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Cancer Research

Toll-like Receptor 3–Mediated Suppression of TRAMP Prostate Cancer Shows the Critical Role of Type I Interferons in Tumor Immune Surveillance

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Abstract

Inflammation has increasingly been recognized as a critical component influencing tumor growth. Recent reports have revealed conflicting evidence for the role of Toll-like receptors (TLR) in modulating tumorigenesis. In our study, we implicate TLR3 in mediating immune surveillance with increased growth of implanted transgenic adenocarcinoma of the mouse prostate (TRAMP) tumors in $TLR3^{-/-}$ compared with $TLR3^{+/+}$ mice. Activation of TLR3 by polyinosinic-polycytidylic acid (polyI:C) leads to induction of multiple inflammatory pathways, including NF-κB, mitogen-activated protein kinases, and interferon (IFN) regulatory factors. We explored the potential of TLR3 stimulation in prostate cancer immunotherapy and showed that treatment with polyI:C can strongly suppress both s.c. implanted TRAMP tumors in syngenic mice as well as orthotopic prostate cancers in TRAMP C57Bl6 × FvB F1 $Tg^{+/-}$ transgenic mice. Treated tumors remained well differentiated to moderately differentiated with increased infiltration of T lymphocytes and natural killer (NK) cells compared with poorly differentiated adenocarcinoma observed in untreated tumors. Like $TLR3^{-/-}$ mice, IFN-α receptor 1 $(IFNAR1)^{-/-}$ mice exhibited reduced tumor surveillance and impaired tumor suppression following polyI:C treatment. We observed that type I IFN–dependent induction of cytokines was responsible for NK activation, with depletion of NK cells leading to increased tumor growth as well as expansion of $CD4^+CD25^+Foxp3^+$ T regulatory lymphocytes. Our study therefore delineates the importance of IFNAR-dependent functions in TLR3-mediated tumor suppression and supports the use of TLR3 agonists for prostate cancer immune-based therapies. *Cancer Res; 70(7); 2595–603. ©2010 AACR.*

Introduction

In advanced prostate cancer, the failure of hormone therapy or chemotherapy to provide durable responses has forced investigations into other modalities such as tumor immunotherapy. Although the mechanisms of tumor surveillance remain unclear, innate immune recognition of tumors and subsequent activation of adaptive immunity within the microenvironment may modulate tumor growth. Areas of malignant glands in prostate cancer are frequently juxtaposed to inflamed areas of prostate tissue, implying that either inflammation propagates the cancer or is responding to it (1). Potential candidate receptors in regulating these inflammatory processes include the family of Toll-like receptors (TLR). The TLR family of pattern recognition receptors consists of 13 mammalian receptors that recognize conserved microbial motifs, or pathogen-associated molecular patterns specific for microbial components (2). Predominantly expressed in innate immune cells, TLRs recognize these motifs and trigger innate immune activation with a subsequent direction of adaptive immunity. Several endogenous ligands such as heat-shock proteins, the chromatin component HMGB1, and other components of injured cells collectively termed danger-associated molecular patterns have also been characterized, suggesting a role for this receptor family in inflammatory responses resulting from tissue damage even in the absence of infection, such as the transformed cell (3).

Most TLRs interact with and recruit the adaptor proteins myeloid differentiation primary response gene 88 (MyD88) and then serine kinase interleukin (IL)-1 receptor–associated kinase (IRAK), leading to the activation of mitogen-activated protein kinases (MAPK), NF-κB, and expression of inflammatory genes (2). Negative regulatory mechanisms exist to balance the robust immune activation to prevent tissue damage and autoimmunity. This includes negative regulation of intracellular signaling pathways at the level of IRAK by IRAK-M or through the activation of T regulatory (Treg) cells (4–6). TLR3 is unique in that it signals exclusively through MyD88-independent pathways and activates IRF-3, thereby inducing type I interferons (IFN) and IFN-inducible genes. This occurs

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through the adaptor protein TIR domain–containing adapter-inducing IFN-β (TRIF), also known as TLR adaptor molecule 1. Best known for the regulation of type I IFNs in antiviral responses, TLR3 has also been implicated in natural killer (NK) cell activation and dendritic cell maturation (7–9). Whereas MyD88-dependent pathways largely regulate CTL induction, NK activation relies on TRIF-dependent pathways (8).

