**Microenvironment and Immunology**

**A Multifunctional Chimeric Chaperone Serves as a Novel Immune Modulator Inducing Therapeutic Antitumor Immunity**

Xiaofei Yu\(^1,2,3\), Chunqing Guo\(^1,2,3\), Huanfa Yi\(^1,2,3\), Jie Qian\(^1,2,3\), Paul B. Fisher\(^1,2,3\), John R. Subjeck\(^4\), and Xiang-Yang Wang\(^1,2,3\)

**Abstract**

Converting the immunosuppressive tumor environment into one that is favorable to the induction of antitumor immunity is indispensable for effective cancer immunotherapy. Here, we strategically incorporate a pathogen (i.e., flagellin)-derived, NF-kB–stimulating "danger" signal into the large stress protein or chaperone Grp170 (HYOU1/ORP150) that was previously shown to facilitate antigen crosspresentation. This engineered chimeric molecule (i.e., Flagrp170) is capable of transporting tumor antigens and concurrently inducing functional activation of dendritic cells (DC). Intratumoral administration of adenoviruses expressing Flagrp170 induces a superior antitumor response against B16 melanoma and its distant lung metastasis compared with unmodified Grp170 and flagellin. The enhanced tumor destruction is accompanied with significantly increased tumor infiltration by CD8\(^+\) cells as well as elevation of IFN-\(\gamma\) and interleukin (IL)-12 levels in the tumor sites. In situ Ad.Flagrp170 therapy provokes systemic activation of CTLs that recognize several antigens naturally expressing in melanoma (e.g., gp100/PMEL and TRP2/DCT). The mechanistic studies using CD11c-DTR transgenic mice and Batf3-deficient mice reveal that CD8\(^+\) DCs are required for the improved T-cell crosspriming. Antibody neutralization assays show that IL-12 and IFN-\(\gamma\) are essential for the Flagrp170–elicited antitumor response, which also involves CD8\(^+\) T cells and natural killer cells. The therapeutic efficacy of Flagrp170 and its immunostimulating activity are also confirmed in mouse prostate cancer and colon carcinoma. Together, targeting the tumor microenvironment with this chimeric chaperone is highly effective in mobilizing or restoring antitumor immunity, supporting the potential therapeutic use of this novel immunomodulator in the treatment of metastatic diseases. Cancer Res; 73(7); 2093–103. ©2013 AACR.

**Introduction**

Cancer immunotherapy aims to achieve immune-mediated control of tumor growth and metastasis by mounting tumor-reactive T-cell responses. Although immunotherapy holds great promise for cancer treatment, its clinical success has so far been limited (1). Despite the inherent ability of the immune system to detect transformed cells, multiple mechanisms are exploited by cancer cells to create an immunosuppressive environment that enable them to escape immune destruction (2, 3). Overcoming immunosuppressive mechanisms and induction of durable antitumor immunity using novel immunomodulators are the goal of cancer immunotherapy.

Studies by others and us have shown that stress/heat shock proteins can be exploited as immunostimulatory adjuvants for generating antitumor immune responses, which is based on their unique ability of integrating both innate and adaptive immune components (4). Grp170, the largest endoplasmic reticulum (ER) chaperone (5), displays an exceptional capacity of holding client proteins or antigens and a superior immunostimulatory vaccine activity when prepared from tumors or complexing with de-argininated NF-B.

**Authors’ Affiliations:**
1Department of Human and Molecular Genetics, Virginia Commonwealth University School of Medicine, Richmond, Virginia; 2VCU Institute of Molecular Medicine, VCU Massey Cancer Center, Virginia Commonwealth University; 3VCU Massey Cancer Center, Virginia Commonwealth University School of Medicine, Richmond, Virginia; and 4Department of Cellular Stress Biology, Roswell Park Cancer Institute, Buffalo, New York

**Corresponding Author:** Xiang-Yang Wang, Department of Human & Molecular Genetics, Virginia Commonwealth University School of Medicine, Richmond, PO Box 980033, VA 23298. Phone: 804-628-2679; Fax: 804-628-1194; E-mail: xywang@vcu.edu

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against cancer (13). Direct manipulation of pathogen-sensing TLR signaling in the tumor environment was also reported to elicit a potent antitumor response (14).

