Induction of hsp70-Mediated Th17 Autoimmunity Can Be Exploited as Immunotherapy for Metastatic Prostate Cancer

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Abstract

A close connectivity between autoimmune and tumor rejection responses is known to exist in the case of melanoma immunotherapy. However, relatively little is known about self-antigens on other types of normal cells, their relation to the development of autoimmune disease, and their possible co-existence as potential tumor rejection antigens on associated tumors. In the current study, we induced inflammatory killing of normal prostate tissue in situ using a fusogenic membrane glycoprotein along with the immune adjuvant hsp70. We show here that, in the prostate, hsp70 induces interleukin (IL)-6, which triggers a CD4+ and CD8-dependent progressive autoimmune reactivity, associated with IL-17 expression. This autoimmune response was also able to induce the rejection of established prostate tumors, but not other histologic types of tumors, growing elsewhere in the animal. These data show that the intimate connectivity between autoimmune and tumor rejection responses extends beyond the classic melanoma paradigm and may be clinically valuable for the treatment of established metastatic disease of the prostate. [Cancer Res 2007;67(24):11970–9]

Introduction

A close link between development of tumor immunity, with concomitant induction of autoimmune reactivities, has been well-documented in both preclinical and clinical model systems. This is particularly true for melanoma, where many melanoma antigens are known to be unaltered self-proteins of melanocytes (1, 2). Hence, successful immune therapies against melanoma also run the risk of developing immune reactivity against normal melanocytes (1, 3). There is frequently—but not always (4)—a correlation between the induction of tumor immunity and concomitant development of autoimmune pathology, most principally vitiligo, correlated with CD8, CD4, and humoral responses to melanoma antigens (1, 2, 5, 6). Strategies that enhance the potency of antitumor T-cell responses often also increase the severity of autoimmune variables (5–7). The positive prognostic importance of the development of autoimmune disease in patients undergoing immunotherapy for melanoma has recently been convincingly shown in a large-scale clinical trial (8).

The established paradigm for tumor vaccination uses tumor cell-derived immunogens to raise T-cell responses against tumor antigens that may then be accompanied by autoimmune antimalanocyte responses (2, 9). Often, such vaccines require a potent adjuvant to promote the breaking of tolerance to tumor-derived antigens. In this respect, we (10–12) and others (13–18) have shown that hsp70 can act as powerful inducer of tumor immunity and converts tolerogenic antigen presentation into immunostimulatory presentation to break tolerance and induce autoimmune disease (19). Heat shock proteins can enhance immunogenicity as chaperones of immunogenic peptides (15–17), cytokines (17, 18), immunogens (20), and their ability to mature dendritic cells (19). We have also shown that hsp70 induces proinflammatory cytokines from monocytes (12) after ligation to receptors including Toll-like receptors (TLR) 2 and 4 (18, 21), and that it also acts directly upon macrophages to suppress secretion of interleukin (IL)-10 and phagocytosis (12), and that hsp70 is a key mediator of immunogenic cell death (10–12), thereby mediating effective tumor vaccination (10–12).

However, obtaining tumor cells, lysates, or derivatives; identifying relevant tumor antigens; and developing vectors that can specifically target systemic tumors in vivo can be time consuming, expensive, and problematic. In this respect, we recently described a completely novel approach to tumor vaccine design by targeting readily accessible normal tissues in situ as a source of immunogen (9). We hypothesized that by inducing "stressful death" of normal cells (melanocytes), it would be possible to generate autoimmune responses, which may then be effective against tumor cells (melanoma) that share antigens with the normal tissue (1, 3). We showed that killing normal melanocytes, in the presence of the potent immune adjuvant hsp70 (10–12, 19, 22, 23), generates T-cell reactivity against established melanomas. Intradermal plasmid DNA injections of a transcriptionally targeted cytotoxic gene [tyrosinase promoter driving the expression of the herpes simplex virus thymidine kinase (HSVtk) gene], along with a plasmid expressing hsp70 (10), induced direct in vivo inflammatory killing of normal melanocytes, broke tolerance to self-antigens, and led to rejection of systemic tumors (21, 24, 25). In this system, hsp70 acts through TLR-4 signaling and local induction of tumor necrosis factor-α to promote migration of antigen presenting cells to the lymph node (21). Progressive autoimmune disease was inhibited (24, 25) because the antimalanocyte CD8+ T-cell response was rapidly suppressed by regulatory T cells (24–26). In addition, in suboptimal therapeutic protocols, the T-cell response selected aggressive, amelanotic, antigen loss B16 tumor variants (24, 25). Moreover,
addition of a plasmid expressing CD40L increased antitumor efficacy, generated potent immunologic memory but also induced aggressive autoimmunity (21).