The role of TLRs in mediating immune surveillance is conflicting, as prior reports have suggested tumor-promoting as well as tumor-suppressing effects. Deficiency in MyD88 confers decreased development of tumors in a mouse model of spontaneous intestinal tumorigenesis and diethylnitrosamine-induced hepatocellular tumors, whereas deficiency in IRAK-M also impairs growth of implanted tumor cells, although these adaptor molecules have opposing effects on TLR signaling (10–12). Nonetheless, TLR agonists have been used as adjuvants to enhance host immunity, with the TLR7 agonist imiquimod approved for use in treatment of basal cell cancer and TLR9 agonists in phase I to III trials against multiple malignancies, including breast, melanoma, and lymphomas (13). Although growth of tumors in the absence of TLR3 has not been studied, the absence of type I IFNs in IFN-α receptor 1 (IFNAR1)$^{-/-}$ mice resulted in increased melanomas in a syngenic mouse model (14).

In this study, we examined the role of MyD88-independent pathways in tumor surveillance and showed increased growth of tumors in the absence of TLR3. Exploring the ability of polyinosinic-polycytidylic acid (polyI:C) to suppress prostate cancer growth, we showed growth retardation of autochthonous TRAMP tumors through both IFN-dependent and IFN-independent pathways. The TLR3-dependent activation of NK cells may in part mediate this effect, whereas the depletion of NK cells resulted in an induction of Treg lymphocytes. This work further implicates and highlights TLR3 as a mediator of tumor immune surveillance and provides mechanistic insight in the balance between inflammation and immune tolerance.

Materials and Methods

Mice. TRAMP Tg$^{+/-}$ mice on a C57Bl6 background (Jackson Laboratories) were generated and genotyped as previously described (15). For the assessment of autochthonous TRAMP tumors by polyI:C, 10-wk-old TRAMP C57Bl6 × FvB F1 Tg$^{+/-}$ males were given i.p. saline or 250 μg of polyI:C (Sigma) reconstituted in 100 μL sterile normal saline weekly for 6 consecutive weeks. TLR3$^{-/-}$ mice backcrossed to a C57Bl6 background over 10 generations and IFNAR1$^{-/-}$ mice backcrossed to a C57Bl6 background for six generations were generated and genotyped as previously described (gift from Shizuo Akira, Osaka University, Osaka, Japan; ref. 16). Six- to 8-wk-old male mice were used for the experiments. Age-matched IFNAR1$^{+/+}$ male littermates and C57Bl6 wild-type males for TLR3$^{-/-}$ mice were used as controls. All mice were kept in specific pathogen-free conditions in the University of California at Los Angeles (UCLA)–Division of Laboratory Animal Medicine facilities according to Animal Research Committee protocols.

Cell culture. TRAMP C2 cell lines (American Type Culture Collection) were grown in DMEM supplemented with 0.005 mg/mL bovine insulin, 10 mmol/L DHEA, 5% fetal bovine serum, and 5% NuSerum IV (Life Technologies Bethesda Research Laboratories). For cell viability determination, 4,000 cells were plated in each well of a 96-well plate overnight and treated with incremental concentrations of polyI:C for 48 h. Incorporation of MTT (Sigma) was assessed by spectroscopy at 600 λ and compared to a standard curve.

Subcutaneous tumor implantation model. TRAMP C2 cells grown to log phase were harvested with trypsinized media and washed in DMEM twice. Following s.c. implantation of 5×10^6 cells in 100 μL normal saline in the shaved flank of male mice, tumor size was determined by measuring the length, width, and depth of tumor and multiplying the product by π/6 to estimate tumor volume in an ellipsoid shape. To assess tumor regression by polyI:C, mice were given i.p. saline or 250 μg of polyI:C weekly for 4 consecutive weeks starting on day 5 following tumor cell implantation.