To test the hypothesis that strategic incorporation of a pathogen-derived "danger" signal into the Grp170 markedly enhances its potency in driving antitumor immunity, we engineered a chimeric chaperone by fusing Grp170 with the defined NF-κB-activating domain of flagellin (15), a major structural protein of the bacterial flagella (16, 17). This hybrid chaperone molecule, termed Flaggp170, not only maintains highly efficient antigen-holding ability, but also possesses a strong capability to activate DCs. We show that adenovirus (Ads)-mediated expression of this Flaggp170 at tumors, as well as distal metastases. The superior antitumor efficacy of Flaggp170 compared with unmodified Grp170 or flagellin is also supported by enhanced T-cell activation both locally (i.e., tumor site) and systemically. Our results indicate that targeting the tumor environment using Flaggp170 can subvert tumor-associated immunosuppressive mechanisms and promote highly immunostimulatory presentation of tumor antigens.

Materials and Methods

Mice and cell lines

C57BL/6 mice, Balb/C mice, Batf3−/− (18), Pmel (19), and CD11c−DTR transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Bone marrow chimeras were generated by reconstitution with bone marrow cells from CD11c−DTR mice as described (20). All experimental procedures were conducted according to the protocols approved by the VCU Institutional Animal Care and Use Committee. B16-gp100 (21) and TRAMP-C2 (22) cells were kindly provided by Dr. A Rakhmilevich (University of Wisconsin-Madison, Madison, WI), and Dr. B Foster (Roswell Park Cancer Institute, Buffalo, NY), respectively. Cells were morphologically authenticated by morphologic inspection and animal grafting for assessing their ability to grow and metastasize as well as tumor histology.

CT-26 cells purchased from American Type Culture Collection (ATCC) authenticated by this established provider were cultured for less than 6 months after resuscitation in the laboratory. All cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% FBS (HyClone) and routinely examined for mycoplasma contamination using a PCR-based mycoplasma detection kit.

Reagents and antibodies

For fluorescence cell sorting (FACS) analysis, fluorescein isothiocyanate (FITC)-conjugated CD3 (17A2), CD8α (53–67), CD11c (HL3), granzyme B (GB11), phycoerythrin (PE)-conjugated IFN-γ (XMG1.2), allophycocyanin (APC)-conjugated CD4 (GK1.5) Abs and isotype control rat IgG2b (RTK4530) were purchased from Biologend. PE-conjugated CD40 (1C10) and CD86 (PO3.1) Abs were purchased from eBioscience. For immunofluorescence staining, rat anti-mouse CD8α (550281) Abs were purchased from BD Pharmingen. AlexaFluor 594-conjugated secondary Abs were from Invitrogen. For Western blotting analysis, phospho-erk (T202/Y204), phospho-JNK (T183/Y185), phospho-P38 (T180/Y182), phospho-P65 (S536), p44/42 MAPK (Erk1/2), and 1xBc (4D4) were purchased from Cell Signaling Technology. Anti-Penta-His Abwas from Qiagen. Neutralizing Abs for IFN-γ (XMGL1.2) and interleukin (IL)-12 (C17.8) were from BioXcell. Murine CD8 T-cell-negative selection kits were purchased from Milteny Biotech. gp10025–33 (KYPQRQDWL), and AH1 (SPSYSVYHQF) peptides were from AnaSpec Inc.. Recombinant Grp170 and Flaggp170 protein were produced using a BacPAK baculovirus expression system (Clontech) as previously described (23).