From these, and other data, it is clear that self-reactive T cells exist in the periphery that have escaped thymic deletion and can, if correctly activated, kill both melanoma cells and normal melanocytes (1, 2, 6, 8). However, there is a relative paucity of data concerning the link between autoimmune responses to other tissues and their relevance to generating immune responses against tumors of the same histologic type. In particular, loss of melanocytes is not life threatening and can be tolerated considerably better than metastatic melanoma (8). In the current

Figure 1. Ad-VSV-G+Adhsp70 treatment of the prostate is associated with ongoing autoimmune destruction. A, C57Bl/6 mice under anesthetics were injected intraprostatically with $10^5$ plaque-forming unit (pfu) of Ad-GFP or Ad-VSV-G (27–29, 31, 46–50), or Ad-hsp70, or with $5 \times 10^6$ pfu Ad-VSV-G+$5 \times 10^6$ pfu Ad-hsp70. After surgery, mice were recovered and were euthanized 1 wk after the intraprostatic injection of viruses. Prostates were analysed histologically using H&E sections. Slides were scored by two independent pathologists blinded to the experimental groups for infiltration and necrosis. Ad-hsp70 induced moderate levels of inflammatory infiltrate (top right) but extensive immune infiltration and necrosis were recorded for prostates injected with Ad-VSV-G+Adhsp70 (bottom right). Syncytia were only seen in prostates injected with Ad-VSV-G (bottom left). Essentially normal prostate architecture was recorded from prostates injected with Ad-GFP (top left). B, 45 d after intraprostatic injections as described in A, animals were euthanized and prostates were recovered and weighed. Mean weights of prostates from animals of different treatment groups are shown.
report, we investigated whether the principles that we observed in the melanocyte/melanoma system also hold true for the prostate. In response to the intentional induction of inflammatory killing of the prostate, we observed progressive autoimmunity destruction of the prostate in response to the intentional induction of inflammatory killing, associated with induction of IL-6 and a Th-17 response but with no detectable Treg induction. Correspondingly, inflammatory killing of normal prostate was highly effective at curing established metastatic prostatic tumors but not tumors of a different histologic type. Our results are significant in several respects. They show that autoimmune disease of the prostate can be induced by specific cytokine responses to one, or a few, key pathogenic-like signals. They show that the intimate connectivity between autoimmunity and antitumor rejection responses extends beyond the classic melanoma paradigm; and they suggest that the principle of inflammatory killing of normal cells to treat neoplastic disease is applicable to tumors other than just melanoma.

Materials and Methods

Cell lines, plasmids, and viruses. Transgenic adenocarcinoma of the mouse prostate (TRAMP)-C2 (TC2) cells are derived from a prostate tumor that arose in a TRAMP mouse and were characterized by Dr. Esteban Celis. These cell lines express a variety of prostate-specific genes, including PSMA, Hoxb-13, and NXX3.1. TC2 cells grow in an androgen-independent manner and have a reduced level of expression of MHC class I which can be up-regulated by IFN-γ, making them susceptible to specific lysis by CTL. We routinely grow TC2 tumors in C57Bl/6 male mice. The murine melanoma B16.F1 tumor cell line has been previously described (27). Cell lines were grown in DMEM (Life Technologies) supplemented with 10% FCS (Life Technologies) and L-glutamine (Life Technologies). All cell lines were grown in DMEM (Life Technologies) supplemented with 10% FCS (Life Technologies) and L-glutamine (Life Technologies).

Histopathology of tumor sections. Prostates were harvested and fixed in 10% Formalin in PBS, then paraffin embedded and sectioned. H&E-stained sections were prepared for analysis of tissue destruction and gross infiltrate. Two independent pathologists examined H&E sections, blinded to the experimental design, and scored the degree of necrosis.

Reverse transcriptase PCR. Organ samples were snap frozen in liquid nitrogen. RNA was prepared with the Qiagen RNA extraction kit. One microgram of total cellular RNA was reverse transcribed in a 20 μL volume using oligo-(dT) as a primer. A cDNA equivalent of 1 ng RNA was amplified using oligo-(dT) as a primer. A cDNA equivalent of 1 ng RNA was amplified using oligo-(dT) as a primer.

Results in multiple different experiments.

**Figure 2.** Prostate responds to hsp70 by IL-6 induction. A, C57Bl/6 mice (two per group) were injected intraprostatically with 10⁹ pfu Ad-GFP, Ad-hsp70, or Ad-VSV-G. Three days later, injected prostates were recovered and used to prepare cDNA, which was analyzed by PCR for IL-6 or glyceraldehyde-3-phosphate dehydrogenase. B, prostates from three different C57Bl/6 mice were recovered, weighed, and two blocks of 30 mg of prostate tissue per mouse were dissociated separately in culture. Explanted cultures were allowed to settle overnight. The following day, one of the two cultures from each animal was incubated with recombinant murine hsp70 (10 μg/mL) for 24 h, while the second duplicate culture was treated with bovine serum albumin. Twenty-four hours later, culture supernatants were recovered and assayed for levels of IL-6 by ELISA as shown. Error bars represent the SD from three wells per sample in the ELISA assay. Results are representative of two separate experiments. C and D, C57Bl/6 mice were injected intraprostatically with 10⁹ pfu Ad-GFP, Ad-VSV-G, or Ad-hsp70 or with 5 × 10⁸ pfu Ad-VSV-G+5 × 10⁸ pfu Ad-hsp70. Eight days later, draining lymph nodes (LN) were used to prepare cDNA. which was analyzed by PCR for IL-6 (C), TGF-β (D), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results are presented as a ratio of the cytokine signal to the GAPDH signal for each treatment over at least three experiments, and a sample gel is shown. Results in A to D are representative of multiple different experiments.
by PCR for a variety of murine cytokines or vector-derived transgenes as described previously (27, 32) (details of the primers upon request).

