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Heterologous Prime/Boost Immunization with p53-based Vaccines Combined with Toll-Like Receptor Stimulation Enhances Tumor Regression

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Summary

The p53 gene product is overexpressed in ~50% of cancers, making it an ideal target for cancer immunotherapy. We previously demonstrated that a modified vaccinia Ankara (MVA) vaccine expressing human p53 (MVA-p53) was moderately active when given as a homologous prime/boost in a human p53 knock in (Hupki) mouse model. We needed to improve upon the inefficient homologous boosting approach, because development of neutralizing immunity to the vaccine viral vector backbone suppresses its immunogenicity. To enhance specificity, we examined the combination of two different vaccine vectors provided in sequence as a heterologous prime/boost. Hupki mice were evaluated as a human p53 tolerant model to explore the capacity of heterologous p53 immunization to reject human p53-expressing tumors. We employed attenuated recombinant *Listeria monocytogenes* expressing human p53 (*LmddA-LLO-p53*) in addition to MVA-p53. Heterologous p53 immunization resulted in a significant increase in p53-specific CD8⁺ and CD4⁺ T cells compared to homologous single vector p53 immunization. Heterologous p53 immunization induced protection against tumor growth but had only a modest effect on established tumors. To enhance the immune response we utilized synthetic double-strand RNA (polyI:C) and unmethylated CpG-containing oligodeoxynucleotide (CpG-ODN) to activate the innate immune system via Toll-like receptors (TLRs). Treatment of established tumor-bearing Hupki mice with poly(I:C) and CpG-ODN in combination with heterologous p53 immunization resulted in enhanced tumor rejection relative to treatment with either agent alone. These results suggest that heterologous prime/boost immunization and TLR stimulation increases the efficacy of a cancer vaccine, targeting a tolerized tumor antigen.

Keywords

p53; cancer vaccine; heterologous prime/boost immunization; modified vaccinia Ankara; listeria; toll-like receptor agonist

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A novel approach to cancer treatment involves the use of vaccines which target tumor associated antigens (TAA). An ideal and widely expressed target for the enhancement of the cellular immune response to malignancy is the p53 protein. About 40–60% of cancers have p53 mutations which abrogate its normal function as a suppressor of cell division, and p53 mutations occur as early events in tumorigenesis.^{1, 2} p53 overexpression is an independent predictor of more aggressive malignancy and failure to respond to standard therapeutic regimens, and ultimately of cancer-related mortality.^{2, 3} Mutations of p53 are associated with elevated nuclear and cytoplasmic concentration of the p53 protein.⁴ Although p53 mutations may represent true tumor-specific antigens, most of the mutations do not occur at sites that correspond to immunological epitopes.⁵ In experimental models, it has been possible to target wild type (wt) p53 because the mutated molecule is associated with a high nuclear and cytoplasmic concentration of the p53 protein, and aside from point mutations, the remainder of the expressed protein is wild type (wt).

What makes the p53 protein such an attractive target for an adaptive immune response is that the intracellular concentration of non-mutated p53 is normally very low, and normal cells have a low p53 turnover rate.⁶ Cells expressing non-mutated p53 at low levels escape damage by an enhanced immune response to overexpressed mutant p53.⁷ Work from our laboratory in several different murine tumor models, each having different mutant p53 sequence characteristics confirms our ability to utilize a single vaccine composed of the non-mutated p53 sequence to prevent outgrowth of p53-over-expressing tumors.^{8–11} The clinical relevance for cancer patients is the observation from our group among others, that p53-specific T-cells can be elicited and shown to lyse autologous tumor.^{11, 12} Targeting a wide variety of aggressive solid tumor malignancies for therapeutic intervention through immunologic mechanisms is achievable utilizing a single p53 sequence that is applicable to patients with tumors having a wide spectrum of mutations at different positions in the p53 protein.

One approach to stimulating strong tumor-specific immunity involves repeated vaccination with a recombinant vaccine vector such as a poxvirus or other attenuated virus expressing a TAA such as p53. In our prior studies repetitive administration of a single vaccine construct could prevent outgrowth of very early nonpalpable tumors however rejection of palpable established tumors was not possible.^{8–11} Boosting immune responses by repeated administration (homologous boosting) of a recombinant vaccine virus can be inefficient. Development of neutralizing immunity to the viral vaccine vector backbone interferes with expression of the TAA as a result of vector clearance by immune mechanisms.^{13, 14} Investigators have developed strategies to circumvent this problem by sequential administration of recombinant vaccines that use vaccine vectors of different sequence composition by a process known as heterologous prime/boost. Heterologous prime/boost minimizes the development of strong vector-specific neutralizing immunity.^{14, 15} Several heterologous prime/boost strategies utilizing recombinant MVA have been reported.¹⁶ Live, attenuated strains of *Listeria monocytogenes* (Lm) such as the Lm Δ dal Δ dat Δ actA (LmddA) strain have been used as vaccine vectors for delivery of foreign antigens.¹⁷ Such vaccines are able to generate strong innate and T cell mediated immune responses toward the targeted antigens. However, the efficacy for tumor rejection of the combination of recombinant MVA with recombinant *Listeria monocytogenes* is still unknown.