Generation of replication-defective recombinant adenoviruses

The chimeric Flaggp170 was generated by fusing the N-terminal (amino acid 1-176) and C-terminal (amino acid 402-505) fragments of flagellin (GenBank accession #AAL20871.1) from Salmonella enterica serovar Typhimurium LT2 to the ATP-binding domain-truncated Grp170 (GenBank accession #AF228709, amino acid 431–994). The flagellin cDNA was amplified from a Salmonella enterica cDNA library using Platinum Pfx DNA polymerase (Invitrogen) and subcloned into the pGEX-KG cloning vector (ATCC) to create the flagellinNC fragment. The truncated Grp170 was amplified from the pCMV-Grp170 vector (6) and in-frame linked to the flagellinNC fragment via the second flexible linker. A 34-aa signal peptide for Grp170 was added to the N-terminus of the fusion construct, and the "KNDEL" ER retention sequence was removed to generate a secretable form of Flaggp170. All constructs were subcloned into shuttle vector pShuttle-CMV in frame with a 6× His-tag in the C-terminus. Recombinant adenoviruses were prepared using AdenoPACK Maxi spin columns (Sartorius Stedom Biotech) as previously described (11).

Tumor study

Tumors were established by injecting 2 × 105 B16-gp100, 5 × 105 CT-26, or 2 × 106 TRAMP-C2 tumor cells s.c. into the right dorsal flank. When tumors reached the size of 3 or 6 mm in diameter, mice were randomized and received intratumorally 1 × 106 p.L. of Ads in 50 μL of PBS. All treatments were administered every other day for a total of 5 doses. Tumor growth was monitored by measuring the perpendicular diameters of tumors. Animals were euthanized when tumors reached the size of approximately 2.0 cm3 or 10% of the total body weight. To generate experimental lung metastases, mice bearing 5-day subcutaneous B16-gp100 tumors were injected intravenously with 1 × 105 B16-gp100 tumor cells. In vivo depletion of CD4+, CD8+, or natural killer (NK) cell subsets was conducted using anti-CD4 (GK1.5, ATCC), anti-CD8 (2.43, ATCC), and anti-NK1.1 (PK136, ATCC) Abs (24). Neutralization of IFN-γ or IL-12 in vivo was carried out before therapy by injecting intraperitoneal XMG1.2 or C17.8 Abs, respectively, at a dose of 200 μg every other day. For analysis of leukocyte infiltration, tumor tissues were digested with collagenase D (10 μg/mL) and DNase I (100 μg/mL), and cell suspensions were filtered through a 70 μm cell strainer as previously described (24). The viable mononuclear cells were isolated using the
Targeting the Tumor Environment with an Immune Chaperone

Figure 1. Flagrp170 possesses antigen chaperoning and presenting activity. A, schematic depiction of a chimeric chaperone Flagrp170. The NF-xB-activating domain derived from flagellin is fused to the ATP-binding domain deletion mutant of Grp170 (BLH). SP, Grp170 signal peptide (33aa); FlagN, N-terminus of flagellin (1-172aa); FL, flexible linker; FlagC, C-terminus flagellin (402-495aa); Flagrp170BLHKNDEL-6×His, the BLH domain of Grp170, from which the KNDEL sequence (401-994aa) was removed and replaced with a His-tag. B, extracellular secretion of Flagrp170 protein following infection of B16 cells with indicated Ads at 100 multiplicity of infection. Culture supernatants (CM) and whole-cell lysates (WCL) were analyzed for transgene expression. Adenovirus-encoding a sequence (431-994aa) was removed and replaced with a His-tag. C, antigen-holding capability of Flagrp170, as indicated by its ability to effectively block luciferase Ads at 100 multiplicity of infection. Culture supernatants (CM) and whole-cell lysates (WCL) were analyzed for transgene expression. Adenovirus-encoding a secretable form of Grp170 was used as a control. C, antigen-holding capability of Flagrp170, as indicated by its ability to effectively block luciferase denaturation at 43°C. D, tumor-secreted Flagrp170 promotes antigen crosspresentation. Gp100-specific CD8+ T cells from Pmel mice were cocultured with BMDCs in the presence of gp100 protein and conditioned media from tumor cells infected with indicated Ads. IL-2 levels in the culture media was assessed using ELISA (*, P < 0.005).