Treg-mediated inhibition of IFNγ secretion from activated T cells. OT-I mice are transgenic mice whose T cells express the Vα2 chain of the transgenic OT-I T-cell receptor that specifically recognizes the SIINFEKL peptide from the chicken ovalbumin protein (ova) in the context of H-2Kb as expressed by B16ova tumor cells (33). For preparation of naive OT-I T cells, spleen and lymph nodes from OT-I-transgenic mice were combined and crushed through a 100-μm filter to prepare a single-cell suspension. RBC were removed by a 2-min incubation in ACK buffer (sterile dH2O containing 0.15 mol/L NH4Cl, 1.0 mmol/L KHCO3, and 0.1 mmol/L EDTA adjusted to pH 7.2–7.4). OT-I T cells were activated by incubation of splenocyte populations with the cognate antigen recognized by the OT-I T cells. Single-cell suspensions from spleen and lymph nodes were adjusted to 1.0 × 107 cells/mL in Iscove’s modified Dulbecco’s medium plus 5% FCS, 105 mol/L 2-ME, 100 units/mL penicillin, and 100 μg/mL streptomycin and stimulated with 1 μg/mL SIINFEKL peptide and 50 IU/mL human IL-2 (Mayo Clinic Pharmacy). This routinely induces large amounts of IFN-γ to be expressed from the activated OT-I T cells.

To assay for the presence of T-cell suppressive (Treg) activity within splenocyte populations from intraprostatically injected mice, 250,000 freshly harvested splenocytes from treatment groups were plated along with 105 naive OT-I CD8+ T cells in the presence of either no added peptide, an irrelevant nonactivating peptide (TRP-2180188 SVYDFFVWL; ref. 34), or with the

Figure 3. IL-17 expression is induced by inflammatory killing of prostate without induction of Treg. A, C57Bl/6 mice were injected intraprostatically with 109 pfu Ad-GFP, Ad- VSV-G, or Ad-hsp70 or with 5 × 109 pfu Ad-VSV-G+5 × 109 pfu Ad-hsp70. Eight days later, the injected prostates were used to prepare cDNA, which was analyzed by PCR for IL-17 or GAPDH. Results are presented as a ratio of the cytokine signal to the GAPDH signal for each treatment over at least three experiments, and a sample gel is shown. B, the cDNA obtained from the experiment described in Fig. 2B (lymph node draining the prostates injected with adenoviral vectors) was also screened for expression of IL-17 as shown. The GAPDH control in this experiment is the same as in Fig. 2B. In addition, C57Bl/6 mice were treated intraprostatically with 109 pfu Ad-GFP, Ad-hsp70, Ad-VSV-G, or 5 × 109 pfu Ad- VSV-G+5 × 109 pfu Ad-hsp70. Eight days later, 105 cells from the draining lymph nodes were cultured for 24 h. Supernatants were assayed for IL-17 using ELISA (eBioscience). C, C57Bl/6 mice were injected intraprostatically with no virus (lane 1) or with 105 pfu of Ad-GFP (lane 2); intradermally with the Tyr-HSVtk/CMV-hsp70 plasmid combination that we have previously shown induces Treg cells (refs. 24, 25; lane 3) or with 5 × 109 pfu Ad-VSV-G+5 × 109 pfu Ad-hsp70 (lanes 4–6). Fourteen days postviral or plasmid injection (lanes 1–3, 7, 8), or 4 or 41 days postviral injections (lanes 4 and 6), splenocytes were recovered from treated mice and 250,000 were plated with 105 naive OT1 CD8+ T cells with H2Kb-restricted ova peptide SIINFEKL in triplicate, and 48 h later, supernatants were assayed by ELISA for IFN-γ. Lane 7, OT-1 with no added splenocytes; lane 8: OT-1 with the nonactivating TRP-2 peptide instead of SIINFEKL. D, C57Bl/6 mice under anesthesia were injected intraprostatically with 0.1 mg of a plasmid expressing the cDNA for chick ovalbumin along with 105 pfu of Ad-VSV-G or Ad-hsp70, or along with 5 × 109 pfu Ad-VSV-G+5 × 109 pfu Ad-hsp70. All groups received an i.p. injection of either a control immunoglobulin (Ig) or the anti-CD25 Treg-depleting PC61 antibody (0.5 mg) 2 d before the intraprostatic plasmid/viral injection. After surgery, the mice recovered. One week later, 500,000 splenocytes per treatment group were harvested and cocultured with the H-2Kb restricted ova peptide SIINFEKL in triplicate, and 48 h later, supernatants were assayed by ELISA for IFN-γ. Results shown are representative of two different experiments.
synthetic H-2Kb–restricted ova peptide SIINFEKL (33) in tissue culture wells. Splenocyte/OT-1 cocultures were stimulated in triplicate and supernatants were assayed for IFN-γ production by ELISA. The degree of suppressive activity in the test splenocyte cultures is reflected by their ability to inhibit the IFN-γ response of the naïve OT-1 T cells when presented with their cognate, activating SIINFEKL antigen. The dependence of any such T-cell suppressive activity on expression of transforming growth factor-β1 (TGF-β1; ref. 35) was assayed using the recombinant human TGF-β1, R&D chimera (R&D Systems), a 159 amino acid extracellular domain of human TGF-β receptor type II fused to the Fc region of human IgG1.