In this report, we describe the evaluation of a heterologous prime/boost vaccine strategy using MVA and the LmddA vector expressing full-length wt human p53 in a human p53 knock-in (Hupki) mouse model which is tolerant to human p53.^{11, 18} Potent cellular immune responses to p53 were generated following heterologous MVA-p53/LmddA-LLO-p53 immunization. Heterologous p53 prime/boost immunization induced protection against tumor growth but had only modest effects on established palpable tumors in this model. To

enhance the immune response we used poly(I:C) and CpG-ODN to activate the innate immune system via TLRs. Treatment of palpable s.c. tumors in Hupki mice with poly(I:C) and CpG-ODN in combination with heterologous prime/boost resulted in potent antitumor effects beyond results obtained in previous approaches.¹¹ These results suggest that heterologous prime/boost immunization and TLR stimulation may be useful in enhanced targeting of tolerized tumor antigen.

MATERIALS AND METHODS

Mice

Human p53 knock-in mice, which carry a partly humanized p53 gene harboring the human core domain sequence, were originally generated in a strain of 129/Sv genetic background mice.¹⁸ These mice were backcrossed onto the H-2d background (BALB/cJ mice) for five generations. p53-genotyping was performed by duplex PCR. Female 8–10-week old Hupki mice were used in these experiments. The 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) guidelines.

Cell Lines

A p53 null murine mammary adenocarcinoma cell line, 4T1,¹⁹ was transfected with mutant human p53 (R to H at position 175) using lipofectin (Invitrogen, Carlsbad, CA). The transfected cell line was cloned and the expression of p53 protein in 4T1p53 was confirmed by immunostaining and Western blot analysis using the anti-p53 mAb PAb122 (BD Pharmingen, San Diego, CA), as described previously.¹¹ Baby hamster kidney cells (BHK-21; Ref. 20) were purchased from American Type Culture Collection (Manassas, VA) and grown in MEM supplemented with nonessential amino acids, L-glutamine, and 10% FCS.

Reagents and Antibodies

Synthetic polyinosinic:polycytidylic acid (poly(I:C)) was purchased from Sigma (St. Louis, MO). CPG 7909, a B-Class CpG ODN of sequence 5'-TCGTCGTTTGTGCGTTTGTGCGTT-3' was synthesized with a wholly phosphorothioate backbone (TriLink™, San Diego, CA). Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or allophycocyanin (APC)-conjugated mAbs to mouse CD4, CD8 and gamma interferon (IFN- γ) were purchased from BD Pharmingen (San Diego, CA). Anti-CD4 (GK1.5)²¹ was purchased from American Type Culture Collection (Manassas, VA). Anti-CD8 (H35)²² was a kind gift from James P. Allison (Memorial Sloan-Kettering Cancer Center, New York, NY). Anti-asialo GM1 was purchased from Wako Chemicals (Richmond, VA).

Generation of Recombinant MVA

Construction of recombinant MVA was performed as described previously.²³ In brief, full-length wt human p53 or CEA were inserted into the MVA shuttle plasmid pLW22 (provided by Linda Wyatt and Bernard Moss, Laboratory of Viral Diseases, NIAID, NIH). MVA-expressing p53 or CEA was generated by homologous recombination by transfecting the pLW22-p53 or pLW22-CEA plasmid into wt MVA-infected BHK-21 cells, as described.^{9–11}

Listeria Constructs

The human p53 gene was cloned as a fusion protein to a truncated, non-hemolytic form of listeriolysin O (LLO), into the *Listeria* shuttle plasmid, pAdv142 which derives antigen expression under the control of *hly* promoter, harbors a copy of *Bacillus subtilis* d-alanine racemase gene and can complement the *dal-dat* and *actA* knock out Lm strain *LmddA*. This complementation is necessary for *in vivo* and *in vitro* plasmid retention by the vaccine strain in the absence of antibiotic selection markers.¹⁷ After verifying the correct sequence, the plasmid, pAdv159 was electroporated into the *LmddA* vaccine vector. Positive colonies of *LmddA*-LLO-p53 were identified by PCR screening and were passaged two times in mice to isolate the most stable colonies.²⁴ Glycerol stocks were prepared in BHI/2% glycerol and stored at -80°C . One vial was titrated in duplicate on BHI plates, to determine the colony forming units/ml. *LmddA*-control, expressing PSA, was generated as described previously.²⁵

Overlapping Peptide Library

A 15-mer overlapping peptide library derived from wt human p53 was composed of 96 peptides, which covered all 393 amino acids of the p53 protein with an 11-amino acid overlap between peptides, as described previously.¹¹ Purity was ascertained by reverse phase HPLC analysis.

