Two Distinct Pathways of Immuno-Modulation Improve Potency of p53 Immunization in Rejecting Established Tumors

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ABSTRACT

The p53 gene product is overexpressed by almost 50% of cancers, making it an ideal target for cancer immunotherapy. We previously demonstrated rejection of established p53-overexpressing tumors without stimulating autoimmunity by immunization with modified vaccinia Ankara-expressing murine p53 (MVAp53). Tumor rejection was enhanced through antibody-mediated CTL-associated antigen 4 (CTLA-4) blockade. We examined the role of synthetic oligodeoxynucleotides (ODN) containing unmethylated cytosine-phosphate-guanine (CpG) motifs (CpG ODN) in enhancing MVAp53-mediated tumor rejection. CpG ODN with MVAp53 resulted in tumor rejection in BALB/c mice bearing poorly immunogenic 11A-1 murine mammary carcinomas or Meth A sarcomas and C57Bl/6 mice bearing MC-38 colon carcinomas. The effect was similar to that seen in tumor-bearing mice treated with MVAp53 along with CTLA-4 blockade. Monoclonal antibody depletion experiments demonstrated that the adjuvant effects of CpG ODN and CTLA-4 blockade were CD8 dependent. CpG ODN were partially natural killer cell dependent and ineffective in Toll-like Receptor 9+/− and inter leukin 6+/− mice, whereas CTLA-4 blockade was partially CD4 dependent and functional in Toll-like Receptor 9+/−and inter leukin 6+/− mice. In addition, when administered with MVAp53, both adjuvants enhanced p53-specific cytotoxicity and demonstrated an additive effect when combined. The combination of CpG ODN and CTLA-4 blockade worked synergistically to reject palpable 11A-1 and MC-38 tumors. These experiments demonstrate the potential for augmenting MVAp53-mediated antitumor immunity using CpG ODN and CTLA-4 blockade. This cell-free immunotherapy approach is a candidate for evaluation in cancer patients.

INTRODUCTION

The p53 gene product is an ideal and widely expressed target for the enhancement of the cellular immune response to malignancy. A large proportion of all human cancers have p53 mutations, p53 mutations occur as early events in tumorigenesis (1). p53 overexpression is an independent predictor of more aggressive cancer (2), of lymph node metastases (3), of failure to respond to standard therapeutic regimens (4), and ultimately of cancer-related mortality (5). In normal cells, p53 is responsible for temporarily arresting cell growth in response to certain types of molecular damage to allow for repair (6). Other types of physiological damage act by way of p53 to effect apoptosis (6). Mutations of p53 abrogate its function as a suppresser of cell division. Although p53 mutations may represent true tumor-specific antigens, most of the mutations do not occur at sites that would correspond to immunological epitopes (7). In experimental models, it has been possible to target p53 because the mutated molecule is associated with a high nuclear and cytoplasmic concentration of the p53 protein (8), and aside from point mutations, the remainder of the expressed protein is wild type (WT). What makes the p53 gene product such an attractive target for an adaptive immune response is that the intracellular concentration of nonmutated p53 is normally very low, and cells expressing normal p53 at low levels will most likely escape an enhanced immune response to overexpressed mutant p53 (9). Physiologically murine p53 is analogous to human p53 with 80% sequence homology. The murine system provides an excellent preclinical model for evaluating immunological approaches to overcoming tolerance to p53.

p53, like most of the tumor-associated antigens that are recognizable by the cellular arm of the immune system, is an autoantigen. An effective immunotherapy approach targeting p53 will need to overcome tolerance to p53 (10). In murine models, it has been possible to eliminate established p53-overexpressing tumors by the systemic administration of epitope-specific CTL (11), epitope-pulsed dendritic cells (DCs; Ref. 12), or mutant p53 epitope with interleukin (IL)-12 (13). In these reports, tumor rejection occurred with the noticeable absence of autoreactivity toward autologous noncancerous murine cells that express normal levels of p53. Prior studies from our laboratory have demonstrated the ability to generate p53-specific responses after immunization with modified vaccinia Ankara (MVA) expressing WT murine p53 (MVAp53; Ref. 14). Immunized mice developed vigorous p53-specific CTL responses and were able to reject small, established p53-overexpressing Meth A tumors. The therapeutic effect was enhanced by the administration of an anti-CTLA-4 monoclonal antibody [mAb (9H10); Ref. 15]. CTLA-4 blockade, which lowers the T-cell activation threshold and removes the attenuating effects of CTLA-4 enabled the rejection of large well-established Meth A tumors with the development of lasting tumor immunity. Tumor rejection occurred with the noticeable absence of autoreactivity toward autologous noncancerous murine cells that express normal levels of p53.