Antigen priming assays—splenocyte preparation and antigen presentation. Splenocytes enriched in lymphocytes were prepared from spleens of treated/vaccinated animals by standard techniques (36). Freshly purified splenocyte populations were washed in PBS and either incubated with target tumor cells (TC2 or B16) typically at ratios of 100:1, 10:1, or 1:1 or, where appropriate, were pulsed with 1 μg/mL of the target peptide for which antigen specificity of response was being tested [SIINFEKL for induced responses to ova (33) or TRP-2180188 SVYDFFVWL (34) as the negative irrelevant antigen control]. Forty-eight to seventy-two hours later, cell-free supernatants were tested for IFN-γ production by ELISA (PharMingen). The synthetic H-2Kb–restricted peptides TRP-2180188 SVYDFFVWL (34) and Ova SIINFEKL (33) were synthesized at the Mayo Foundation Core facility.

ELISA analysis for IFNγ secretion. For ELISA, cell-free supernatants were collected from sample wells and tested by specific ELISA for IFN-γ or IL-6 (BD OptEIA; IL-6; BD Biosciences) or IL-17 (R&D Systems) according to the manufacturers’ instructions.

In vivo studies. All procedures were approved by the Mayo Foundation Institutional Animal Care and Use Committee. C57Bl/6 mice or B6.129S2-IL6tm1Kopf/J [IL-6 knockout (IL-6KO); Jackson; No.002650] were purchased from The Jackson Laboratory at ages 6 to 8 weeks. To establish s.c. tumors, 2 × 106 B16 cells or 2 × 104 TC2 cells, in 100 μL of PBS were injected into the flank of mice. Intraprostatic injections (50 μL) were performed on mice under anesthetic, typically at day 6 after tumor establishment. For survival studies, tumor diameter in two dimensions was measured thrice weekly using calipers, and mice were killed when tumor size was >1.0 × 1.0 cm in two perpendicular directions.

Immune cell depletions were performed by i.p. injections (0.1 mg per mouse) of anti-CD8 (Lyt 2.43) and anti-CD4 (GK1.5), both from the Monoclonal Antibody Core Facility, Mayo Clinic; and IgG control (ChromPure Rat IgG; Jackson ImmunoResearch) at day 4 after tumor implantation and then weekly thereafter. For Treg depletion, 0.5 mg of PC-61 antibody (Monoclonal Antibody Core Facility; Mayo Clinic) per mouse was given i.p. 4 days after tumor implantation and 2 days before the first viral injection. Fluorescence-activated cell sorting analysis of spleens and lymph nodes confirmed subset specific depletions.

Statistics. Survival data from the animal studies was analyzed using the log-rank test (37), and the two-sample unequal variance Student’s t test analysis was applied for in vitro assays. Statistical significance was determined at the level of P value of <0.05.

Results

Inflammatory killing of normal prostate induces ongoing autoimmune destruction. Previously, we used plasmids expressing the HSVtk suicide gene and hsp70 to target killing of normal melanocytes in the skin (21, 24, 25). Because HSVtk requires active division of target cells for cytotoxicity, in the current studies, we used an adenoviral vector expressing VSV-G, the fusogenic membrane glycoprotein (F MG) from VSV (27), to induce killing of normal prostate cells. We have previously shown that killing induced by fusion of cells using viral F MG can be potently immunogenic through the fusion of cells into multinucleated syncytia (27, 28). In addition, we used a second adenoviral vector to express the murine hsp70 gene (30).

Direct intraprostatic injection of an adenovirus-expressing GFP (Ad-GFP) did not induce any detectable lasting damage to the prostates of C57Bl/6 mice either in terms of the architecture of the organ or immune infiltration (Fig. 1A). Injection of Ad-hsp70 alone induced an inflammatory response associated with a dense inflammatory infiltrate and some loss of normal architecture (Fig. 1A). Significant immune infiltration was also observed with injection of Ad-VSV-G and Ad-hsp70 caused severe infiltration, necrosis, and tissue destruction (Fig. 1A) consistent with our experience of intradermal injection of plasmids expressing HSVtk and hsp70 (24, 25). Unlike those studies, however, the dense infiltration with immune cells was persistently present in prostate tissue and did not significantly resolve up to 3 weeks postinjection (data not shown). This persistent inflammation was associated with an ongoing autoimmune destruction of the prostate as reflected by a progressive decrease of the wet weight of prostates recovered from treated animals (Fig. 1B; P < 0.01 for Ad-GFP and Ad-VSV-G+Ad-hsp70).

Hsp70 induces IL-6 from prostate tissue. A screen of injected prostates by reverse transcriptase PCR (RT-PCR) for different cytokines indicated that IL-6 was consistently induced in prostates injected with Ad-hsp70 (whether or not Ad-VSV-G or Ad-GFP were also injected; Fig. 2A). These results were confirmed at the protein level by treating explanted and dissociated prostate with recombinant hsp70 (Fig. 2B; P < 0.001 for all three prostates tested when compared plus or minus hsp70). We also assayed the lymph nodes draining the injected prostates to investigate the profile of cytokine expression induced by local inflammatory killing, which will directly influence the outcome of T-cell priming. Lymph node draining the injected prostates again showed IL-6 expression in mice injected with the Ad-hsp70 vector (but not in mice injected with other adenovirus vectors, indicating that IL-6 is not a response to the adenovirus per se; Fig. 2B). Importantly, TGF-β was expressed in the majority of the lymph node, largely irrespective of the adenovirus vectors that were injected into the associated organs (Fig. 2B).