Intracellular Cytokine Assays

A prime immunization was injected i.p. and consisted of either 5×10^7 plaque-forming units (pfu) of recombinant MVA or 1×10^8 colony-forming units (cfu) of recombinant *Listeria* on Day 0. A homologous or heterologous boost was administered on day 14. One week after the boost immunization, mice were sacrificed and splenocytes were incubated in the presence of irradiated and peptide library (10 $\mu\text{g}/\text{ml}$ each peptide) pulsed LPS blasts, as described.¹¹ After a 7-day *in vitro* stimulation, the splenocytes were assayed for intracellular IFN- γ production. The cells were exposed to 10 $\mu\text{g}/\text{ml}$ of peptide library for 1 h after which GolgiPlug (BD Pharmingen, San Diego, CA) was added. Following an overnight incubation, the cells were washed and labeled with FITC-conjugated antibody to mouse CD4 and PE-conjugated antibody to mouse CD8. The cells were then permeabilized and labeled with APC-conjugated antibody to IFN- γ for 30 min at 4°C . The cells were washed and analyzed on a FACSCanto flow cytometer (Becton Dickinson, San Jose, CA).

In Vivo Tumor Challenge Experiments

To develop a tumor protection model, 8–10-week old female Hupki mice (H-2d) were first immunized with TLR agonists. In brief, 50 μg of poly(I:C) and 15 nmol of CpG-ODN were administered by i.p. injection on days 0 and 5. On day 1, the mice were immunized i.p. with 5×10^7 pfu of recombinant MVA or 1×10^8 cfu of recombinant *Listeria* or PBS control. A boost immunization was administered i.p. on day 8. One week after the boost immunization, the mice were challenged with 5×10^5 4T1p53 cells by subcutaneous injection into the right lower flank. To develop an established tumor model, mice were challenged with 2.5×10^5 4T1p53 cells by subcutaneous injection. When the tumors were palpable and reached 4 to 5 mm in diameter in all mice, 50 μg of poly(I:C) and 15 nmol of CpG-ODN were administered by i.p. injection on days 6 and 11. On day 7, mice were immunized i.p. with 5×10^7 pfu of recombinant MVA or 1×10^8 cfu of recombinant *Listeria* or PBS control. A boost immunization was administered by i.p. on day 14. Tumors were measured twice weekly in three dimensions with calipers. Growth curves, representing mean tumor size, were truncated when the first mouse in the respective group died. Survival curves were truncated after 50 days, because death from tumor was not seen after 45 days in this tumor model. The dosing and timing of administration of MVA-p53 or *LmddA*-LLO-p53 with poly(I:C) and

CpG-ODN were derived empirically by titrating the dose and schedule to achieve optimal tumor rejection and survival.

In Vivo Monoclonal Antibody Injections

Mice treated with heterologous p53 immunization plus poly(I:C) and CpG-ODN were depleted by i.p. injection of 200 µg of anti-CD8 mAb or anti-CD4 mAb, or anti-asialo GM1 (dilution 1/20; 0.2 ml) every 3 days until sacrifice of the animals.^{9, 10} The control group did not receive monoclonal antibodies. This regimen was shown to deplete (>95%) CD8, CD4, or NK cells based on flow cytometry of splenocytes from treated animals (data not shown).

Statistical Methods

Comparisons with more than two groups were done by ANOVA using GraphPad Prism 5 software. Values of the results were expressed as means and SEs. Differences were considered to be statistically significant when $P < 0.05$. The survival time was evaluated using Kaplan-Meier plots and Log-rank (Mantel-Cox) tests.