Recent advances in vaccine immunology have demonstrated that synthetic oligodeoxynucleotide (ODN) containing unmethylated cytosine-phosphate-guanine (CpG) motifs are potent immunostimulatory agents that can enhance vaccine potency (16). CpG ODN directly activates innate immunity [macrophages, DCs, and natural killer (NK) cells], which indirectly stimulates adaptive immunity and both humoral and cellular immunity (16) through an interaction with Toll-like Receptor 9 (TLR9). CpG ODN is a highly effective vaccine adjuvant, at least as effective as Freund’s adjuvant but with higher TH 1 activity and less toxicity (17). In an experimental murine tumor treatment model, CpG ODN when administered in the presence of CTLA-4 blockade augmented the antitumor effect of a peptide vaccine (18). Complete tumor rejection, however, was not seen with the peptide vaccine. To determine the feasibility of a more generalizable approach to the immunotherapy of tolerized tumor antigen, we examined whether the administration of CpG ODN in the presence or absence of CTLA-4 blockade could enhance the effect of the cell-free MVAp53 vaccine. We found that CpG ODN could enhance the effect of
MVAp53, resulting in the rejection of a number of established p53-overexpressing tumors in two different strains of mice. The adjuvant effect of CpG ODN differed from the adjuvant effect of CTLA-4 blockade in that the CpG ODN effect was partially NK cell dependent and required the presence of TLR9 and IL-6. The differing pathways of adjuvanticity were further supported by the synergistic effect of CpG ODN and CTLA-4 blockade on MVAp53 immunization.

MATERIALS AND METHODS

Animals

Female 6-8-week-old BALB/c, C57Bl/6, and B6.129S2-IL6tm1Skop1 (IL-6−/−) mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and TLR9−/− mice were a kind gift of Dr. Shizuo Akira (Osaka University, Osaka Japan). Mice were maintained in a specific pathogen-free environment. All studies were approved by the Research Animal Care Committee of the City of Hope National Medical Center and performed under the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International guidelines.

Cell Lines

CV-1 (19), TK− (20), and baby hamster kidney cells (BHK-21; Ref. 21; Ref. 21) were purchased from American Type Culture Collection (Manassas, VA) and grown in MEM supplemented with nonessential amino acids, L-glutamine, and 10% FCS. 11A-1 (22) was a kind gift of Dr. R. L. Ullrich (Medical Branch, University of Texas, Galveston, TX) and was grown in conditioned complete medium. MC-38 (23) was a kind gift from Dr. S. A. Rosenberg (National Cancer Institute, Bethesda, MD). Meth A sarcoma cells (Meth A; Ref. 24) were a kind gift of Dr. L. J. Old (Memorial Sloan-Kettering Cancer Center, New York, NY). p53 null 101 cells, a kind gift of Dr. Susan Kane (City of Hope National Medical Center, Duarte, CA), were stably transducted with full-length murine p53 (10.1/p53; Ref. 14). Meth A was passaged as an ascitic tumor. Cells were harvested, counted, and washed with PBS before use. Four days after inoculation with 2 × 10⁴ 11A-1, 1 × 10⁴ MC-38, or 1 × 10⁴ Meth A cells, small organized tumor nodules were seen on histological section (data not shown).

Antibodies

Anti-CD4 (GK1.5; Ref. 25) and anti-NK1.1 (PK136; Ref. 26) were purchased from American Type Culture Collection. Anti-CD8 (H35; Ref. 27) and anti-CD4 (H10; Ref. 15) were kind gifts from James P. Allison (University of California, Berkeley, CA). Antibodies were produced using a CELLine Device (BD Biosciences, Bedford, MA). IgG antibodies were purified by absorbance over protein G-Sepharose (Amersham Biosciences, Upsala, Sweden) followed by elution with 0.1 M glycine-HCl (pH 2.7). The product was then dialyzed against PBS and concentrated using a Centriplus centrifugal filter device (Millipore, Bedford, MA). Control Syrian hamster IgG was a kind gift of Dr. L. J. Old (Memorial Sloan-Kettering Cancer Center, New York, NY). Anti-CD8 (H35; Ref. 27) and anti-NK1.1 (PK136; Ref. 26) were purified using a Protein A spin column (Pharmacia, Piscataway, NJ). Antibodies were stored in PBS at -20°C before dilution in aqueous 0.9% sodium chloride solution before injection.

