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In Vivo Manipulation of Dendritic Cells Overcomes Tolerance to Unmodified Tumor-Associated Self Antigens and Induces Potent Antitumor Immunity

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Most tumor-associated Ags are self proteins that fail to elicit a T cell response as a consequence of immune tolerance. Dendritic cells (DCs) generated ex vivo have been used to break tolerance against such self Ags; however, in vitro manipulation of DCs is cumbersome and difficult to control, resulting in vaccines of variable potency. To address this problem we developed a method for loading and activating DCs, in situ, by first directing sufficient numbers of DCs to peripheral tissues using Flt3 ligand and then delivering a tumor-associated Ag and oligonucleotide containing unmethylated CG motifs to these tissues. In this study, we show in three different tumor models that this method can overcome tolerance and induce effective antitumor immunity. Vaccination resulted in the generation of CD8+ T and NK cell effectors that mediated durable tumor responses without attacking normal tissues. These findings demonstrate that unmodified tumor-associated self Ags can be targeted to DCs in vivo to induce potent systemic antitumor immunity. The Journal of Immunology, 2005, 174: 2645–2652.

The identification of tumor Ags to which autologous T cells respond has stimulated efforts to develop vaccines incorporating these Ags for immunotherapy (1). CTLs are a major focus of this effort because of their well-documented ability to eradicate tumor cells in vivo (2). A number of different antigenic constructs have been incorporated into vaccines for the induction of CTL-mediated antitumor immunity, including full length proteins, synthetic peptides, and naked plasmid DNA. However, for the most part these vaccines have been comprised of relatively immunogenic foreign or mutated Ags (3–5). In contrast, autologous nonmutated tumor Ags have proven to be poorly immunogenic as a consequence of peripheral tolerance as well as central tolerance (6–8).

Dendritic cells (DCs) (3) offer the potential to overcome tolerance against self Ags and activate CTLs specific for such Ags (9, 10). Human as well as animal studies have demonstrated that these extremely potent APCs can induce immunity to otherwise weakly immunogenic tumor Ags, and clinical trials of DC-based vaccines have shown promise in patients with a variety of tumors (11–13). However, all of these trials have involved cumbersome ex vivo preparation, loading, and activation of DCs, resulting in vaccines of variable consistency and potency. Given these difficulties we and others have begun to develop methods to load and activate DCs in vivo (14–16). For example, our group reported that treatment of mice with Flt3 ligand (FL) to expand the number of accessible DCs, followed by exposing the cells in situ to a mixture containing OVA and a DC-activating agent (a CpG-containing immunostimulatory DNA sequence), resulted in the generation of potent OVA-specific CTLs that could eradicate tumors that had been engineered to express this protein (14).

Because the majority of tumor-associated Ags are nonmutated self proteins (17, 18), a more rigorous assessment of this immunotherapeutic approach is needed to determine whether it can be used to induce a T cell response to such Ags leading to regression of tumors that express the same Ags. In the current study, we addressed this question in a variety of tumor models including B16 melanoma, CT26 colon cancer, and carcinoembryonic Ag (CEA) MC-38 colon cancer. In each model, the vaccine consisted of CpG and either an unmmodified self peptide or peptide expressed by the tumor. The results show that immunization of FL-treated mice with these Ags in combination with CpG leads to regression of pre-existing tumors as well as protection against a new tumor challenge.

Materials and Methods

Cells and cell culture

The murine B16 (H2b) melanoma cell line, CT26 (H2b) colon cancer cell line and EL-4 (H2b) thymoma cell line were maintained in complete medium that consisted of RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FBS (Invitrogen Life Technologies) and 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM l-glutamine (all reagents from Invitrogen Life Technologies). Murine MC-38 cells (H2b) transfected with human CEA were kindly provided by Dr. F. J. Primus (Vanderbilt University Medical Center, Nashville, TN) and maintained in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM l-glutamine, and 1 mg/ml G418.

Recombinant cytokines

Recombinant human FL was purchased from PeproTech. Recombinant human IL-2, mouse IL-4, and mouse GM-CSF were obtained from R&D Systems.