Lymph nodes draining the prostates undergoing inflammatory killing contain IL-17. The ongoing autoimmune inflammation and destruction of the prostate, combined with the detection of both IL-6 and TGF-β in the lymph node draining the injected prostates, suggested that Ad-VSV-G+Ad-hsp70 treatment of prostate may generate progressive autoimmunity through induction of a Th-17 response, differentiation of which is characterized by a combination of TGF-β and IL-6 (38–40). Consistent with this hypothesis, mRNA for IL-17 was detected in both prostates injected with Ad-VSV-G+Ad-hsp70 (Fig. 3A) and also in the draining lymph node (Fig. 3B). This result was confirmed at the protein level in lymph node (Fig. 3B; P < 0.001 for treatment with Ad-VSV-G+Ad-hsp70 compared with all the other three treatments).

It has been reported that in the presence of TGF-β, the presence or absence of IL-6 acts a critical mediator between the differentiation of CD4 cells into either Th-17 (IL-6 present) or Treg (IL-6 absent; refs. 38–40). Because TGF-β is present in lymph node draining the injected prostates, and IL-6 was induced in response to hsp70 (Figs. 2 and 3), we tested for the generation of Treg responses. Splenocytes recovered from normal mice (uninjected) cannot significantly suppress IFN-γ secretion from activated T cells in the presence of their cognate antigen (Fig. 3C, lanes 1 and 7; P > 0.05). We have previously shown, however, that splenocytes from mice undergoing inflammatory killing of normal melanocytes contain suppressor activity associated with the generation of Treg and TGF-β [refs. 24, 25; Fig. 3C, lanes 3 and 7; P < 0.002 (Tyr-HSV/
In contrast, splenocytes from mice injected intraprostatically with Ad-VSV-G+Ad-hsp70 were unable to exert any suppression of activated T cells in this assay (as represented by the positive control of OT-1 cells alone; lane 7) even when spleens were harvested at different times after prostatic injection (Fig. 3C and lanes 4–7; P > 0.05), suggesting that inflammatory killing of normal prostates does not induce significant Treg responses.

The repertoires of known tissue/tumor-associated antigens in prostate cancer are much less well-characterized than for the melanoma model. Therefore, we used the ova as a model antigen to characterize how inflammatory killing of normal cells affects the generation of antigen-specific responses. Thus, when a plasmid expressing the ova protein was cojected into the prostates of C57Bl/6 mice along with different adenoviral treatments, both Ad-VSV-G (P < 0.02 compared with Ad-hsp70/CMV-ova) and, more potently, Ad-VSV-G+Ad-hsp70 (P < 0.01) primed easily detectable antiova responses in splenocytes from those mice (Fig. 3D). This antiova reactivity was neither enhanced nor diminished in mice in

Figure 4. IL-6 mediates the balance between Th17 and Treg immune responses after hsp70-mediated inflammatory killing of the normal prostate. A, IL-6KO B6.129S2-IL6tm1Kopf/J mice under anesthetics were injected intraprostatically with 10^9 pfu of Ad-GFP or with 5 x 10^8 pfu Ad-VSV-G+5 x 10^8 pfu Ad-hsp70. After surgery, the mice recovered and were euthanized 1 wk after the intraprostatic injection of viruses. Prostates were analysed histologically using H&E sections. Slides were scored by two independent pathologists blinded to the experimental groups for infiltration and necrosis. No significant changes were recorded in either the levels of immune infiltration or tissue damage between the two injected groups. Results representative of three different mice per group. B, C57Bl/6 (lanes 5–8) or B6.129S2-IL6tm1Kopf/J (lanes 1–4) mice were injected intraprostatically with 10^9 pfu of Ad-GFP (lanes 3, 4, and 6) or with 5 x 10^8 pfu Ad-VSV-G+5 x 10^8 p.f.u Ad-hsp70 (lanes 1, 2, and 5). On day 8, 250,000 splenocytes were plated with 10^6 naïve OT-1 CD8+ T cells with H-2Kb-restricted ova peptide SIINFEKL in triplicate, and 48 h later, supernatants were assayed by ELISA for IFN-γ. Lane 5, OT-1 with splenocytes from a C57Bl/6 mouse injected with Ad-VSV-G+Ad-hsp70; lane 6, OT-1 with splenocytes from a C57Bl/6 mouse injected with Ad-GFP in the prostate; lane 7, OT-1 with no added splenocytes; lane 8, splenocytes from a C57Bl/6 mouse with no added OT-1. C, C57Bl/6 (lanes 5 and 6) or B6.129S2-IL6tm1Kopf/J (IL-6KO; lanes 1–4) mice were injected intraprostatically with 10^9 pfu Ad-GFP (lanes 3, 4, and 6) or with 5 x 10^8 pfu Ad-VSV-G+5 x 10^8 pfu Ad-hsp70 (lanes 1, 2, and 5). Eight days later, prostates were used to prepare cDNA that was analyzed by PCR with primers specific for IL-6, IL-17, or TGF-β. PCR for GAPDH showed equal loading (data not shown).
which Treg had previously been depleted by antibody treatment before viral injections (no significant difference between lanes 1 and 4).