ODN

Synthetic ODN 1826 with CpG motifs underlined (5′-TCCATGACGTTC-C-3′) and non-CpG ODN 1826 (5′-TCCAGGACTTCTCAGGTT-3′; Ref. 29) were synthesized with nuclease-resistant phosphorothioate backbones by Trilink (San Diego, CA). The Na+ salts of the ODN were resuspended at 5 mg ml−1 in 10 mM Tris (pH 7.0) and 1 mM EDTA and stored as 50-μl aliquots at −20°C before dilution in aqueous 0.9% sodium chloride solution before injection.

In Vivo Tumor Challenge Experiments

MVA plus Anti-CTLA-4 mAb. Six-week-old BALB/c mice received s.c. injections in the left flank of 2×10⁶ 11A-1 cells. Six-week-old C57Bl/6, TLR9−/−, or IL-6−/− mice received injections s.c. in the left flank of 1 × 10⁶ MC-38 cells. Anti-CTLA-4 mAb or the control hamster antibody was injected i.p. on days 4, 7, and 10 at 100, 50, and 50 μg/dose, respectively. On day 5, mice were immunized i.p. with 5 × 10⁶ plaque-forming units (pfu) of MVAp53, 5 × 10⁶ pfu of MVApp65, or PBS. s.c. tumors were measured twice weekly in three dimensions with calipers. Growth curves truncate when the first mouse in the respective group dies. The dosing and timing of administration of MVAp53 with anti-CTLA-4 mAb was derived empirically by titrating dose and schedule to achieve optimal tumor rejection and survival.

MVA plus CpG ODN. Mice were challenged with tumor as above. Fifteen nmol of CpG ODN or the non-CpG ODN control were injected i.p. on days 4, 9, and 14. On day 5, the mice were immunized i.p. with 5 × 10⁶ pfu of MVAp53, 5 × 10⁶ pfu of MVApp65, or PBS. The s.c. tumors were measured twice weekly in three dimensions with calipers. The dosing and timing of administration of MVAp53 with CpG ODN were derived empirically by titrating dose and schedule to achieve optimal tumor rejection and survival.

Combined Anti-CTLA-4 mAb and CpG ODN. Six-week-old BALB/c mice received injections s.c. in the left flank of 2×10⁶ 11A-1 cells. Anti-CTLA-4 mAb or the control hamster antibody was injected i.p. on days 14, 17, and 20 at 100, 50, and 50 μg/dose, respectively. Fifteen nmol of CpG ODN were injected i.p. on days 14, 19, and 24. On day 15, the mice were immunized i.p. with 5 × 10⁶ pfu of MVAp53, 5 × 10⁶ pfu of MVApp65, or PBS. The combined anti-CTLA-4 mAb and CpG ODN treatment schedule for MC-38 tumors in C57Bl/6 mice was as described above for MVA plus anti-CTLA-4 mAb and MVAp53 plus CpG ODN.

In Vivo mAb Injections

Mice treated with MVA + CTLA-4 mAb or MVA + CpG ODN were depleted of CD8, CD4, or NK cells by i.p. injection of 200 μg of the relevant mAb or control mAb on days 4, 6, 8, and 15 with a maintenance dose every 7 days until sacrifice of the animals. This regimen was shown to deplete (>95%) BALB/c mice of CD4, CD8, or NK 1.1 cells based on flow cytometry of peripheral blood from treated animals (data not shown).

Cytotoxicity Assays

Mice were immunized with 100 μg of anti-CTLA-4 mAb or 15 nmol of CpG ODN or both followed by an i.p. injection of 5 × 10⁶ pfu of MVAp53. Anti-CTLA-4 mAb treatment was repeated 1 and 3 days after MVAp53 immunization, and CpG ODN treatment was repeated 4 days after MVAp53 immunization. After 2 weeks, spleens were harvested and disassociated, and splenocytes were washed and counted. Splenocytes were stimulated in vitro for 6 days with syngeneic lipopolysaccharide treated blasts infected with adenovirus expressing WT p53. Na+-CrO4-labeled 1.0 or 1.1 p53 target cells were added to 96-well plates with the effectors, in triplicate, at various effector to target ratios, in 200 μl of complete medium. The plates were incubated for 4 h at 37°C, and the supernatant was harvested and analyzed. Percent specific lysis was calculated using the formula: percent specific release = (experimental release – spontaneous release)/(total release – spontaneous release) × 100.