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6 Abbreviations used in this paper: DC, dendritic cell; CEA, carcinoembryonic Ag; CpG, oligonucleotide containing unmethylated CG motifs; FL, Flt3 ligand; LN, lymph node; PDC, plasmacytoid DC; Tg, transgenic; TRP, tyrosinase-related protein.
Mice
Six- to 8-wk-old male C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory. Male and female CEA transgenic (Tg) (C57BL/ 6-TGN(CEAGe)18FIP) mice, kindly provided by Dr. F. J. Primus, were bred at the animal care facility of Stanford University (Stanford, CA). Animal experiments were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Ags and CpG
Peptides used in this study were obtained from Sigma-Genosys. All peptides were >95% pure as indicated by analytical HPLC. Lyophilized peptides were diluted in DMSO and stored at −20°C until use. Purified human CEA was purchased from Protein Sciences. Oligonucleotide containing unmethylated CG motifs (CpG) (TCCATGACGTTCCTGACGTT) was synthesized and phosphorothioate-stabilized by Oligos, Etc. The oligonucleotide was reconstituted in sterile pyrogen-free water and then diluted in PBS for in vivo injections.

Vaccination
FL (10 μg) was administered i.p. daily for the number of days indicated. Control mice were injected in the same manner with PBS. In addition to the last FL injection, mice received a single s.c. injection of Ag (300 μg) or CpG (30 μg) alone, or CpG (30 μg) mixed with Ag (300 μg).

Abs and flow cytometry
Three-color analyses were performed on a FACS Calibur (Becton Dickinson). Mouse mAbs to CD8α (Ly-2; IgG2a), CD4 (GK1.5; IgG2b), B220 (RA3-6B2; IgG2b), CD11b (M1/70; IgG2b), CD11c (HL3; IgG1), GR-1 (RB6-8C5; IgG2b), CD86 (GL1; IgG2a), NK1.1 (NK1.1) (PK136, IgG2a), and TCR β-chain (H57-597; IgG2a) were purchased from BD Pharmingen.

Plasmacytoid DC (PDC) activation
Groups of three BALB/c mice received nine daily injections of FL or PBS starting from day 0, followed at days 6 and 9 by a s.c. injection of either CpG (100 μg) or PBS in the right footpad. Two days after the last CpG injection (day 11 post-FL) mice were sacrificed and the right draining popliteal and inguinal lymph nodes (LN) were harvested and injected with collagenase D (1 mg/ml) (Boehringer-Mannheim) in RPMI 1640 and 10% FCS for 20 min at 37°C. Digested LNs were filtered through a stainless-steel sieve to obtain a cell suspension. The percentage and activation status of LN PDCs were determined by flow cytometry.

Assessment of in vitro cytotoxic activity
For CTL measurements, mice were euthanized 10 days after immunization and their LNs were removed. The cells were resuspended in vitro in 24-well plates in 2 ml of culture medium with 10 μg/ml each peptide or irradiated tumor cells in the presence of 10 IU/ml IL-2. After 6 days of culture, viable effector cells were isolated using a lympholyte-M (Cedarlane Laboratories) gradient. Cytotoxic activity of the effector cells was assessed in standard 4-h51Cr-release assays. One million target cells were incubated with 50 μCi of 51Cr with or without peptide (10 μM) for 1 h at 37°C. Cells were then washed three times and incubated for 4 h with different numbers of effector cells. Thereafter, 50 μl of supernatant was harvested and radioactivity was counted. Percent specific lysis was calculated according to the following formula: (cpm experiment − cpm spontaneous)/(cpm maximum − cpm spontaneous) × 100. Spontaneous lysis was measured from wells containing only target cells, whereas maximum lysis was measured from the wells containing target cells incubated with 10% SDS.

Tetramer analysis
H-2Kbm2/TPR2 tetramers were obtained from Beckman Coulter. Single cell suspensions were prepared from the draining LN of the immunized mice. These cells were resuspended in 100 μl of medium containing 5 μl of anti-CD8-FITC mAb (mAb), 5 μl of anti-TCRβ-APC mAb (both purchased from BD Pharmingen) and 10 μl of K2/TPR2 tetramer for 30 min at room temperature before analysis by flow cytometry.