RESULTS

Heterologous p53 Prime/boost Immunization Induced Dramatic p53-specific CD8⁺ and CD4⁺ T Cell Responses

As an initial test, we immunized 8 week old Hupki mice with MVA-p53 (5×10^7 pfu) and/or *LmddA*-LLO-p53 (1×10^8 cfu) in a homologous or heterologous prime/boost strategy. Hupki mice are phenotypically normal but have the majority of murine p53 replaced by human p53.¹⁸ We have previously demonstrated that these mice are tolerant of human p53.¹¹ The initial i.p. vaccination was followed two weeks later by an additional boost vaccination. Splenocytes from immunized mice were restimulated *in vitro* with the human p53-specific peptide library for one week prior to conducting an ICC assay to detect IFN- γ release. As shown in Figure 1, heterologous prime/boost with MVA-p53 followed by *LmddA*-LLO-p53 resulted in a dramatic p53 specific IFN- γ release far in excess of homologous prime/boost with MVA-p53 or *LmddA*-LLO-p53. Interestingly, the reverse heterologous prime/boost (*LmddA*-LLO-p53, MVA-p53) was not as effective, especially in stimulating CD8⁺ T cell immunity. The level of response to the heterologous vector regimen is far greater than what we have observed with our prior p53 vaccine strategies.¹¹ To evaluate the induction of CD4⁺ and CD8⁺ T cell responses when heterologous p53 immunization is combined with TLR agonists, we also evaluated p53-specific IFN- γ responses associated with heterologous p53 immunization with poly(I:C) and CpG-ODN. However, dual TLR stimulation did not augment the induction of CD4⁺ and CD8⁺ T cell responses specific to p53 (data not shown).

Heterologous p53 Immunization Elicits Potent Antitumor Effects in a Tumor Protection Model

Immunization of mice solely with rMVA expressing human p53 resulted in modest p53 specific CD8⁺ cellular immunity (Figure 1) and rejection of several established experimental murine tumors which over express p53.^{9–11} Female Hupki mice, 8–10-week old, were immunized i.p. with 5×10^7 pfu of MVA-p53, 1×10^8 cfu of *LmddA*-LLO-p53, or PBS. Initial immunization was followed 7 days later by a homologous or heterologous boost immunization. 5×10^5 poorly immunogenic and highly aggressive 4T1p53 cells were inoculated 7 days following the boost immunization. There was improved tumor control with heterologous immunization using the same vector order as in the best immunogenicity responding group (Figure 1) compared to homologous immunization (Figure 2A). Of note, either homologous group (MVA or Lm) was only marginally effective, greater than the PBS

control but lower than the heterologous prime/boost group. The survival analysis of mice from this trial showed a similar benefit of the heterologous and *Listeria* homologous vaccines (Figure 2B), and a small reduction in survival in groups where homologous boosting with MVA was exclusively employed. However, none of the groups caused complete tumor rejection, which prompted us to explore addition of TLR adjuvants to the regimen for possible improvement in survival.

Improvement in Potency of Heterologous Versus Homologous p53 Vector Immunization When Combined with Poly(I:C) and CpG-ODN in a Tumor Protection Model

Recent advances in vaccine immunology have demonstrated that ligands of both TLR3 and TLR9 are potent immunostimulatory agents that can enhance vaccine potency.^{26, 27} Mice were immunized using either a homologous or heterologous regimen augmented with TLR stimulation. The regimen for this immunization test is shown in Figure 3A. 50 µg of poly(I:C) and 15 nmol of CpG-ODN were administered by i.p. injection on days 0 and 5. On day 1, Hupki mice were immunized i.p. with 5×10^7 pfu of MVA-p53, MVA-control viruses or PBS. For the heterologous immunization, mice were immunized i.p. with 1×10^8 cfu of *LmddA*-LLO-p53, *LmddA*-control or PBS on day 8. Mice received s.c. injections of 5×10^5 4T1p53 cells on day 15. The p53-specific heterologous regimen (Figure 3B) exceeded homologous (Figure 3A) vector combinations in eliciting antitumor effects when combined with poly(I:C) and CpG-ODN. The control MVA and Lm vector combinations, either homologous or heterologous, were equally ineffective at controlling tumor growth regardless of TLR agonist stimulation (Figures 3A and B). The differential lower tumor growth in the effective p53-specific heterologous vector combination translated into improved survival versus any of the other groups (Figure 3C). This result is visualized using a Kaplan-Meier plot which shows the effect on survival that is 100% at 50 days in the p53-specific heterologous vector group, whereas all other groups either had all subjects expired by 38 days post-tumor inoculation or had a lone survivor (homologous MVA-p53 + adjuvants, Figure 3C). Nonetheless, there was a significant difference in survival between the heterologous group and the MVA homologous group ($p < 0.05$ by log-rank test).