Statistical Methods

For experiments in which the growth of some tumors necessitated early sacrifice, growth curves were compared by the time to a fixed size using a logrank test. Contrasts of single groups to all others were conducted after a significant omnibus test. For cell depletion experiments, all mice were followed for a fixed amount of time, and final tumor size was compared by the Wilcoxon rank-sum test, after a significant Kruskal-Wallis test if there were more than two groups. For survival experiments, a logrank test was used.
RESULTS

Adjuvant Effect of Anti-CTLA-4 in Murine Breast and Colon Cancer Models. Prior studies from our laboratory demonstrated the adjuvant effect of CTLA-4 blockade with mAb on immunization with an MVA vaccine-expressing WT mup53 (14). These studies were performed using the well-characterized murine fibrosarcoma line, Meth A, which is considered to be an immunogenic tumor cell line (24). To determine whether effective immunization using MVAp53 with CTLA-4 blockade can be generalized to other tumor types, we replicated prior studies, using the murine breast cancer cell line 11A-1 (22) in BALB/c mice. 11A-1(H-2b) is a tumorigenic mammary carcinoma cell line that overexpresses p53 mutated at codon 173. It can be lysed by p53-specific CTL generated in MVAp53 immunized mice (data not shown). It is poorly immunogenic because mice immunized with 10^4 irradiated 11A-1 tumor cells fail to reject a subsequent challenge with 11A-1. Groups of mice with 4- to 5-day-old 11A-1 tumors immunized with MVAp53 in the presence of CTLA-4 blockade with mAb developed delayed tumor growth (Fig. 1A). Animals treated with CTLA-4 blockade alone or with a control MVA vaccine developed rapidly progressing lethal tumors (P = 0.00044, comparing MVAp53 with CTLA-4 blockade with control groups). Similar results were obtained in C57BL/6 mice bearing MC 38 colon carcinoma tumors (Fig. 1B, P = 0.0001, comparing MVAp53 with CTLA-4 blockade with control groups). MC38(H-2b) overexpresses p53 mutated at codon 242 and is recognized by WT p53-specific CTL (30, 31).

Adjuvant Effect of CpG ODN. CpG ODN treatment has been shown to be an effective adjuvant in a number of experimental tumor vaccine models (16). We evaluated the adjuvant effect of CpG ODN on MVAp53 immunization. Animals with 4-day-old 11A-1 tumors were immunized with MVAp53, CpG ODN, or the combination of MVAp53 and CpG ODN. Although MVAp53 and CpG ODN each separately resulted in minimal attenuation of tumor growth, all animals developed progressively lethal tumors. The combination of CpG ODN and MVAp53 immunization resulted in significantly diminished tumor outgrowth (Fig. 2A, P = 0.00002). In fact, six of eight animals treated did not develop palpable tumors and developed lasting tumor immunity, rejecting a rechallenge with 11A-1 at 60 days (data not shown). A similar pattern of tumor rejection was seen after treatment of early established Meth A(H-2b) tumors in BALB/c mice (Fig. 2B, P = 0.0015). Meth A is a fibrosarcoma cell line that overexpresses p53 mutated at codons 132, 168, and 234. To demonstrate that the adjuvant effect of CpG ODN with MVAp53 immunization is not strain specific, the immunization strategy was repeated in C57BL/6 mice bearing early MC38 colon cancers. The combination of CpG ODN and MVAp53 immunizations resulted in significant suppression of tumor growth in this model as well (Fig. 2C, P = 0.0004).