Quantitative PCR. To assess the induction of gene expression, mice implanted with TRAMP cells were subjected to four weekly treatments of saline or 250 μg of polyI:C, and sacrificed 4 h before the final treatment. RNA from splenocytes prepared by Trizol (Invitrogen) per the manufacturer's instructions was used to generate cDNA for quantitative real-time PCR using iScript reverse transcription-PCR (RT-PCR; Bio-Rad). A Bio-Rad iCycler was used to analyze the samples under the following conditions: 95°C (5 min), 55 cycles of 95°C (20 s), 55°C (30 s), and 72°C (10 s). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. The quantitative real-time PCR total volume was 19 μL and consisted of 2 μL cDNA, 8 μL H_2O, 8 μL of 2× SYBR Green Master Mix (Applied Biosystems), and 1 μL of a 10 μmol/L oligo set for the following genes: IL-6, 5′-CACAGAGGATACCACTCCCAACA, 3′-TCCACGATTTCCCAGAGAACA; Mx-1, 5′-AAACCTGATCCGACTTCACTTCC, 3′-TGATCGTCTTCAAGGTTTCCTTGT; IP-10, 5′-CCAGTGAGAATGAGGGCCATA, 3′-TCGTGGCAATGATCTCAACAC; IL-15, 5′-CACTTTTTAACTGAGGCTGGCATT, 3′-TCCAGTTGGCCTCTGTTTTAGG; IL-1β, 5′-GAGCTGAAAGCTCTCCACCTCA, 3′-TCGTTGCTTGGTTCTCCTTGTAC; TNF-α, 5′-GGTGCCTATGTCTCAGCCTCTT, 3′-CGATCACCCCGAAGTTCAGTA; TGFβ, 5′-GCTGCTGACCCCCACTGATA, 3′-CGTCTCCTTGGTTCAGCCAC; IL-12, 5′-CCCATTCCTACTTCTCCCTCAA, 3′-CCTTTCTGGTTACACCCCTCCT; GAPDH, 5′-ACTCCACTCACGGCAAATTCA, 3′-CGCTCCTGGAAGATGGTGAT.

Intracellular staining and flow cytometry. Single-cell suspensions prepared from spleens were used for flow cytometry. Intracellular staining was performed according to the manufacturer's instructions (BD Bioscience). Splenocytes were stained with CD4 (RM4-5), CD25 (PC61.5), and control IgG2a and IgG1 antibodies at 1:500 dilution for 20 min, and stained with FoxP3 (FJK-16s) or IgG2a antibodies at 1:200 dilution for 30 min. To assess the induction of effector cells following polyI:C stimulation *in vivo*, age-matched 6-wk-old C57Bl6 male mice were given an i.p. dose of 250 μg of polyI:C at 0, 24, and 48 h before harvest. Labeled splenocytes were analyzed using BD

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FACSCalibur flow cytometry and CellQuest Pro software. Antibodies include antimouse CD8 (53-6.7), CD4 (RM4-5), CD25 (PC61.5), Foxp3 (FJK-16s), NK1.1 (PK136), and the respective IgG2a and IgG1 controls (eBioscience).

Antibody depletion. NK cell depletion monoclonal antibody clone PK136 was expanded in hybridoma media (Life Technologies Bethesda Research Laboratories) and purified by precipitation in 50% saturating ammonium sulfate followed by dialysis against PBS. Following implantation of TRAMP C2 tumor cells in 6-wk-old male C57Bl6 mice, groups of mice were given 100 μg IgG control antibody or 100 μg PK136 by i.p. injection starting on days 2, 3, and 4 after tumor challenge and weekly starting at day 11. Subsequently, i.p. saline or 250 μg of polyI:C was administered weekly for 4 consecutive weeks starting on day 5, with mice sacrificed 4 h following the final treatment. Splenocytes were analyzed by flow cytometry, whereas cytokine levels were measured from serum. NK cell depletion was confirmed by flow cytometry to be >95% (data not shown).

ELISA. Serum harvested from cardiac puncture of euthanized animals was used for detecting circulating levels of TGFβ and IL-6 per the manufacturer's instructions (R&D Systems).

Immunohistochemistry. Prostate and seminal vesicles were extracted and fixed in 10% buffered formalin, paraffin embedded, and sectioned at 5 μm. H&E staining was performed and slides were visualized using an Olympus BX41 microscope and captured using an Olympus DP71 digital camera and software. For immunohistochemistry, paraffin-embedded sections were dissolved in xylene and rehydrated in graded ethanol. For anti-CD3 (A0452, DAKO) and anti-NK (Asialo GM1, Cedarlane) staining, antigen retrieval by steaming in 0.01 mol/L sodium citrate (pH 6.0) at 95°C for 25 min was followed by primary antibody staining at 1:100 and 1:1,000 dilution, respectively, for 60 min. Secondary antibody staining was performed using an anti-rabbit horseradish peroxidase (HRP)–labeled polymer (DAKO) for 30 min. Anti-CD45R (RA3-6B2, eBioscience) staining was completed at a 1:50 dilution for 2 h. For anti-Foxp3 (FJK-16s, eBioscience) staining, antigen retrieval by steaming in 0.001 mol/L EDTA (pH 8.0) at 115°C for 3 min was completed followed by primary antibody staining at a 1:1,000 dilution for 1 h. Secondary antibody staining for both anti-CD45R and anti-Foxp3 included two steps consisting of a 30-min incubation using a 1:200 dilution of a rabbit anti-rat antibody (E0468, DAKO), followed by incubation with an anti-rabbit HRP-labeled polymer (DAKO) for 30 min. Staining was completed by incubation with 3,3′-diaminobenzidine for 10 min and hematoxylin counterstaining.