Histopaque (Sigma-Aldrich) gradient, and analyzed using a FACScaliber (BD Biosciences). In some cases, tumors were homogenized in 3 volumes of ice-cold PBS and then centrifuged to remove the debris. Cytokine levels in the clarified tumor homogenates were assessed using ELISA kits.

Real-time PCR
Total RNA was extracted using TRizol Reagent (Invitrogen Corp.). Reverse transcription and real-time reverse transcription-PCR were conducted using primers and carboxyfluorescein (FAM)-labeled probe sets from Applied Biosystems (25). Gene expression was quantified relative to the expression of β-actin, and normalized to that measured in Ad.CMV-treated group by standard 2(−ΔΔCT) calculation.

Immunohistochemistry
For histologic analysis of lung metastasis, the tracheas were cannulated after mice were sacrificed under anesthesia, and the lungs were inflated with 10% formalin. The tissues were fixed, embedded in paraffin, and subjected to hematoxylin and eosin (H&E) staining. Apoptotic cells in the tumor tissues were examined using a terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) kit from Roche. Images were taken at ×100 or ×200 magnification using an Olympus BX41 fluorescence microscope (Olympus) and analyzed using NIH Image J 1.45 software. The number of immune cells and apoptotic cells per square millimeter was quantified from 5 randomized fields per tumor section.

T-cell activation assays
Bone marrow-derived cells (BMDC) were infected with Ads for 24 hours and pulsed with 20 μg/ml of gp100 protein. Serially diluted DCs were incubated with 1 × 10^5 purified Pmel CD8+ T cells for 3 days. 3H-thymidine (3H-Tdr, 0.5 μCi/well) was added to the wells for the final 16 hours of culture. T-cell proliferation was measured using 3H-Tdr incorporation assays. Cytokine levels in the supernatants were determined using ELISA kits. For intracellular cytokine staining, gp100,25-33 peptide (1 μg/ml)-stimulated splenocytes or tumor-infiltrating leukocytes were treated with phosphor 12’myristate 13’acetate (10 nmol/L) plus ionomycin (1 μmol/L) in the presence of brefeldin A (5 μg/mL) for 5 hours. Cells were stained with anti-CD8 Abs and permeabilized using a Cytofix/ Cytoperm Kit (BD Biosciences). The cells were then stained with Abs for cytokines or granzyme B. In vivo CTL assays were conducted as described previously (20).

Statistical analysis
Data are expressed as mean ± SD values. Statistical significance between groups within experiments was determined by the Student t test or ANOVA test. Survival was compared using the Kaplan–Meier method. Values of P < 0.05 were considered to be statistically significant.
Results

Construction and characterization of a novel chimeric immune chaperone

We engineered a chimeric molecule containing the ATP-binding domain truncated Grp170 previously shown to be a chaperoning competent (7, 10) and the NF-κB–activating domain of flagellin, which has been mapped to its evolutionarily conserved N- and C-terminus (ref. 15; Fig. 1A). The hypervariable region of flagellin that triggers production of neutralizing Abs was deleted using overlapping PCR. The resulting fragment, which consists of its N- and C-terminus of flagellin connected by a flexible linker, exhibits entirely the NF-κB stimulating activity, and is substantially less immunogenic and toxic than flagellin (26). Infection of cells with adenovirus encoding Flagrp170 (Ad.Flagrp170) resulted in expression of a secretable form of the chimeric chaperone due to the absence of the ER retention sequence (Fig. 1B). Similar to native Grp170, Flagrp170 protein prepared from a Baculovirus expression system prevented the denaturation of reporter protein/antigen (e.g., luciferase) under heat shock conditions (Fig. 1C), implicating an excellent antigen-holding ability of this chimeric molecule. Flagrp170 secreted by B16 tumor cells was also highly efficient in promoting crosspresentation of melanoma antigen gp100 (Fig. 1D).