Contribution of CD4, CD8, and NK Cells to Vaccine Effect. The cellular requirements for the adjuvant effect of CTLA-4 blockade on MVAp53 immunization was previously evaluated in the Meth A tumor model in BALB/c mice. The antitumor effect was entirely dependent on CD8^+ cells and only partially dependent on CD4^+ cells, with no dependence on NK cells (14). To determine whether the adjuvant effect of CpG ODN on MVAp53 immunization follows a similar pattern, BALB/c mice with 4-day-established 11A-1 tumors received injections of depleting doses of CD4^+, CD8^+, or NK cell-specific antibodies. The adjuvant effect of CpG ODN on MVAp53 immunization could be largely abrogated by the administration of depleting CD8 mAb (Fig. 3A, P = 0.004). In contrast, CD4 depletion had no effect on the antitumor efficacy of CpG ODN with MVAp53, whereas depletion of NK cells partially abrogated the vaccine effect (P = 0.007, comparing NK with CD4 and control antibody depletions). Similarly, the therapeutic effect of the combination of CTLA-4 blockade with MVAp53 immunization on 11A-1 tumors in BALB/c mice could be minimized by administering depleting doses of anti-CD8 mAb (Fig. 3B, P = 0.004). Similar to prior findings in the Meth A tumor model, the antitumor effect of CTLA-4 blockade and MVAp53 administration was partially blocked by the administration of depleting anti-CD4 mAb (P = 0.008) but not by the administration of an NK-depleting mAb. The partial effect of either CD4 or NK cell subsets on tumor growth is striking, because the respective adjuvants both cause equivalent levels of tumor rejection. This result suggests that their mode of action is through differing immunological mechanisms.

Contribution of TLR9. The cell subset depletion studies suggest that the mechanism of adjuvant activity of CTLA-4 blockade and CpG ODN is different. CpG ODN activity results from the stimulation of B-cells and plasmacytoid DCs through an interaction with the TLR9 receptor (17). A bias toward the Th1 cytokine milieu is caused by CpG ODN treatment, and NK cell proliferation is stimulated, which may account for the partial effect on tumor rejection. To further delineate the divergent pathways involved in the CpG ODN and CTLA-4 blockade adjuvant effects, tumor challenge experiments were conducted in TLR9⁻/⁻ mice. TLR9⁻/⁻ mice fail to immunologically respond to CpG ODN administration (32). As expected, TLR9⁻/⁻
mice bearing early established MC-38 tumors failed to respond immunologically by rejecting tumor, similar to their WT relatives (Fig. 2C) to CpG ODN with MVAp53 immunization (Fig. 4A). In contrast, CTLA-4 blockade with MVAp53 immunization resulted in tumor suppression in TLR9−/− mice (P = 0.0009) to a similar extent to that seen in WT C57BL/6 mice (Fig. 1B).

**Contribution of IL-6.** CpG ODN and CTLA-4 blockade inhibit CD25+ CD4+ suppressor or regulatory T cells (Treg), and this effect may contribute to their adjuvant activity in the tumor models described here. CTLA-4 blockade is thought to have a direct inhibitory affect on Treg, most of which constitutively express CTLA-4 (33). In contrast, CpG ODN inhibit Treg activity through the secretion of IL-6 by DCs (34). To evaluate the role of IL-6 on the CpG ODN and CTLA-4 blockade adjuvant effects, tumor challenge experiments were conducted in IL-6−/− mice. IL-6−/− mice bearing early established MC-38 tumors failed to immunologically respond to MVAp53 immunization with CpG ODN by rejecting tumor (Fig. 4B). In contrast, CTLA-4 blockade with MVAp53 immunization resulted in tumor suppression in IL-6−/− mice (P = 0.02) to an extent similar to that seen in WT C57BL/6 mice (Fig. 1B).

**Fig. 2.** Effect of MVAp53 and CpG ODN treatment on established murine tumors. BALB/c mice received injections s.c. in the left flank of 2 × 10⁶ 11A-1 cells (A; P = 0.00002 comparing MVAp53 w/CpG ODN with all other groups) or 1 × 10⁶ Meth-A cells (B; P = 0.0015 comparing MVAp53 w/CpG ODN with all other groups); and C57BL/6 mice received injections of 1 × 10⁶ MC-38 cells (C; P = 0.0004 comparing MVAp53 w/CpG ODN with all other groups). For all panels, 15 nmol of CpG ODN (CpG) were injected i.p. on days 4, 9, and 14. On day 5, the mice were immunized i.p. with 5 × 10⁷ pfu of MVAp53, 5 × 10⁷ pfu of MVApp65, or PBS. The s.c. tumors were measured twice weekly in three dimensions with calipers. Each line represents the mean and SD of eight mice. In C, the non-CpG-ODN 1982 was also evaluated.

