Moss, B., Rosenberg, S. A., and Restifo, N. P. gp100/pm17 is a murine tumor rejection antigen: induction of “self”-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. *J. Exp. Med.*, *188*: 277–286, 1998.
- Wang, R. F., Appella, E., Kawakami, Y., Kang, X., and Rosenberg, S. A. Identification of TRP-2 as a human tumor antigen recognized by cytotoxic T lymphocytes. *J. Exp. Med.*, *184*: 2207–2216, 1996.
- Kawakami, Y., Robbins, P. F., Wang, R. F., and Rosenberg, S. A. Identification of tumor-regression antigens in melanoma. *Import. Adv. Oncol.*, *3*: 21, 1996.
- Bakker, A. B., Schreurs, M. W., de Boer, A. J., Kawakami, Y., Rosenberg, S. A., Adema, G. J., and Figdor, C. G. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. *J. Exp. Med.*, *179*: 1005–1009, 1994.
- Bloom, M. B., Perry-Lalley, D., Robbins, P. F., Li, Y., el-Gamil, M., Rosenberg, S. A., and Yang, J. C. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. *J. Exp. Med.*, *185*: 453–459, 1997.
- Xiang, R., Lode, H. N., Chao, T. H., Ruelmann, J. M., Dolman, C. S., Rodriguez, F., Whitton, J. L., Overwijk, W. W., Restifo, N. P., and Reisfeld, R. A. An autologous oral DNA vaccine protects against murine melanoma. *Proc. Natl. Acad. Sci. USA*, *97*: 5492–5497, 2000.
- Darjy, A., Guzman, C. A., Gerstel, B., Wacholz, P., Timmis, K. N., Wehland, J., Chakraborty, T., and Weiss, S. Oral somatic transgene vaccination using attenuated *S. typhimurium*. *Cell*, *91*: 765–775, 1997.
- Paglia, P., Medina, E., Arioli, I., Guzman, C. A., and Colombo, M. P. Gene transfer in dendritic cells, induced by oral DNA vaccination with *S. typhimurium*, results in protective immunity against a murine fibrosarcoma. *Blood*, *92*: 3172–3176, 1998.
- Kaufmann, H. S. Immunity of intracellular bacteria. *Annu. Rev. Immunol.*, *11*: 129–163, 1993.
- Matzinger, P. Tolerance, danger and the extended family. *Annu. Rev. Immunol.*, *12*: 991–1054, 1994.
- Becker, J. C., Pancook, J. D., Gillies, S. D., Furukawa, K., and Reisfeld, R. A. T cell-mediated eradication of murine metastatic melanoma induced by targeted interleukin 2 therapy. *J. Exp. Med.*, *183*: 2361–2366, 1996.
- Xiang, R., Lode, H. N., Gillies, S. D., and Reisfeld, R. A. T cell memory against colon carcinoma is long-lived in the absence of antigen. *J. Immunol.*, *163*: 3676–3683, 1999.
- Barouch, D. H., Santra, S., Steenbeke, T. D., Zheng, X. X., Perry, H. C., Davies, M. E., Freed, D. C., Craiu, A., Strom, T. B., Shiver, J. W., and Letvin, N. L. Augmentation and suppression of immune responses to an HIV-1 DNA vaccine by plasmid cytokine/Ig administration. *J. Immunol.*, *161*: 1875–1882, 1998.
- Barouch, D. H., Craiu, A., Kuroda, M. J., Schmitz, J. E., Zheng, X. X., and Santra, S. Augmentation of immune responses to HIV-1 and simian immunodeficiency virus DNA vaccines by IL-2/Ig plasmid administration in rhesus monkeys. *Proc. Natl. Acad. Sci. USA*, *97*: 4192–4197, 2000.
- Geissler, M., Bruss, V., Michalak, S., Hockenjos, B., Ortmann, D., Offensperger, W. B., Wands, J. R., and Blum, H. Intracellular retention of hepatitis B virus surface proteins reduces interleukin-2 augmentation after genetic immunizations. *J. Immunol.*, *163*: 4284–4292, 1999.
- Sin, J. L., Ciccarelli, R. B., Higgins, T. J., Weiner, D. B., and Kim, J. J. *In vivo* modulation of vaccine-induced immune responses toward a Th1 phenotype increases potency and vaccine effectiveness in a herpes simplex virus type 2 mouse model. *J. Virol.*, *73*: 501–509, 1999.
- Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Sznol, M., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Seipp, C. A., Einhorn, J. H., Rogers-Freezer, L., and White, D. E. Impact of cytokine administration on the generation of antitumor reactivity in patients with metastatic melanoma receiving a peptide vaccine. *J. Immunol.*, *163*: 1690–1695, 1999.
- Haraguchi, M., Yamashiro, S., Yamamoto, A., Furukawa, K., Takamiya, K., Lloyd, K. O., and Shiku, H. Isolation of GD3 synthase gene by expression cloning of GM3  $\alpha$ -2,8-sialyltransferase cDNA using anti-GD2 monoclonal antibody. *Proc. Natl. Acad. Sci. USA*, *91*: 10455–10459, 1994.
- Becker, J. C., Pancook, J. D., Gillies, S. D., Mendelsohn, J., and Reisfeld, R. A. Eradication of human hepatic and pulmonary melanoma metastases in SCID mice by antibody-interleukin 2 fusion proteins. *Proc. Natl. Acad. Sci. USA*, *93*: 2702–2707, 1996.
- Lode, H. N., Xiang, R., Pertl, U., Forster, E., Schoenberger, S. P., Gillies, S. D., and Reisfeld, R. A. Melanoma immunotherapy by targeted IL-2 depends on CD4(+) T-cell help mediated by CD40/CD40L interaction. *J. Clin. Invest.*, *105*: 1623–1630, 2000.
- Pardoll, D. M., and Topalian, S. L. The role of CD4<sup>+</sup> T cell responses in antitumor immunity. *Curr. Opin. Immunol.*, *10*: 588–594, 1998.