Adenovirus-mediated Flagrp170 expression enhances activation of DCs

Activation of NF-κB is essential for the optimal function of DCs in vivo (27). Compared with Ad.CMV and Ad.Grp170, infection with Ad.Flagrp170 or Ad.Flagellin efficiently induced activation of NF-κB in splenic CD11c+ DCs (Fig. 2A) and BMDCs (Supplementary Fig. S1A), indicated by phosphorylation of p65 and degradation of IκB. Mitogen-activated protein kinases (MAPK), for example, P38, Erk1/2, and c-jun_NH2_kinase (JNK) was also activated in DCs infected with Ad.Flagrp170 or Ad.Flagellin. Luciferase reporter assays validated that intracellular Flagrp170 overexpression can stimulate NF-κB activity (Supplementary Fig. S1C).

The enhanced signaling activation by Flagrp170 correlated with substantially increased production of inflammatory cytokines, including TNF-α, IL-6, and IL-12p70. This was confirmed by ELISA (Fig. 2B) and qRT: quantitative real-time PCR (qRT-PCR) (Supplementary Fig. S1B). Ad.Flagrp170 and Ad.Flagellin resulted in a sharp elevation of costimulatory molecules on DCs, that is, CD40 (Fig. 2C) and CD86 (Supplementary Fig. S1D), whereas Ad.Grp170 only modestly increased their levels. Ad.Flagrp170-infected DCs were most effective in stimulating antigen (i.e., gp100)-specific CD8+ T cells in vitro, determined by T-cell proliferation (Fig. 2D) and IL-2 production (Supplementary Fig. S1E). Although Ad.Flagellin stimulated a stronger cytokine production than Ad.Grp170, Ad.Grp170-infected DCs were more efficient than Ad.Flagellin-infected DCs in stimulating T cells, suggesting that overexpression of chaperones in DCs might improve their antigen processing and/or cross-presenting capability. Intriguingly, TLR5 overexpression in DCs had little effect on activation of NF-κB and MAPKs by Ad.Flagrp170 or Ad.Flagellin (Supplementary Fig. S2A and S2B), suggesting that direct intracellular delivery of Flagrp170 via adenovirus may trigger intracellular signaling pathways independent of the surface TLR5.
In contrast to the marked response in DCs, Ad.Flagrp170 failed to activate these signaling pathways in B16 tumor cells (Supplementary Fig. S3A). Tumor modification with Ad. Flagrp170 substantially reduced its growth rate in vivo, not in vitro (Supplementary Fig. S3B). The observed tumor suppression was associated with increased tumor infiltration by activated CD8+ T cells (Supplementary Fig. S3C–S3E), which is consistent with our previous report on Grp170-enhanced tumor immunogenicity (8).

**In situ Ad.Flagrp170 therapy generates a profound systemic antitumor response**

Treatment of mice bearing B16-gp100 melanoma with Ad. CMV intratumorally had a negligible effect on tumor growth. Ad.Flagrp170 therapy resulted in a profound inhibition of this aggressive tumor compared with other treatment (Fig. 3A–C), leading to significantly prolonged survival of mice (Fig. 3B). We also examined the dose effect of Ad.Flagrp170 by treating mice for 5, 7, or 9 times. Administration of Ad.Flagrp170 more than 5 times did not result in additional therapeutic benefits (Supplementary Fig. S4A). Mice that responded to Ad.Flagrp170 therapy did not show any noticeable autoimmune symptoms (e.g., vitiligo) or apparent toxicity in major organs (data not shown).

To evaluate whether local Ad.Flagrp170 therapy generated systemic protective immunity, experimental lung metastasis was established simultaneously in mice bearing subcutaneous B16 tumors (Fig. 3D). We showed that mice treated with Ad. Flagrp170 had the fewest lung metastases among all treatment groups (Fig. 3E and 3F). Histologic analysis showed that lung tissues from these mice had much smaller and fewer metastatic lesions (Fig. 3G).