**Fig. 3.** Effect of depletion of the CD4+ T cells, CD8+ T cells, or NK cells on immunized mice. A, BALB/c mice received injections s.c. in the left flank of 2 × 10⁶ 11A-1 cells on day 0. Anti-CTLA-4 mAb was injected i.p. on days 4, 7, and 10 at 100, 50, and 50 μg/dose, respectively. On day 5, the mice were immunized i.p. with 5 × 10⁷ pfu of MVAp53. The mice were depleted of CD8+ T cells, CD4+ T cells, or NK cells by i.p. injection of 200 μg of the relevant mAb (CD4, CD8, or NK) or control Ab (isotype matched Ab) on days 4, 6, 8, and 15 and then every 7 days thereafter (see A for labels). Tumors were measured twice weekly in three dimensions with calipers. Each curve represents the mean and SD of eight mice (P = 0.004 comparing CD8+ depleted with all other groups; P = 0.008 comparing CD4+ depleted with anti-NK cell and control groups).

**Fig. 4.** Effect of depletion of the CD4+ T cells, CD8+ T cells, or NK cells on immunized mice. A, BALB/c mice received injections s.c. in the left flank of 2 × 10⁶ 11A-1 cells. Fifteen nmol of CpG ODN were injected i.p. on days 4, 9, and 14. On day 5, the mice were immunized i.p. with 5 × 10⁷ pfu of MVAp53. The mice were depleted of CD8+ T cells, CD4+ T cells, or NK cells by i.p. injection of 200 μg of the relevant mAb (CD4, CD8, or NK) or control Ab (isotype matched Ab) on days 4, 6, 8, and 15 and then every 7 days thereafter (see A for labels). Tumors were measured twice weekly in three dimensions with calipers (P = 0.004 by two-sided Wilcoxon test comparing CD8+ depleted with all other groups; P = 0.007 comparing anti-NK with anti-CD4 and control antibody). B, on day 0, BALB/c mice received injections s.c. in the left flank of 2 × 10⁶ 11A-1 cells. Anti-CTLA-4 mAb was injected i.p. on days 4, 7, and 10 at 100, 50, and 50 μg/dose, respectively. On day 5, the mice were immunized i.p. with 5 × 10⁷ pfu of MVAp53. The mice were depleted of CD8+ T cells, CD4+ T cells, or NK cells by i.p. injection of 200 μg of the relevant mAb or control mAb on days 4, 6, 8, and 15 and then every 7 days thereafter (see A for labels). Tumors were measured twice weekly in three dimensions with calipers. Each curve represents the mean and SD of eight mice (P = 0.004 comparing CD8+ depleted with all other groups and P = 0.008 comparing CD4+ depleted with anti-NK cell and control groups).
Adjuvant Effect of Anti-CTLA-4 and CpG ODN on CTL Responses. Because the mAb depletion studies suggest a primary role for CD8+ cells in the MVAp53 + anti-CTLA-4 or CpG ODN-mediated tumor rejection (Fig. 3), we wished to determine whether MVAp53 immunization with anti-CTLA-4 and/or CpG ODN results in enhanced antigen-specific CTL responses. Splenocytes from immunized mice were evaluated for CTL activity using as targets cells with or without endogenous p53 overexpression. Anti-CTLA-4 mAb and CpG ODN administration enhanced p53-specific CTL activity (Fig. 5). Furthermore, the effect of anti-CTLA-4 mAb and CpG ODN was additive, resulting in enhanced CTL activity when both adjuvants were used together.