Tumor challenge experiments
Mice were injected s.c. with 1 × 106 or 1 × 105 B16 tumor cells or 1 × 105 CT26 tumor cells. In tumor protection experiments, mice were inoculated with tumor cells 1 day after completion of the vaccination protocol. In tumor therapy experiments, mice were first inoculated with tumor cells and 3 days later daily FL injections were started, and following their completion CpG and the indicated Ag were coinjected s.c. Tumor volume was expressed as the square of the smallest diameter of the tumor multiplied by the value of its largest diameter multiplied by 0.52.

Lymphocyte depletion experiments
To deplete specific lymphocyte subpopulations, 200 μg of anti-CD4, anti-CD8, or anti-asialo GM1 mAb (Biogenesis) was resuspended in 500 μl of PBS and injected i.p. into each mouse on the day before Ag immunization, followed by three injections on days 1, 3, and 10 after Ag immunization. Depletion of each cell type was confirmed by FACS analysis of peripheral blood lymphocytes.

FIGURE 1. Generation of Ag and tumor-specific CTLs in mice treated with FL + TPR2 + CpG. A, Experimental design. Group of five C57BL/6 mice were treated with nine daily injections of FL followed by 1 s.c. injection of TPR2180–188 peptide mixed with CpG. Control mice were injected in the same manner with PBS instead of FL. Ten days after the peptide injection, draining LN cells were harvested and assessed for CTL activity. B, Lysis of EL4 cells pulsed with TPR2180–188 peptide by LN cells restimulated with TPR2180–188 peptide for 6 days in the presence of IL-2. EL4 cells were preincubated with 10 μg/ml TPR2180–188 peptide for 1 h and then labeled with 51Cr for 1 h. Aliquots of 105 labeled target cells were then incubated for 4 h with the restimulated LN cells at the indicated ratios. Data shown are representative of three experiments with similar results. B, LN cells derived from FL + TPR2180–188 + CpG-treated mice; C, LN cells derived from FL + CpG-treated mice; ▲, LN cells derived from PBS + TPR2180–188 + CpG-treated mice; ■, LN cells derived from FL + TPR2180–188 + CpG-treated mice; ●, LN cells derived from PBS + TPR2180–188 treated mice. C, Lysis of B16 melanoma cells by restimulated LN cells. The restimulated LN cells were incubated for 4 h with 51Cr-labeled B16 cells at the indicated E:T ratios for evaluation of specific lytic activity. ●, LN cells derived from FL + TPR2180–188 + CpG-treated mice; △, LN cells derived from FL + CpG-treated mice; ▲, LN cells derived from PBS + TPR2180–188 + CpG-treated mice; □, LN cells derived from PBS + TPR2180–188 + CpG-treated mice; △, LN cells derived from PBS-treated mice. Data represent the average of triplicate cultures ± SD. D, Characterization of TPR2-specific CD8+ T cells in draining LNs. Single cell suspensions of draining LN cells were stained with anti-CD8-FITC Ab, anti-TCRβ-APC Ab, and K2/TPR2 tetramers. Naive mice were used as controls to determine background staining. Numbers in the upper right quadrants represent the percentage of TPR2-specific T cells in total CD8+ T cells.
blood. Mice treated with anti-CD4 or anti-CD8 had <1% of CD4 or CD8 T cells, respectively. Mice treated with anti-asialo GM1 had <2% of NK1.1+ cells.

Statistical analysis

SigmaStat statistical software package (Jandel Scientific) was used for data analysis. The Kaplan-Meier plot for tumor survival was assessed for significance with the log-rank test. For other data, the significance of differences between the experimental groups was analyzed using the nonparametric Tukey test. For all analyses, the level of significance was set at a probability of 0.05 to be considered significant.

Results

Induction of immunity to tyrosine-related protein 2 (TRP2) peptide in C57BL/6 mice