**Ad.Flagrp170 induces high levels of Th1 cytokines and CD8+ T cells in the tumor sites**

Intratumoral IL-12p70 levels were significantly elevated in Ad.Flagrp170- or Ad.Flagellin-treated mice compared with those receiving Ad.CMV or Ad.Grp170 (Fig. 4A). However, only...
Ad.Flagrp170 treatment induced the highest IFN-γ expression in the tumor sites. Interestingly, Ad.Flagrp170 was also more effective in inducing IFN-γ expression than Ad.Flagellin (Fig. 4A). Similar results were seen when qRT-PCR was used to examine the transcription levels of these cytokines (Supplementary Fig. S4B).

Ad.Flagrp170 therapy resulted in a marked increase in the frequency of tumor-infiltrating CD8⁺ T cells that express IFN-γ and granzyme B, as determined by intracellular cytokine staining and FACS analysis (Fig. 4B and Supplementary Fig. S4C). Tumor-infiltrating CD4⁺ T cells also displayed increased levels of IFN-γ (Supplementary Fig. S4D). Immunohistochemistry staining showed that the enhanced tumor infiltration by CD8⁺ T cells correlated positively with the increased cell death within the tumors (Fig. 4C).

**Ad.Flagrp170 therapy augments systemic activation of CTLs recognizing endogenous melanoma antigens**

In addition to the immunostimulation in the tumor site, Ad.Flagrp170 therapy led to the strong systemic activation of tumor-reactive T cells, as indicated by enhanced IFN-γ and IL-2 production in splenocytes and lymph node cells upon stimulation with B16 tumor lysates (Fig. 5A) or H-2D<sup>β</sup>-restricted gp100<sub>25-35</sub> (Fig. 5B) (28). Increased frequency of IFN-γ–producing gp100<sub>25-35</sub>-specific T cells was also shown using intracellular cytokine staining (Fig. 5C). In vivo CTL assays indicated that Ad.Flagrp170 treatment resulted in the strongest cytotoxicity against gp100<sub>25-35</sub>-positive cell targets (Fig. 5D). Ad.Flagrp170 therapy also generated CD8⁺ T cells that recognize another melanoma antigen, Tyrosinase-related protein 2 (TRP2, Supplementary Fig. S4E; ref. 29).

Given the robust antitumor response, we sought to determine the involvements of immunoeffector cells or molecules. Depletion of CD8⁺ or NK cells, not CD4⁺ cells, profoundly attenuated the antitumor activity of Flagrp170 (Fig. 5E). In addition, neutralization of endogenous IL-12 or IFN-γ also abolished the therapeutic efficacy of Ad.Flagrp170 (Fig. 5F).

**CD8α⁺ DCs are required for Ad.Flagrp170-induced activation of CTLs**

Ad.Flagrp170 treatment also recruited CD11c<sup>+</sup>CD8α⁺ myeloid cells to the tumor sites (Supplementary Fig. S5A). Considering an important role of CD8α⁺ DCs in T-cell cross-priming, we used CD11c-DTR transgenic mice and Batf3<sup>−/−</sup> mice that lack CD8α⁺ DCs to define its potential involvement in Ad.Flagrp170-induced antitumor immune response. First, we developed bone marrow chimera in which wild-type mice were reconstituted with the bone marrow from CD11c-DTRtg mice. We showed that depletion of CD11c<sup>−</sup> in diphtheria toxin (DT)-treated chimera mice partially abrogated the therapeutic efficacy of Ad.Flagrp170 (Supplementary Fig. S5C and Fig. 6A). IFN-γ production by the tumor-infiltrating CD8⁺ T cells and IFN-γ levels in the tumor sites were significantly reduced in mice depleted of CD11c<sup>−</sup> DCs (Fig. 6B and C). Similar
observations were also made in Batf3−/− mice deficient in CD8α+ DCs (Fig. 6E and G). The loss of CD8α+ DCs also impaired the recruitment of tumor-infiltrating CD8+ T cells (Fig. 6F). In agreement with the results in the tumor sites, peripheral splenic CD8+ T cells from Ad.Flagr170-treated mice without CD11c DCs (Fig. 6D) or CD8α− DCs (Fig. 6H) also showed significantly reduced IFN-γ and IL-2 expression.