Effect of CpG ODN and CTLA-4 Coadministration. The divergent cellular and molecular requirements for the adjuvant effect of CpG ODN and CTLA-4 blockade (Fig. 3) and observations made in TLR9−/− and IL-6−/− mice (Fig. 4) suggest that the adjuvant effects function through separate pathways. The cytotoxicity data suggested that the effects of anti-CTLA-4 mAb and CpG ODN might be additive. A more rigorous tumor model was designed to evaluate the potential additive effects of CpG ODN and CTLA-4 blockade on MVAp53 immunization. BALB/c mice were challenged with 11A-1 tumor and followed for 2 weeks until palpable tumors were present. The mice were then treated with MVAp53 vaccine and adjuvant. MVAp53 immunization with either CTLA-4 blockade or CpG ODN adjuvants resulted in prolonged survival, but all animals eventually succumbed to progressive tumor growth. The combination of CTLA-4 blockade and CpG ODN administration with MVAp53 immunization resulted in tumor rejection and prolonged survival in the majority of treated animals (Fig. 6, A and B). In terms of survival, the combination of CTLA-4 blockade and CpG ODN provides better adjuvant activity than either CpG ODN alone (P = 0.02) or CTLA-4 blockade alone (P = 0.01). The effect of combined CTLA-4 blockade and CpG ODN administration provides a greater benefit in terms of survival at 60 days than the simple addition of the effects of both adjuvants separately. A similar pattern was seen in C57BL/6 mice bearing MC 38 tumors (Fig. 6, C and D). In that tumor model, the combination of CTLA-4 blockade and CpG ODN also provided better adjuvant activity and survival than either CpG ODN alone (P = 0.002) or CTLA-4 blockade alone (P = 0.001). Differences between the various groups are better resolved by measuring survival as an end point as growth curves truncate when the first animal in each group died. The combined effect in both tumor models is not simply a dose-additive effect, because the CpG ODN and anti-CTLA-4 mAb were both already administered at doses of maximal efficacy. This data suggests that the differing requirements of each of the two adjuvants for their activity result in a true synergistic effect on tumor rejection.
growth, when both are administered with MVAp53. Although tumor rejection occurred after MVAp53 immunization with CpG ODN and anti-CTLA-4 mAb, no evidence of visible autoimmunity or toxicity was observed.

DISCUSSION

MVA is an ideal vector for the generation of a therapeutic response to overexpressed p53. Although MVA is able to efficiently replicate DNA in mammalian cells, it is avirulent because of the loss of two important host range genes among at least 25 additional mutations and deletions that occurred during its 570 serial passages through chicken embryo fibroblasts (35). In animal experimental tumor models, MVA-based vaccines stimulate tumor-specific CTL activity and effect regression of established tumors (14). Most impressive is their ability to overcome tolerance to autoantigen in mice (14). There are numerous distinct advantages to immunization with whole protein expressed in MVA. In contrast to peptide immunization, multiple epitopes can be expressed, and a polyclonal host response can be stimulated. Antigen-specific cognate help, which is essential to the propagation of a CTL response, can be achieved through expression of a protein in MVA. In addition, expression of whole protein can result in the stimulation of responses to otherwise cryptic epitopes.

Immunization with vaccinia viral constructs results in uptake and presentation of viral proteins by DCs (36). In draining lymph nodes, the DCs present antigen to naive CD8+ T cells, resulting in T cell activation and the subsequent propagation of an immune response (36). The current studies were designed to determine the feasibility of augmenting the response to MVAp53 by addressing both the initiation of the response and its propagation. To enhance priming of p53-specific responses, we have used CpG ODN. Immune activation by CpG ODN initiates with specific binding to the TLR9 receptor in B cells and plasmacytoid DCs. TLR9 ligation in DCs results in secondary activation of lymphocytes, macrophage, monocyte, NK-cell, and T-cell populations through the elaboration of cytokines generating a Th1 cytokine milieu (37). This results in increased NK activity as well as improved antigen presentation and T cell help that can augment

Fig. 6. Antitumor effect of combined CpG ODN and CTLA-4 mAb administered with MVAp53. BALB/c mice (n = 8) received injections s.c. in the left flank of 2 × 10^6 11A-1 cells. Anti-CTLA-4 mAb (CTLA-4 mAb) was injected i.p. on days 14 (delayed treatment), 17, and 20 at 100-, 50-, and 50-μg doses, respectively. Fifteen nmol of CpG ODN (CpG) were injected i.p. on days 14, 19, and 24. On day 15, the mice were immunized i.p. with 5 × 10^7 pfu of MVAp53, 5 × 10^7 pfu of MVApp65, or PBS. Tumors were measured twice weekly in three dimensions with calipers. Survival (A) and growth (B) curves were generated. For growth, each curve represents the mean and SD of the eight mice. C57BL/6 mice (n = 8) received injections s.c. in the left flank of 1 × 10^6 MC-38 cells. Anti-CTLA-4 mAb was injected i.p. on days 4, 7, and 10 at 100-, 50-, and 50-μg doses, respectively. Fifteen nmol of CpG ODN were injected i.p. on days 4, 9, and 14. On day 5, mice were immunized i.p. with 5 × 10^7 pfu of MVAp53, 5 × 10^7 pfu of MVApp65, or PBS (see A for labels).