Taken together, these data indicate that inflammatory killing of normal prostates does not induce a significant Treg response.

Loss of IL-6 converts a Th17 response into a Treg response in vivo. Taken together, these data suggest that the IL-6 response of prostate tissue to hsp70 expression drives the resultant immune response against tissue-associated self-antigens down a Th17 pathway. To test the central importance of IL-6, we repeated several of these experiments in IL-6KO mice. Whereas injection of Ad-VSV-G+Ad-hsp70 into the prostates of C57Bl/6 mice led to progressive chronic destruction of the prostates associated with intense immune infiltration (Fig. 1), no significant damage or infiltration was observed in similarly injected prostates of IL-6KO mice, and there was no difference between injection of Ad-VSV-G+Ad-hsp70 or Ad-GFP (Fig. 4A). Similarly, there was no significant difference between the wet weights of prostates of IL-6KO mice injected with either Ad-VSV-G+Ad-hsp70, Ad-GFP, or PBS 60 days after viral injection (data not shown)–in contrast to the reduction in prostate weights of up to 50% seen in C57Bl/6 mice (Fig. 1).

However, we did observe a dramatic difference in the ability of splenocytes from IL-6KO mice, injected intraprostatically with Ad-VSV-G+Ad-hsp70, to suppress IFN-γ secretion from activated T cells. Whereas splenocytes from Ad-VSV-G+Ad-hsp70-injected C57Bl/6 mice contained no detectable suppressive activity in this assay (Fig. 3C), splenocytes from IL-6KO mice were potently inhibitory to activated T cells when the prostates had undergone inflammatory killing with Ad-VSV-G+Ad-hsp70—but not with intraprostatic injection of Ad-GFP (Fig. 4B, lanes 1 and 2) compared with lanes 3 and 4; P < 0.01, in all cases). The mechanism of this suppression was shown to be dependent in large part on TGF-β. Thus, when splenocytes from IL-6KO mice, treated intraprostatically with Ad-VSV-G+Ad-hsp70, were cocultured with activated OT-1 T cells; IFN-γ production from the OT-1 T cells was significantly inhibited as shown in Fig. 4B. These splenocytes were also cocultured with activated OT-1 in the presence of 50 ng/mL of 341-BR TGF-β sRII/Fc (R&D Systems), a 159 amino acid extracellular domain of human TGF-β receptor type II fused to the Fc region of human IgG1, to neutralize TGF-β. In two such experiments, 341-BR TGF-β sRII/Fc increased the amount of IFN-γ secreted by activated OT-1 and in the presence of splenocytes from IL-6KO mice treated with Ad-VSV-G+Ad-hsp70, by ~5- to 6-fold (mean values of 130 pg/mL in the absence of 341-BR TGF-β sRII/Fc to 710 pg/mL in its presence)—approaching the levels of IFN-γ produced by splenocytes of IL-6KO mice injected with Ad-GFP as the negative control (815 pg/mL). These data indicate that the suppressive effects of splenocytes from IL-6KO mice on activated T cells is mediated, in part at least, by TGF-β.

To confirm the transition of the immune response to inflammatory killing from a Th17 autoimmune response to Treg protective immunity in these IL-6KO mice, we studied the microenvironment of the injected prostates. As before, prostates of C57Bl/6 mice injected with Ad-VSV-G+Ad-hsp70 contain readily detectable levels of IL-6 and IL-17 but only minimal TGF-β (Fig. 4C). However, in the absence of IL-6, no IL-17 could be detected in injected prostates and these organs now contained abundant TGF-β, indicative of a much more immunosuppressive tissue microenvironment (Fig. 4C). These data show that by removing IL-6, the prostate-specific IL-6/Th-17 immune response to inflammatory killing of normal cells was converted into a Treg response.