The goal of this study was to determine whether effective antitumor immunity could be induced by loading and activating DCs in situ with tumor-associated self Ags. Initially, we assessed the ability of a synthetic peptide derived from the melanocyte protein TRP2 (SVYDFFVWL; TRP2180–188), and known to contain a CTL epitope, to induce CTLs in C57BL/6 mice. Although TRP2 is a potential immunotherapeutic target for human as well as murine melanoma (4, 19), it has been reported to be poorly immunogenic due to peripheral tolerance (20). C57BL/6 mice were given FL injections for 9 consecutive days to increase the number of immature DCs in peripheral tissues, and on the final day they received a single s.c. injection of a mixture of CpG and TRP2180–188 (Fig. 1A). Ten days after immunization the draining LN cells were isolated and restimulated for 6 days with TRP2180–188, before the cells were assayed for CTL activity against TRP2180–188-pulsed EL-4 cells and unpulsed B16 melanoma cells. As shown in Fig. 1, B and C, both targets were efficiently killed by T cells obtained from the vaccinated mice, while little or no cytotoxic activity was observed in mice that received CpG + TRP2 without FL. Using MHC class I/TRP2 peptide tetramers to stain T cells in LNs, we found dramatic expansion of TRP2-specific CD8+ T cells in vaccinated mice compared with unvaccinated mice (2.96% vs 0.7%) (Fig. 1D). These T cells likely are responsible for the T cell activity detected in vitro.

Effect of immunity to TRP2 on the growth of B16 melanoma

We further investigated whether the TRP2-specific immunity induced by FL + TRP2 + CpG could provide protection against lethal B16 melanoma challenge. One day after mice were vaccinated, they were challenged with a single s.c. injection of B16
malignant melanoma cells (Fig. 2A). The TRP2<sub>180–188</sub> vaccine induced strong inhibition of tumor growth, whereas injection of FL + CpG without the Ag, or TRP2<sub>180–188</sub> + CpG without FL, had only a small effect on tumor growth (Fig. 2A). Sixty percent of the mice treated with FL + TRP2<sub>180–188</sub> + CpG survived 80 days after tumor challenge without developing any tumors compared with none of the mice treated with FL + CpG without TRP2<sub>180–188</sub> or with TRP2<sub>180–188</sub> + CpG without FL (Fig. 2B). To evaluate the therapeutic effect of FL + TRP2<sub>180–188</sub> + CpG, we injected mice with B16 tumor cells 3 days before the start of FL injections and 12 days before injection of TRP2<sub>180–188</sub> + CpG. Eighty percent of mice vaccinated in this manner survived at least 80 days after tumor challenge compared with none of the mice treated with FL + CpG without TRP2 or with TRP2 + CpG without FL (Fig. 2C), indicating that this vaccination strategy had a therapeutic as well as prophylactic effect on B16 melanoma.

**Effect of FL + Ag + CpG on CT26 colon cancer**

Next, we evaluated the effect of the same vaccination strategy against CT26 tumors in BALB/c mice. A CTL epitope, AH1 (SPSVVYHQE), in addition to gp70<sub>320–333</sub> peptide was added. Both peptides are derived from the murine leukemia virus gp70 envelope protein expressed by CT26 cancer cells (21), and were used as immunogens. As shown in Fig. 3, injection of a mixture of AH1 peptide and CpG induced strong AH1-specific CTL, while no CTL activity was observed in control mice. Addition of gp70<sub>320–333</sub> did not induce a stronger CTL response suggesting that gp70<sub>320–333</sub> was not an immunodominant epitope. To evaluate the therapeutic effect of vaccination, we injected FL-treated mice with AH1 + CpG 10 days after they were inoculated with CT26 tumor cells (see Fig. 4A). On the day mice were injected with Ag the average tumor volume was 58.2 mm<sup>3</sup>. Tumors in the mice injected with FL + AH1 + CpG continued to grow for several days after vaccination, reaching an average volume of 84.7 mm<sup>3</sup> on day 15 following tumor inoculation, and then regressed thereafter. The tumors regressed completely by 30 days, while treatment of mice with FL + CpG or AH1 + CpG had only a minor antitumor effect (Fig. 4B). Moreover, 100% of the mice treated with FL + AH1 + CpG survived to day 100 posttumor inoculation compared with 20% of mice treated with FL + CpG and none of the mice treated with AH1 + CpG without FL (Fig. 4C). Also, all of the mice treated with FL + AH1 + CpG rejected a second tumor challenge without any additional immunizations, indicating that vaccination resulted in strong and durable immunologic memory (data not shown).

**Role of CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, and NK cells in the anti-CT26 tumor response**

To test whether CD4<sup>+</sup>, CD8<sup>+</sup> T cells, or NK cells were required for protective immunity against CT26 tumors in vivo, we depleted...
these cells in mice using specific Abs. Depletion of CD8+ T cells or NK cells reduced the antitumor effect of the vaccine, whereas depletion of CD4+ T cells had no effect on antitumor immunity (Fig. 4D). Thus, CD8+ T cells and NK cells are the effector cells responsible for the tumor rejection induced by our vaccine.