The survival plot shows the proportion of surviving animals in each group as a function of days post tumor challenge. Tumors were measured twice weekly in three dimensions with calipers. Survival (C) and growth (D) curves were generated. For growth, each curve represents the mean and SD of the eight mice. (In A, P = 0.02 comparing combined CpG ODN and anti-CTLA-4 with CpG ODN alone and P = 0.01 comparing combined CpG ODN and anti-CTLA-4 with anti-CTLA-4 alone. In C, P = 0.002 comparing combined CpG ODN and anti-CTLA-4 with CpG ODN alone and P = 0.001 comparing combined CpG ODN and anti-CTLA-4 with anti-CTLA-4 alone.)
humoral and cell-mediated immune responses. In addition, TLR ligation results in the production of IL-6 by DCs, which helps overcome the suppressive effect of CD4+CD25+ Treg cells (34).

CpG ODN administration, when given alone, has been shown to exert modest antitumor effects in murine tumor models (38). CpG ODN has also been shown to be an effective adjuvant for a variety of experimental tumor vaccines in mice. CpG ODN can enhance the effect of peptide (39), protein (40), DCs (41), idiotype (42), and granulocyte macrophage colony-stimulating factor-secreting tumor cell vaccines (43). The ability of CpG ODN to prime for T_h1 responses and stimulation of NK cells probably accounts for the adjuvant activity in these vaccine approaches and in our own approach using MVAp53. This is supported by the mAb depletion experiments, which demonstrated that the adjuvant effect was mediated primarily by CD8+ cells and to a lesser degree by NK cells. The absence of adjuvant activity of CpG ODN in IL-6−/− mice suggests that CpG ODN could be mediating its adjuvant effect, in part, through the IL-6-dependent pathway of Treg cell inhibition. Nonetheless, the tumor models used here were too vigorous to support a therapeutic effect of CpG ODN alone without accompanying MVA p53 administration.

CpG ODN AND ANTI-CTLA-4 ANTIBODY

The studies presented here were designed to evaluate the ability of CpG ODN to break immunological tolerance to p53. Small numbers of factor-producing tumor cell vaccines, CTLA-4 results in rejection of established poorly immunogenic melanoma, mammary carcinoma, and prostate carcinoma grafts (45–47). This occurs through a process that involves breaking tolerance to tumor-associated autoantigens. Because p53 is an autoantigen widely expressed throughout development (48), tolerance to p53 might limit the effectiveness of p53-directed immunotherapies. Functional (10) and tetramer studies (49) in mice have clearly demonstrated tolerance to p53 at the CTL level. To achieve successful p53-directed immunotherapy, it will be necessary to break immunological tolerance to p53. Small numbers of self-reacting T cells escape during the processes involved in the immune tolerance. In mice, MVAp53 immunization alone can generate modest p53-specific CTL and rejection of early established tumors that overexpress p53 (14). Rejection of poorly immunogenic tumors and well-established progressing tumors requires the addition of adjuvants, which can promote tumor rejection. CpG ODN is a highly effective means of priming antigen-specific immune responses. Blocking the inhibitory signal provided by CTLA-4 using mAb also helps break peripheral T-cell tolerance. CpG ODN and CTLA-4 blockade with mAb help expand auto-reactive p53-specific CTL. The differences in the cellular and molecular requirements for the adjuvant effects of CTLA-4 blockade and CpG ODN suggest that each acts through a separate pathway. The additive effect of the combined use of both adjuvants further supports the diverse mechanisms of activity.

The studies presented here were designed to evaluate the ability of a cell-free vaccine with a number of immune adjuvants to break tolerance to p53, resulting in tumor rejection. Effective tumor rejection was demonstrated in three different tumor models across two different strains of mice. We used unmanipulated murine tumorigenic cell lines, which overexpress murine p53 by virtue of point mutations. The models are made more vigorous by the very high cell number in the tumor inoculum and the delay to treatment. These studies provide evidence of the ability to immunize mice against p53, resulting in regression of well-established tumor deposits. These findings support the need for the clinical evaluation of MVAp53-based vaccines with CTLA-4 blockade and CpG ODN adjuvants.

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