Heterologous p53 Immunization Results in Modest Antitumor Effects in an Established Tumor Model

Initial studies showed that heterologous p53 immunization dramatically enhanced p53-specific CD4⁺ and CD8⁺ T cell responses (Figure 1). To model the clinical intervention in patients that is a more common scenario than immuno-prophylaxis, we evaluated the p53-specific heterologous prime/boost strategy in a therapeutic model. Hupki mice were inoculated with 2.5×10^5 cells of 4T1p53 by s.c. injection. When the palpable tumors reached 4 to 5 mm in diameter, mice were immunized i.p. with 5×10^7 pfu of MVA-p53 or PBS control. Booster immunization was performed 7 days after the prime immunization. Unlike the profound resistance to tumor growth seen in the prophylaxis model, heterologous p53 immunization with *LmddA*-LLO-p53 resulted in a modest therapeutic antitumor effect on established 4T1p53 tumors (Figure 4).

Enhancement of Antitumor Effect with Poly(I:C) and CpG-ODN

Because heterologous p53 immunization alone was not sufficient to induce a strong antitumor response in a therapeutic model, we tested whether the addition of TLR agonist would result in enhanced priming of p53-specific antitumor responses and increased survival. Hupki mice were first injected with 2.5×10^5 4T1p53 cells. Once palpable tumors developed, 50 µg of poly(I:C) and 15 nmol of CpG-ODN were injected i.p. on days 6 and 11. On day 7 of the regimen, mice were immunized i.p. with 5×10^7 pfu of MVA-p53 or MVA-control. For each booster immunization, mice were immunized i.p. with 1×10^8 cfu of *LmddA*-LLO-p53 or *LmddA*-control on day 14 of the regimen. As shown in Figure 5A,

treatment with poly(I:C) and CpG-ODN in combination with heterologous p53-specific immunization resulted in potent antitumor effects relative to immunizations with control vectors with adjuvants or p53-specific vectors without adjuvants. Combined administration of poly(I:C) and CpG-ODN resulted in a more potent antitumor effect than either agent alone (data not shown). Moreover, mice receiving heterologous p53 immunization and TLR agonists demonstrated a 50% complete regression of established 4T1p53 tumors as shown in a Kaplan-Meier plot (Figure 5B). On day 60, tumor-free mice were re-challenged with 5×10^5 4T1p53 cells titrated as a lethal dose at a different site from the first s.c. inoculation. All mice rejected the tumor challenge (data not shown). The cellular requirements for the adjuvant effect of poly(I:C) and CpG-ODN on heterologous p53 immunization was also evaluated in this established 4T1p53 tumor model. Hupki mice with 4T1p53 established tumors received injections of depleting doses of CD8⁺, CD4⁺, or NK cell specific antibodies. The antitumor effect was significantly dependent on CD8⁺, CD4⁺, and NK cells. The adjuvant effect of poly(I:C) and CpG-ODN on heterologous p53 immunization could be largely abrogated by the administration of depleting CD8 mAb and CD4 mAb (Figure 5C). Depletion of NK cell also partially abrogated the vaccine effect ($P < 0.05$).

DISCUSSION

We have generated and extensively characterized an attenuated poxvirus referred to as modified vaccinia Ankara (MVA) expressing either human or murine p53.^{9–11} MVA engineered with recombinant genes (rMVA) have shown promise as a vaccine in rodents and macaques for infectious disease and cancer. Most impressive are their properties of overcoming tolerance to autoantigen in mice.^{9–11} Lack of viral assembly, avirulence and limited short-lived persistence in mammals, even in those extensively immunocompromised,²⁸ makes it suitable for use in patients with malignancy. MVA infection leads to effective antigen presentation by human antigen presenting cells,²⁹ and the generation of TAA-specific CTL.³⁰ Preliminary studies of rMVA vaccines in humans have demonstrated safety, immunogenicity, and evidence of clinical benefit in patients with cancer.³¹