Intriguingly, although IL-12 levels in the tumor tissues decreased in the absence of CD11c+ (Fig. 6C, right) or CD8α− DCs (Fig. 6G, right), there was no statistically significant difference. These findings suggest that CD11c+ DCs may not be a major source of IL-12 following Ad.Flagr170 therapy, and other myeloid cells (e.g., CD11c− or F4/80+ cells) could also contribute (Supplementary Fig. S5B).

**Ad.Flagr170 therapy efficiently inhibits prostate cancer and colon carcinoma**

Ad.Flagr170 also displayed superior antitumor activity in 2 additional tumor models with different genetic background: TRAMP-C2 prostate cancer (Fig. 7A and B) and CT-26 colon carcinoma (Fig. 7D). It was strikingly that Ad.Flagr170 efficiently inhibits prostate cancer and colon carcinoma. Other myeloid cells (e.g., CD11c− or F4/80+ cells) could also contribute (Supplementary Fig. S5B).
produced significantly more IFN-γ (Fig. 7C) or IL-2 (Supplementary Fig. S6A) than those from other mice. The same observation was made in CT-26 tumor-bearing mice when lymphocytes were stimulated with AH1 (gp70(423–431)) peptides (Fig. 7E), the immunodominant epitope of CT-26 tumor. These results were also validated using ELISPOT that determines the frequency of antigen-specific T cells (Supplementary Fig. S6B).

In the model of CT-26 tumor of Balb/C origin, 80% of mice receiving Ad.Flagrp170 were cured of tumors. These tumor-free mice were resistant to secondary tumor challenge 60 days later, and showed a robust recall T-cell response to AH1 (Fig. 7F), indicating the establishment of immune memory.

Discussion

Tumor escape may result from loss of immunogenicity of cancer cells and/or the establishment of an immunosuppressive state within the tumor microenvironment (3, 30). It is critical to devise new therapeutic strategies capable of effectively and safely restoring immunomediated recognition of the tumor. In the present study, we show that a novel chimeric immunocopherone, Flagrp170, displays a superior immunostimulating activity at the tumor site. The antitumor potency of \emph{in situ} Ad.Flagrp170 therapy has been validated using 3 different murine cancer models, including melanoma, prostate cancer, and colon carcinoma. In addition, local Ad.Flagrp170 therapy also augments systemic protective immunity, dramatically reducing distant pulmonary metastases. Therefore, Flagrp170 represents a highly potent, nontoxic immune modulator that may be used to overcome cancer-induced immunotolerance.

Flagrp170 maintains the highly efficient antigen holding and presenting capability of Grp170. This engineered chaperone also gains a novel immunostimulating feature through a defined NF-κB–activating domain embedded in this chimeric molecule. Several lines of evidence support a significant improvement of Flagrp170 over unmodified Grp170 in immunomodulation. Adenoviral expression of Flagrp170 in DCs markedly induced upregulation of costimulatory molecules (i.e., CD40 and CD86) and production of inflammatory cytokines (e.g., TNF-α and IL-12p70) that are known to promote DC
activation and T-helper 1 (T₄₁) immunity. Flagrp170 expression also resulted in activation of NF-κB and MAPK signaling in DCs. In fact, engaging NF-κB pathways in DC is essential for its optimal functions, including antigen processing and presentation (27, 31–33). Coupling of antigen and "danger" molecules in the same delivery cargo is pivotal for DC-mediated antigen presentation (34, 35). Thus, this chimeric chaperone uniquely combines both actions of facilitating crosspresentation of tumor antigens and stimulation of signaling pathways that are crucial for the functional activation of antigen-presenting cells.

The administration of Flagrp170 in situ has a strong immunomodulating impact on the tumor environment. Among all treatment groups, the IFN-γ levels in the Flagrp170-treated tumors are the highest, even though the IL-12 levels are comparable in tumors treated with Ad.Flagrp170 or Ad.Flagellin. The increased IFN-γ may be attributed to the recruited tumor-infiltrating T cells and possibly NK cells as well following Ad.Flagrp170 therapy. In addition to immunooactivation in the tumor site, Ad.Flagrp170 therapy results in systemic mobilization of tumor-reactive T cells, as indicated by activation of lymphocytes in the spleens and lymph nodes. These activated CD8⁺ T cells recognize several endogenous antigens naturally expressing in melanoma, including gp100 and TRP-2.