**Induction of immunity to CEA in CEA-Tg mice**

Additional experiments were conducted in CEA-Tg mice to determine whether our vaccination method could be used to overcome tolerance to CEA, a 185-kDa secretory glycoprotein produced mainly by cells lining the gastrointestinal tract as well as a variety of human tumors (22). These mice express CEA in a tissue-specific manner, as observed in humans, where the colon is the main site of CEA production (23). CEA-Tg mice were given FL for 9 consecutive days, followed by injection of Cpg and CEA protein, and 10 days later LN cells were analyzed for specific CTL activity against MC-38.CEA cells (Fig. 5A). In these experiments the mice were vaccinated with CEA protein or CEA peptides, including CEA526–533, which has been reported to be an H-2Db-restricted CTL epitope in C57BL/6 mice (data not shown). As shown in Fig. 5B, incorporation of CEA protein into the vaccine resulted in induction of CTL activity against MC-38.CEA cells, whereas incorporation of CEA peptides failed to do so. LN cells from the mice immunized with CEA protein did not have CTL activity against EL-4 cells pulsed with CEA526–533 and CEA 492–500, suggesting that these epitopes are not immunodominant in CEA-Tg mice (Fig. 5C). We further investigated whether the CEA-specific immunity induced by FL + CEA + Cpg could protect CEA-Tg mice against challenge with MC-38.CEA cells. One day after mice were vaccinated, they were inoculated with a single s.c. injection of 2 × 106 MC-38.CEA cells. As shown in Fig. 6, tumors did not grow in the FL + CEA + Cpg-treated mice, whereas injection of FL + Cpg without the Ag had only a small effect on tumor growth.

**Effect of FL + Cpg on PDCs**

Cpg has been shown to mediate its cellular effect on APCs, including DCs, by binding to a specific TLR, TLR9, in such cells. Among APCs, TLR9 expression predominates in PDCs, a subset of DCs known to secrete high amounts of IFN-α in response to virus stimulation (25). Although in mice TLR9 expression is not limited to PDCs, in humans TLR9 is expressed exclusively by PDCs (26, 27). To test whether FL + Cpg can mobilize and activate PDCs in situ, we treated mice with FL for 9 days followed by 2 s.c. injections of Cpg, which increased the number of PDCs in the skin draining (popliteal and inguinal) LNs by >100-fold compared with untreated control mice. Importantly, Cpg induced local activation of FL-mobilized PDCs as assessed by up-regulation of CD86 and CD80 molecules on PDCs in mice treated with FL + Cpg compared with mice treated with FL alone (Fig. 7).

**Discussion**

In an earlier study (14), we showed in FL-treated mice that exposure of DCs in situ to a foreign Ag (OVA) in combination with a DC activator (Cpg) induces a systemic OVA-specific immune response sufficient to cause the regression of B16 tumor cells that had been engineered to express OVA. However, the effect of this immunization strategy on unmodified tumors that express only self proteins was not explored. In the current study, injection of FL-treated mice with unaltered tumor-associated peptides or proteins (TRP2, AH1, and CEA) together with Cpg induced potent Ag and tumor-specific immunity in two different strains of mice and in three different tumor models including B16 melanoma, CT26 colon cancer, and CEA transduced MC-38 colon cancer. Moreover, a single vaccination led not only to regression of existing tumors but also protected against a second tumor challenge delivered after a delay of several weeks. These results indicate that if a sufficient number of DCs are loaded with tumor-associated peptides or proteins (TRP2, AH1, and CEA) together with Cpg induced potent Ag and tumor-specific immunity in two different strains of mice and in three different tumor models including B16 melanoma, CT26 colon cancer, and CEA transduced MC-38 colon cancer. Moreover, a single vaccination led not only to regression of existing tumors but also protected against a second tumor challenge delivered after a delay of several weeks. These results indicate that if a sufficient number of DCs are loaded with tumor-associated self Ags and then activated in situ, tolerance to these Ags can be overcome and a durable therapeutic antitumor response induced.