Prostate autoimmunity correlates closely with tumor rejection. We are particularly interested in whether the autoimmune response induced by inflammatory killing of normal cells can be exploited to treat tumors of the same histologic type sharing common antigens with the tumor (9, 21, 24, 25). TC2 cells are murine prostatic cancer cells syngeneic to C57Bl/6 mice. Direct intraprostatic injection of control adenoviruses into animals bearing 6 days established TC2 tumors growing s.c. was unable to effect on the growth of the tumors (Fig. 5A; P > 0.05 for Ad-VSV-G compared with Ad-hsp70 or Ad-GFP). However, the combination of Ad-VSV-G+Ad-hsp70 induced a potent tumor rejection response, which could cure between 50% and 80% of mice depending on the experiment (Fig. 5A; P < 0.001 for Ad-VSV-G+Ad-hsp70 compared with Ad-VSV-G; Ad-hsp70 or Ad-GFP alone). This rejection response was highly prostate specific because mice treated in the same way with Ad-VSV-G+Ad-hsp70 were unable to reject s.c. B16 melanoma tumors (Fig. 5A). Moreover, animals that rejected the primary TC2 tumors were also protected against rechallenge with a tumorigenic dose of TC2 cells 70 days after the initial challenge (data not shown). Consistent with both the inability to reject nonprostate-derived tumors and the generation of long term immunologic memory splenocytes from mice treated with Ad-VSV-G+Ad-hsp70 contained cells specific for prostate antigens expressed on TC2 cells (Fig. 5B) but not for antigens expressed on B16 cells (data not shown). The tumor rejection response induced by inflammatory killing of normal prostate was dependent on both CD8+ and CD4+ cells (Fig. 5C, top; P = 0.001 for control immunoglobulin-treated group compared with either CD4+ or CD8+ T-cell–depleted groups). These results contrast to those in the melanocyte/melanoma model where CD4+ T cells were dispensable for therapeutic effects (9, 21, 24, 25) but are consistent with the role of CD4+ Th17 cells in driving both the autoimmune and antitumor immune responses that we observe in this system (38-40). Consistent also with our observation that IL-6 is a critical mediator of the differentiation of a Th-17 antiprostate autoimmune response (Fig. 4), TC2 tumors could not be cured by inflammatory killing of normal prostates in IL-6KO mice (Fig. 5C, bottom) unlike the result in C57Bl/6 mice (Fig. 5A). There was, however, a small but significant (P < 0.02) prolongation of survival between groups injected with Ad-GFP and Ad-VSV-G+Ad-hsp70, suggesting that factors other than just IL-6 may also be important after hsp70-mediated immune activation in the prostate. This is consistent also with the observation that splenocytes from IL-6KO mice, treated with intraprostatic injections of Ad-VSV-G+Ad-hsp70, secreted IFN-γ in response to coculture with TC2 tumor cells (but not B16 cells) but at greatly reduced levels than was the case for splenocytes from C57Bl/6 mice (Fig. 5D). Finally, IL-17 was undetectable by RT-PCR and ELISA from lymph nodes draining the injected prostates in IL-6KO mice, which was not the case for C57Bl/6 mice (Fig. 5D, Fig. 3, and data not shown). These data show that the inflammatory killing of normal prostate can be exploited as an antitumor immunotherapy, and that there is a close correlation between the cytokine mediators of autoimmune responses with those determining antitumor rejection.

Discussion

We show here that hsp70-mediated IL-6 expression in the prostate acts as an adjuvant to fusogenic cell killing mediated by the VSV-G glycoprotein to promote differentiation of a Th-17–like
immune response that is associated with progressive autoimmune attack of the normal prostate. Both hsp70 and cell killing are required to produce optimal autoimmune reactivity, although we did observe moderate autoimmune inflammatory responses with Ad-VSV-G alone. In contrast, normal cell killing in the presence of hsp70 expression did not generate detectable Treg responses, consistent with the presence of both IL-6 and TGF-\(\beta\) in the lymph nodes draining the injected prostate tissue. In addition, prior depletion of Treg from mice injected intraprostatically with a known immuno-}

**Figure 5.** Inflammatory killing of normal prostate induces potent antitumor rejection responses. A, 2 \(\times 10^5\) B16 or 2 \(\times 10^5\) prostate TC2 cells were seeded s.c. in C57BL/6 mice. On day 6, mice under anesthetics were injected intraprostatically with 10⁵ pfu of Ad-GFP or Ad-VSV-G (27–29, 31, 46–50), or Ad-hsp70 or with 5 \(\times 10^6\) pfu Ad-VSV-G+5 \(\times 10^5\) pfu Ad-hsp70. After surgery, the mice recovered and survival (tumor, 1.0 cm) after seeding of tumors is shown for all TC2-bearing adenovirus-treated mice. Mice bearing B16 tumors s.c. treated with Ad-VSV-G+hsp70 intraprostatically are also shown. Results shown are representative of multiple experiments. B, mice under anesthesia were injected intraprostatically with 10⁵ pfu of Ad-GFP or Ad-VSV-G or Ad-hsp70 or with 5 \(\times 10^6\) pfu Ad-VSV-G+5 \(\times 10^5\) pfu Ad-hsp70. After surgery, the mice recovered. One week later, 500,000 splenocytes per treatment group were harvested and cocultured with 50,000 TC2 or B16 (data not shown) target cells pulsed with IFN-\(\gamma\) to increase levels of MHC class I. Forty-eight hours later, supernatants were harvested and assayed by ELISA for IFN-\(\gamma\). Cocultures of splenocytes with B16 targets produced no IFN-\(\gamma\) over background (data not shown). Results shown are representative of two different experiments. C, 2 \(\times 10^5\) prostate TC2 (top), cells were seeded s.c. in C57BL/6 mice. On day 4, mice received injections of control IgG, or CD4⁺ T cell- or CD8⁺ T cell-depleting antibodies (see Materials and Methods). On day 6, mice under anesthetics were injected intraprostatically with 5 \(\times 10^6\) pfu Ad-VSV-G+5 \(\times 10^5\) pfu Ad-hsp70. After surgery, the mice recovered and survival (tumor, 1.0 cm) after seeding of tumors is shown. Results are representative of two different experiments. Bottom, 2 \(\times 10^6\) prostate TC2 cells were seeded s.c. in B6.129S2-Il6tm1Kop/J (IL-6KO) mice. On day 6, mice under anesthetics were injected intraprostatically with 10⁵ pfu of Ad-GFP or with 5 \(\times 10^6\) pfu Ad-VSV-G+5 \(\times 10^5\) pfu Ad-hsp70. After surgery, the mice recovered and survival (tumor, 1.0 cm) after seeding of tumors is shown. D, when the B6.129S2-Il6tm1Kop/J (IL-6KO) mice of experiment C above were euthanized due to tumor size, 500,000 splenocytes per treatment group were harvested and cocultured with 50,000 TC2, or B16 target tumor cells were pulsed with IFN-\(\gamma\) to increase levels of MHC class I. Splenocytes from a C57BI/6 mouse injected intraprostatically with Ad-VSV-G+Ad-hsp70 were used as a positive control as shown. Forty-eight hours later, supernatants were harvested and assayed by ELISA for IFN-\(\gamma\). In addition, lymph nodes draining the injected prostates were recovered and assayed by ELISA for IL-17 (lymph node IL-17). The only positive sample (>3 pg/mL) came from splenocytes from the C57BI/6/treated mouse incubated with TC2 targets (35 pg/mL).
melanocytes (albeit through transfer of the HSVtk and hsp70 genes) induced a potent Treg response, which restrained the concomitant CD8+ T-cell–dependent response directed against self-antigens of melanocytes (and B16 melanomas); moreover, suppression of this Treg response was required to induce autoimmune manifestations (24, 25). Only when the potency of the antiself–T-cell response was augmented by additional costimulation were overt signs of autoimmunity observed (21). It seems likely that the differences in immunologic outcome between these two models (progressive autoimmune destruction of the normal prostate versus suppression of autoimmunity against melanocytes) may result from differences in the constellation of immune cells residing within different organs and the receptors that they express for hsp70. In this respect, we are currently studying the expression of known hsp70 receptors (41–43), as well as additional costimulatory molecules, on cells which might be expected to respond to hsp70 (such as antigen-presenting cells and macrophages; refs. 12, 18, 41) from the normal prostate.