Ab depletion study clearly shows that CD8⁺ T cells and NK cells are involved in the Flagrp170-induced immune control of B16 melanoma. In vivo neutralization study also underscores the importance of T₄₁ cytokines, that is, IFN-γ and IL-12, in the antitumor efficacy of Ad.Flagrp170. Furthermore, the current work shows that CD11c⁺ cells or CD8⁺ DCs are not a major source of increased IL-12 following Flagrp170 treatment and other myeloid cell subsets could also contribute to these cellular processes. However, depletion of CD11c⁺ cells or lack of CD8⁺ DCs attenuates the antitumor response generated by Ad.Flagrp170 therapy, which correlates with significantly reduced IFN-γ production by CD8⁺ T cells, emphasizing the requirement of these DCs for Flagrp170-enhanced antigen crosspresentation and activation of tumor-specific CTLs. Although these findings provide mechanistic insights into the therapeutic activity of Flagrp170, future studies are necessary to further examine how NK cells regulate the antitumor effect of Flagrp170 in vivo and define the cell types responsible for IL-12 production in vivo.

We used the defined NF-κB–activating domain for construction of the chimeric chaperone because this region was shown to substantially reduce the immunogenicity and toxicity of full-length flagellin (26). Given the presence of only such a small portion of flagellin in the construct, it is striking that Flagrp170 was much more efficacious than Ad.Flagellin in targeting the tumor environment with an immune chaperone.
generating antitumor immunity in different models tested. We reason that the highly efficient capability of Flagrl70 to capture and chaperone tumor antigens for enhanced cross-priming as described previously (8–10) contributes to the robust T-cell activation after Ad.Flagrl70 therapy. Intriguingly, DCs treated with Ad.Flagrl70 or Ad.Grp170 are more effective than DCs treated with Ad.Flagellin in stimulating antigen-specific T cells in vitro, suggesting that overexpression of chaperones (e.g., Flagrl70 or Grp170) in DCs could potentially enhance their antigen-processing/presenting functions (36). Indeed, earlier studies have shown that Grp170 can interact with peptides translocated by transporter associated with antigen processing and may participate in polypeptide trafficking (37, 38).

In summary, our studies show the profound antitumor effects of a multifunctional chimeric chaperone when delivered to the tumor environment. Flagrl70 is capable of "conditioning" immunosuppressive/tolerogenic tumor environment for boosting therapeutic antitumor immunity. However, the question remains with regard to the precise molecular and cellular events triggered by the presence of Flagrl70 within the tumor sites. Despite that flagellin is a known ligand for TLR5, our studies suggest that direct intracellular delivery of Flagrl70 by adenovirus may trigger signaling activation independent of TLR5. Indeed, a TLR5-independent effect of flagellin in innate and adaptive immunity has been reported (39, 40). The possibility of enhanced tumor immunogenicity following Ad. Flagrl70 modification also remains to be determined (8).

Thus, more studies are warranted to further investigate the molecular actions of Flagrl70 in vivo and the feasibility of exploiting this novel immunomodulator to break tolerance against tumor-associated self-antigens.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X. Yu, P. Fisher, J.R. Subjeck, X.-Y. Wang
Development of methodology: X. Yu, C. Guo, H. Yi, J.R. Subjeck
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.R. Subjeck, X.-Y. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Yu, X.-Y. Wang

Writing, review, and/or revision of the manuscript: X. Yu, P. Fisher, J.R. Subjeck, X.-Y. Wang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Yi
Study supervision: X.-Y. Wang

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A Multifunctional Chimeric Chaperone Serves as a Novel Immune Modulator Inducing Therapeutic Antitumor Immunity

Xiaofei Yu, Chunqing Guo, Huanfa Yi, et al.


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