The vaccination strategy evaluated in this study involves three components: a DC growth factor that increases the number of locally accessible DCs, a tumor-associated Ag, and a DC-activating

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**FIGURE 5.** CTL response induced after immunization of FL-treated CEA-Tg mice with CEA + Cpg. A, Experimental design. Groups of five mice were treated with nine daily injections of FL followed by one s.c. injection of CEA protein or CEA peptide mixed with Cpg. Ten days after Ag immunization, the draining LN cells were harvested, restimulated with irradiated MC-38.CEA tumor cells for 6 days in the presence of IL-2, and assessed for CTL activity. B, Lysis of MC-38.CEA tumor cells by restimulated LN cells from mice treated with FL + CEA + Cpg or FL + Cpg only. C, Lysis of EL-4 cells pulsed with 51Cr for 1 h. Aliquots of 106 labeled target cells were then incubated for 4 h with the restimulated LN cells at the indicated ratios. Data shown are representative of three experiments with similar results.

**FIGURE 6.** Effect of FL + CEA + Cpg vaccination on MC-38.CEA tumor cell growth in CEA-Tg mice. Mice were inoculated with MC-38.CEA tumor cells 1 day after completion of the indicated vaccination protocol.
DC ACTIVATION OVERCOMES TUMOR TOLERANCE

FIGURE 7. In situ mobilization and activation of PDCs by FL + CpG. Groups of three mice received nine daily injections of FL or PBS starting from day 0, followed at days 6 and 9 by a s.c. injection of either CpG (FL + CpG) or PBS (FL + PBS). Two days after the last CpG injection, draining LN cells were harvested. A, Contour plots showing the phenotype of PDCs. B–C, Contour plots (B) and bar graphs (C) showing the percentage of LN B220+ CD11clo Gr1+ PDCs in each treatment group. D, Contour plots showing the percentage of LN B220+ CD11clo Gr1+ PDCs that are CD8α+ or CD86+ in each treatment group. Data shown are representative of two experiments with similar results.

agent. Studies in humans as well as mice have shown that injection of recombinant FL results in massive release of DCs from the bone marrow into the circulation (28, 29). Although these DCs are distributed widely to LNs and peripheral tissues, in the absence of activation signals they remain morphologically and functionally immature (14, 29). Our finding, both in this study and in a previous study (14), that FL alone had no antitumor activity is consistent with the immature state of FL-mobilized DCs, although others have observed effects of FL on certain slow-growing tumors in mice (30). In fact, we took advantage of the ability of immature DCs to take up Ags in their environment by targeting tumor-associated Ags together with CpG to the FL-mobilized DCs that had accumulated in the skin and s.c. tissues. It is important to emphasize that in these studies all three vaccine components (FL, CpG, and Ag) were required for induction of effective antitumor immunity. This suggests that there is a minimum number of DCs that must be loaded and activated in situ to elicit systemic antitumor immunity, and further, that this minimum threshold may not be achievable without increasing the number of DCs accessible to local injection beyond normal levels. An alternative approach to achieving this threshold is to inject DCs or DC attracting chemokines directly into tumors (30, 31), which offers the potential to induce immunity to multiple tumor Ags. However, many tumors are not accessible to direct injection and the tumor environment often contains factors that inhibit DC activation (32).

Our results show that the therapeutic antitumor effect mediated by FL + Ag + CpG against CT26 tumors was mainly dependent on CD8 and NK lymphocytes, because in CD8 or NK cell-depleted mice that received all three vaccine components, tumors grew at the same rate as in untreated mice. Interestingly, FL treatment can induce modest NK cell proliferation, but does not induce NK activation (33). In addition, FL-deficient mice have a striking defect in NK cell function (34), suggesting a major role for FL in NK cell development and maturation. Previous studies have shown that CpG does not activate human NK cells directly but augments NK activity by inducing the secretion of IL-12, IFN-α and -β, and TNF-α (35). This suggests that FL and CpG work together through DCs to accentuate NK activation.