Despite advances in vaccine immunology and design, effective vaccines capable of affecting tumor rejection or preventing recurrence of high risk malignancies, remain elusive. One of the ways through which cancer vaccines can be optimized is the development and administration of differing classes of viral or bacterial delivery vehicles or vectors to deliver the same antigen.³² Towards that goal, we also have generated a recombinant attenuated *Listeria monocytogenes* (Lm) vaccine expressing human p53 (Hup53). *Listeria* is a gram-positive facultative intracellular bacterium which is highly efficient at inducing cell mediated immunity in infected hosts.³³ Because *Listeria monocytogenes* has access to both phagosomal and cytosolic compartments, antigens delivered by it can be presented in the context of both MHC Class I and II molecules.³⁴ This dual property, which is rare for most intracellular bacteria, results in the induction of a strong cell mediated immune responses of Th1-type, triggers a cascade of cytokine and chemokine release including IFN- γ , IL-1 α , IL-12, IL-18, and TNF- α ³⁴ which ultimately result in vigorous induction of antigen specific CD8⁺ T cells.^{25, 35} In numerous preclinical animal models, recombinant *Listeria* has been demonstrated to be an efficient vaccine vehicle capable of breaking tolerance toward self-antigens and cause regression of established tumors in an antigen dependent manner.^{25, 35} Listeriolysin O (LLO) is secreted by *Listeria* and perforates the phagosomal membrane allowing the bacterium to escape the vacuole and enter the cytoplasm. LLO is a powerful stimulator of the T cell response in vertebrates including mice.³⁶ Prior studies have indicated that when fused to a truncated form of LLO, antigens expressed and secreted by *Listeria* are significantly more immunogenic than when secreted as stand alone proteins.³⁵ Proteins fused to LLO are trapped in the cytoplasm and immediately directed into the proteasome degradation pathway.³⁷ Several candidate TAAs have been expressed in *Listeria* vaccines

and tumor rejection has been demonstrated in a number of experimental tumor models.^{25, 35} A phase I trial of recombinant *Listeria* expressing the E7 antigen demonstrated that the vaccine could be administered safely with preliminary evidence of modest clinical benefit in patients with advanced cervical cancer.³⁸

A prime/boost strategy helps to overcome the common problem of humoral or cellular immunity that is generated against the vector backbone sequence following multiple homologous immunizations, which is especially significant for poxvirus vaccines.^{13, 14, 32} Access to two highly potent vaccine vectors allowed us to evaluate Hupki mice as a well defined preclinical murine model, the immunogenicity and potential for tumor rejection mediated by a heterologous prime/boost strategy targeting p53. In these studies, we first tested the ability of heterologous p53 prime/boost immunization using MVA-p53 and *LmddA*-LLO-p53. As shown in Figure 1, heterologous p53 prime/boost immunization dramatically induced p53-specific CD8⁺ and CD4⁺ T cell responses. This data provided a compelling rationale to further investigate the capacity for this dual vaccine strategy to generate both immunogenicity and tumor rejection. In a tumor protection model utilizing the poorly immunogenic and highly aggressive 4T1 tumor cell line, heterologous p53 immunization resulted in significant protection against tumor growth when compared to control as shown in Figure 2. Although heterologous p53 immunization elicits dramatic p53-specific T cell responses, heterologous p53 immunization only showed a trend towards an enhanced antitumor effect when compared to homologous p53 immunizations. Similarly, the effect on a palpable established tumor was only modest (Figure 4).

Recent advances in vaccine immunology have demonstrated that synthetic oligodeoxynucleotide (ODN) containing unmethylated cytosine-phosphate-guanine (CpG) motifs and double-strand RNA are potent immunostimulatory agents that can enhance vaccine potency. CpG-ODN directly activate innate immunity [macrophages, DCs, and natural killer (NK) cells], which indirectly stimulates adaptive immunity and both humoral and cellular immunity 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 T_H1 activity and less toxicity.³⁹ 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.^{10, 11} The synthetic dsRNA, poly(I:C), is also an excellent adjuvant which induces cell-mediated immune responses through TLR3.⁴⁰ Poly(I:C) has been shown to induce specific humoral and cellular immune responses, including Th1 and CTL responses. It was shown to induce the stable maturation of functionally active human DCs.⁴¹ *In vitro*, poly(I:C) directly promotes the survival of activated CD4⁺ T cells.⁴² Intraperitoneal injection of poly(I:C) into mice dramatically boosted the number of antigen-specific CD8⁺ T cells against an s.c. injected peptide.⁴³ This increase in specific CD8⁺ T cells was associated with an increase in CD8⁺ T cell function. There is also an increased survival of activated CD8⁺ T cells resulting from an inhibition of apoptosis resulting in a long-lasting memory response.⁴³ Poly(I:C) administered with peptide resulted in significant peptide specific IFN- γ responses, enhanced functional dendritic cells in draining lymph node, significant proliferation of splenocytes, and enhanced antitumor responses.⁴⁴