These data also suggest that strategies aimed at blocking the action of key cytokines should be clinically very useful in treatment of progressive autoimmune disorders—such as IL-6 and IL-17 [or associated cytokines (IL-23)] in (hsp70) induced prostatitis (as we observe with IL-6 KO mice). Additionally, these results support a role for the adoptive transfer of Treg populations to suppress proautoimmune T-cell responses (44). Indeed, such a strategy has recently been shown to be effective in treatment of ongoing autoimmune disease in the pancreas in nonobese diabetic mice (45). We are currently investigating the possibility of inducing Treg specific for prostate antigens and their use in nullifying the progressive autoimmune prostatitis we have reported here.

Finally, the major impetus for these studies was our interest in the connectivity between the generation of autoimmune responses and antitumor immunity (24, 25) in tissue types other than the classically “immunogenic” melanoma model (8, 9). Significantly, we have shown here that the IL-6/IL-17–mediated autoimmune response raised against normal prostate is also extremely effective at rejecting established prostate tumors growing elsewhere in the animal. This response is CD4+ and CD8+ T-cell–dependent and is highly antigen specific because it did not effect on a tumor of a different histologic type (Fig. 5A and B). Therefore, normal prostate tissue expresses antigens that can also serve as targets for tumor rejection responses, and these responses can be primed through inflammatory killing of normal prostate cells. The nature of these antigens is currently unclear; however, our ability to isolate splenocyte populations that are activated against prostate, but not melanoma, tumor cells offers an excellent opportunity for us to identify normal prostate antigens which are shared with prostate tumors and which may serve as vaccine targets. These data also suggest that the approach of normal cell killing could be clinically valuable to treat associated cancers. Because surgical or immunologic prostatectomy is non–life threatening, intentional induction of killing of normal prostate could provide a valuable immunologic adjuvant approach to the treatment of metastatic prostate cancer, especially given the potency of the antitumor effects described in the current study. Direct injection of the appropriate vectors into the normal prostate, using established techniques for brachytherapy, could be used either with or without subsequent surgical prostatectomy to induce priming of protective antitumor immunity against both the local tumor as well as against preexisting metastatic disease. Our data here derive from the TC2-transplantable tumor cell line injected s.c. In the human clinical situation, tumors are likely to have been established for longer periods and to have metastasized to different locations than those reflected in our model. Experiments are currently under way to test the efficacy of inflammatory killing of the normal prostates of TRAMP transgenic mice to treat spontaneously arising metastatic prostate cancers, thereby mimicking more closely treatment of human disease.

The expected toxicity associated with induction of ongoing prostatitis could be resolved by prostatectomy if necessary. To address the issue of systemic autoimmune toxicity induced by the inflammatory killing of normal prostates, a pathology review of major organs and tissues is currently under way. Although no abnormalities have been observed at the level of epithelial damage, or immune infiltrations, of normal organs and tissues in mice 3 weeks after intraprostatic injection of the Ad-VSV-G+Ad-hsp70 vectors, longer term toxicology studies are currently under way to determine whether any autoimmune manifestations become apparent at 6 months posttreatment.

In summary, we show here that in the prostate, hsp70 induces IL-6, which triggers a CD4+ and CD8-dependent progressive autoimmune reactivity, associated with IL-17 expression and no significant Treg response. These data are significant in that they confirm that the intimate connectivity between autoimmune and antitumor rejection responses extends beyond the classic melanoma paradigm and may be clinically valuable for the treatment of established metastatic prostate cancer.

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References


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Induction of hsp70-Mediated Th17 Autoimmunity Can Be Exploited as Immunotherapy for Metastatic Prostate Cancer

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