Although B16 melanoma is an aggressive, rapidly fatal tumor, other vaccine constructs have demonstrated significant protection against this tumor, including TRP2180 –188-pulsed DCs (36), TRP2-transduced DCs (37), TRP2180 –188 linked to foreign protein (38), recombinant adenovirus encoding different melanoma Ags (39), and GM-CSF-producing irradiated tumor cell vaccine (40). The majority of these vaccines provided protection against subsequent tumor challenge, while therapeutic immunity was more elusive. An exception is the combination of GM-CSF-producing B16 cells together with anti-CTLA-4 Ab, which demonstrated a therapeutic as well as prophylactic effect (41). In this system, CD4 T cells were found to be required for prophylaxis, whereas they were dispensable in a therapeutic setting. CD8 T cells and NK cells were the only required effector cells in this setting, while B cells were not required for either effective treatment or prophylaxis. The requirement for CTLs in the therapeutic but not prophylactic setting could reflect the presence of a greater tumor burden in the therapeutic setting that may require a higher number of CTLs for elimination. We have not analyzed the effector cells responsible for the antitumor effect against B16 tumors induced in the current study. However, it seems probable that as for CT26 tumors and OVA-transduced B16 tumors (14), the anti-B16 tumor effect of FL + TRP2 + CpG is mediated mainly by CD8 and NK cells.

None of the vaccinated animals exhibited signs of autoimmune disease or tissue inflammation despite being immunized against Ags expressed by normal tissues. This is not necessarily surprising in the case of TRP2 vaccinated mice in light of a previous study that showed that induction of TRP2-specific CTL was not associated with an autoimmune response against nontumoral melanin-producing cells (36). In contrast, in studies of melanoma bearing mice that were successfully treated with irradiated GM-CSF-producing B16 tumor cells, 50% of the mice developed diffuse vitiligo that was dependent on CD8 T cells (41). It is possible that the TRP2-specific T cells generated by our vaccine had relatively low affinity for TRP2 and therefore failed to engage normal melanocytes. It is also possible that a strong humoral response against TRP2 is required for vitiligo, and the absence of anti-TRP2 Abs in our mice, which were vaccinated with a small synthetic TRP2 epitope, may have prevented the occurrence of autoimmune disease.

Perhaps the most interesting result in this study was the ability of our vaccination scheme to overcome tolerance to CEA in CEA-Tg mice. In patients with a wide range of tumors, but most commonly in tumors of the gastrointestinal tract, CEA is overexpressed and the levels of CEA in the blood of such patients is used clinically as an indicator of tumor burden (22). Tolerance to CEA is believed to have been a major factor in the failure of CEA vaccines to induce either immune responses or tumor regression in clinical trials (42–44). Interestingly, we were able to induce cross-reactive immunity to CEA in a subset of patients with advanced colon cancer by vaccinating the patients with DCs pulsed in vitro with a synthetic CEA peptide that had been modified in an effort to avoid tolerance (29). To evaluate the potential to induce immunity to native CEA in a setting of immune tolerance we used CEA-Tg mice in which CEA is expressed in the gastrointestinal tract in a manner similar to humans (23). Remarkably, following treatment of these mice with FL, administration of native CEA protein in combination with CpG induced CTL and protected the mice against CEA+ tumors.
In humans, responsiveness to CpG containing oligonucleotides is restricted to B cells and PDCs that are the only cells that express the CpG receptor, TLR9 (45). Immature PDCs have been reported to accumulate in malignant ovarian tumors and associated ascites (46). Moreover, these cells were shown to secrete large amounts of IL-10 and inhibit tumor-specific T cell proliferation. These and other data suggest that immature PDCs can induce T cell tolerance (47) and therefore the presence of these cells in tumors might enhance tumor spread. By contrast to immature PDCs, CpG- or CD40L-activated PDCs induce strong CD8 and CD4 T cell responses (48). Thus, the ability of FL + CpG to mobilize and activate PDCs in situ may help overcome immune tolerant mechanisms that play a role in tumor progression. Additional TLRs including TLR7 are expressed by both murine and human DCs and PDCs (26) and a ligand for TLR7 has been shown to activate both subsets of DCs (49). It will be interesting to determine whether these agents, in combination with tumor Ags, can also induce a therapeutic antitumor response in FL-treated mice.

In summary, we have described a vaccination strategy based on in vivo manipulation of DCs that induces strong immunity against common tumor-associated Ags and durable tumor responses. These results provide the basis for evaluating a similar vaccination strategy in patients with cancer.

Disclosures
The approach described in this manuscript is the subject of a patent owned by Stanford University. The patent is based on work previously published by this group. M. Merad and E. G. Engleman are among the inventors listed on the patent.

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