CpG-ODN and poly(I:C) can be used together to develop improved antitumor immune responses. Injection of CpG-ODN and poly(I:C), which engage TLR9 and TLR3, respectively, have been shown to promote tumor immune responses against established experimental melanoma in a murine model.^{26, 27} Recently, the mechanism of the synergistic combination of TLR stimulation has been described. Dual TLR stimulation results in the activation of both the MyD88- and TRIF-dependent signal transduction cascades. Optimal dual TLR stimulation stimulates the production of type I IFNs and other proinflammatory cytokines, which were able to enhance tumor immunity.⁴⁵ The ligation of TLR9 and TLR3

with CpG-ODN and poly(I:C), by MyD88- and TRIF-dependent pathways, respectively, results in phenotypic maturation of dendritic cells and synergistic induction of durable, high level IL-12p70 secretion characteristic of type-I polarized dendritic cells or macrophages.^{45–48} In addition, the antitumor effect of IL-12 functions in a dose-dependent manner.⁴⁹

In this study, we tested the combination TLR strategy administered with heterologous p53 immunization. Treatment with poly(I:C) and CpG-ODN in combination with heterologous p53 immunization resulted in potent antitumor effects relative to treatment with either agent alone against established tumors in a therapeutic murine tumor model. This antitumor effect might not be entirely antigen-specific, because it is likely that TLR stimulation also enhanced innate immunity such as NK cell-mediated cytotoxicity.⁵⁰ However, TLR stimulation administered with heterologous p53 immunization resulted in significant antitumor effect when compared to administration with homologous p53 immunization in a tumor protection model (Figure 3A, 3B). Furthermore, the survival benefit in the series of both protective and therapeutic tumor models was seen in the mice receiving heterologous p53 immunization combined with TLR stimulation (Figure 3C, 5B). This result is particularly impressive given the highly aggressive and poorly immunogenic nature of 4T1 which is far more difficult to eradicate than any other tumor model we have previously evaluated.^{8–11} The ability to eliminate a palpable 4T1 tumor further substantiates the therapeutic potential of this regimen. Immunization with recombinant poxvirus expressing tumor antigens, like p53, can result in the induction of p53-specific B-cell and T-cell immunity.⁵¹ However, there does not appear to be a correlation between p53-specific humoral immunity and stage of disease or clinical response or progression of disease^{51, 52}, so we chose not to focus on p53-specific antibody responses in this study. TLR agonists did not enhance p53-specific cellular immune responses associated with heterologous p53 immunization (data not shown). The dependence on both CD4+ and CD8+ cells in addition to NK cells as demonstrated in the in vivo depletion studies, supports the conclusion that tumor responses were the result of the complementary induction of both antigen specific and innate immunity (Figure 5C). These synergistic TLR agonists are promising candidates to enhance the antitumor efficacy of heterologous p53 immunization. These studies support the use of dual TLR agonists in combination with a heterologous prime/boost strategy for maximal therapeutic benefit against highly tolerized tumor antigens.

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FIGURE 1.

Immunogenicity test of prime/boost combination vaccines in Hupki mice. Hupki mice were immunized by i.p. injection and boosted with homologous or heterologous p53 expressing vectors. ICC assays for IFN- γ were performed on splenocytes from immunized mice. Bars represent the percentage of CD4⁺ and CD8⁺ T cells expressing IFN- γ in response to the human p53 peptide library. Gray bar; CD4⁺ T cell response to human p53. Black bar; CD8⁺ T cell response to human p53. MVA indicates modified vaccinia Ankara; *LmddA*, *Listeria monocytogenes* *dal*-, *dat*-, and *actA*-deleted strain; LLO, listeriolysin O. Each bar represents the mean and standard error of three separate experiments.

FIGURE 2.

Protective antitumor effect of the heterologous p53 vaccine. A, Hupki mice were immunized i.p. with 5×10^7 pfu of MVA-p53, PBS, or MVA-control on day 0. For the heterologous immunization, mice were immunized i.p. with 1×10^8 cfu of *LmddA*-LLO-p53, PBS, or *LmddA*-control on day 7. Mice received s.c. injections of 5×10^5 4T1p53 cells on day 14. Tumors were measured twice weekly in three dimensions with calipers. Bars represent SE. B, Kaplan-Meier graph representing cumulative survival of mice in the indicated treatment groups. * $P < 0.05$ for comparing PBS/PBS to MVA-p53/*LmddA*-LLO-p53 groups by one-way ANOVA. ** $P < 0.05$ for comparing PBS/PBS to MVA-p53/*LmddA*-LLO-p53 groups by Log-rank (Mantel-Cox) test. MVA indicates modified vaccinia Ankara; *LmddA*, *Listeria monocytogenes dat*-, *dat*-, and *actA*-deleted strain; LLO, listeriolysin O. Each experiment was performed with four mice per group and repeated three times. Representative results of three separate experiments, each with similar outcomes are shown.

A

B

FIGURE 3.

Antitumor effect of combined poly(I:C) and CpG-ODN administered with homologous and heterologous p53 immunization. 50 μ g of poly(I:C) and 15 nmol of CpG-ODN were administered by twice i.p. injection on days 0 and 5. On day 1, Hupki mice were immunized i.p. with 5×10^7 pfu of MVA-control or MVA-p53, 1×10^8 cfu of *LmddA*-control or *LmddA*-LLO-p53. For the boost immunization, mice were immunized as homologous or heterologous immunization on day 8. Mice received s.c. injections of 5×10^5 4T1p53 cells on day 15. Tumors were measured twice weekly in three dimensions with calipers. Bars represent SE. A, homologous immunization with poly(I:C) and CpG-ODN. B, heterologous immunization with poly(I:C) and CpG-ODN. C, Kaplan-Meier graph representing cumulative survival of mice in the indicated treatment groups. * $P < 0.05$ for comparing MVA-control/*LmddA*-control with poly(I:C)/CpG to MVA-p53/*LmddA*-LLO-p53 with poly(I:C)/CpG groups by one-way ANOVA. ** $P < 0.05$ for comparing MVA-p53/MVA-p53 with poly(I:C)/CpG to MVA-p53/*LmddA*-LLO-p53 with poly(I:C)/CpG groups by Log-rank (Mantel-Cox) test. MVA indicates modified vaccinia Ankara; *LmddA*, *Listeria monocytogenes* *dat*-, *dat*-, and *actA*-deleted strain; LLO, listeriolysin O. Each experiment was performed with four mice per group and repeated three times. Representative results of three separate experiments, each with similar outcomes are shown.

FIGURE 4.

Therapeutic antitumor effect of heterologous p53 vaccine. Hupki mice received s.c. injections of 2.5×10^5 4T1p53 cells. On day 7, mice were immunized i.p. with 5×10^7 pfu of MVA-p53 or PBS control. For the heterologous immunization, mice were immunized i.p. with 1×10^8 cfu of *LmddA*-LLO-p53 on day 14. Tumors were measured twice weekly in three dimensions with calipers. Bars represent SE. MVA indicates modified vaccinia Ankara; *LmddA*, *Listeria monocytogenes dal*-, *dat*-, and *actA*-deleted strain; LLO, listeriolysin O. Each experiment was performed with four mice per group and repeated three times. Representative results of three separate experiments, each with similar outcomes are shown.

A

B

FIGURE 5.

Antitumor effect of combined poly(I:C) and CpG-ODN administered with heterologous p53 immunization in established 4T1p53 tumor model. A, Hupki mice received s.c. injections of 2.5×10^5 4T1p53 cells. 50 μ g of poly(I:C) and 15 nmol of CpG-ODN were injected i.p. on days 6 and 11. On day 7, mice were immunized i.p. with 5×10^7 pfu of MVA-p53 or MVA-control. For the heterologous immunization, mice were immunized i.p. with 1×10^8 cfu of *LmddA*-LLO-p53 or *LmddA*-control on day 14. Tumors were measured twice weekly in three dimensions with calipers. Bars represent SE. B, Kaplan-Meier graph representing cumulative survival of mice in the indicated treatment groups. Mice rejected 4T1p53 tumor were re-challenged with 5×10^5 4T1p53 cells as lethal dose on day 60. All mice rejected challenge tumors within 10 days after tumor inoculation. Each experiment was performed with four mice per group and repeated three times. Representative results of three separate experiments, each with similar outcomes are shown. C, Effect of depletion of CD8⁺ T cells, CD4⁺ T cells, or NK cells. Mice were injected 2.5×10^5 4T1p53 cells and treated as in 5A. Mice were treated with i.p. injections of 200 μ g of anti-CD8 mAb (H35) or anti-CD4 mAb (GK1.5), or anti-asialo GM1 (dilution 1/20) with a maintenance dose every 3 days until sacrifice. * $P < 0.05$ for comparison of MVA-control/*LmddA*-control with poly(I:C)/CpG to MVA-p53/*LmddA*-LLO-p53 with poly(I:C)/CpG groups by one-way ANOVA. ** $P < 0.05$ comparing PBS/PBS to MVA-p53/*LmddA*-LLO-p53 with poly(I:C)/CpG groups by Log-rank (Mantel-Cox) test. *** $P < 0.05$ comparing MVA-p53/*LmddA*-LLO-p53 with poly(I:C)/CpG to CD4⁺ and CD8⁺ and NK depletion groups by Log-rank (Mantel-Cox) test. MVA indicates modified vaccinia Ankara; *LmddA*, *Listeria monocytogenes* *dat*⁻, *dat*⁻, and *actA*-deleted strain; LLO, listeriolysin O.