Results

TLR3 regulates prostate tumor growth. To examine the role of TLR3 in tumor surveillance, we first implanted syngenic tumor cells in TLR3$^{-/-}$ mice and TLR3$^{+/+}$ counterparts to observe tumor growth in the absence of exogenous stimulation of TLR3. Tumors from s.c. implanted TRAMP cells in TLR3$^{-/-}$ mice grew markedly larger compared with TLR3$^{+/+}$ mice (Fig. 1A). Because tumors exhibited enhanced basal growth in the absence of TLR3, we then assessed the therapeutic

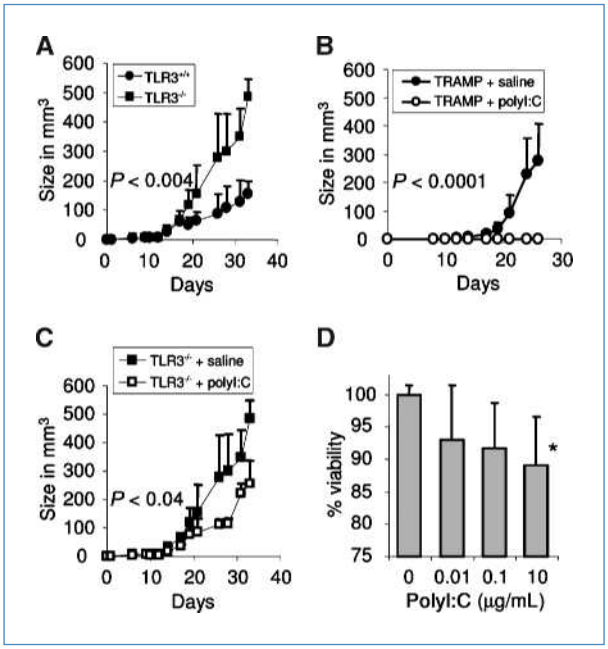

Figure 1. Modulation of TRAMP tumor growth by TLR3. A, s.c. implanted TRAMP C2 cells in TLR3$^{+/+}$ or TLR3$^{-/-}$ mice were assessed for tumor growth. Points, mean of three mice; bars, SD. Data are representative of two independent experiments. Wild-type C57Bl6 mice (B) or TLR3$^{-/-}$ mice (C) s.c. implanted with TRAMP C2 cells were challenged with i.p. saline or polyI:C on days 5, 12, 19, and 26 following implantation and assessed for tumor growth. Points, mean of three mice; bars, SD. Data are representative of two independent experiments. D, TRAMP C2 cells stimulated with polyI:C for 48 h as indicated were assessed for viability by MTT incorporation. Columns, mean of experiments performed in quadruplicate; bars, SD. Data are representative of two independent experiments. *, $P < 0.06$ for polyI:C at 0 versus 10 μg/mL. *P* values were determined by two-tailed Student's *t* test.

potential of TLR3 agonists. Ligands to TLR3 include dsRNA or the synthetic polymer polyI:C. Serial i.p. administration of polyI:C in mice with s.c. implanted TRAMP cells completely suppressed tumor growth, using a dose previously shown to induce NK activity and be well tolerated (Fig. 1B; ref. 8).

PolyI:C signals through TLR3 to activate NF-κB, MAPK, and IRF-3 to induce inflammatory mediators, but may also activate alternative receptors such as cytoplasmic pattern recognition receptors retinoic acid–inducible protein I and melanoma differentiation–associated protein 5 (17). Furthermore, tumor suppression by polyI:C may be induced not only by acting on immune cells but also through direct action of polyI:C on TRAMP cells. To investigate whether polyI:C therapy works through host immune cells, we examined the effect of polyI:C treatment on the tumor growth of implanted TRAMP cells, containing a TLR3$^{+/+}$ background, in TLR3$^{-/-}$ hosts. Only a limited suppression of tumor growth was seen with sequential treatments by polyI:C, supporting a major contribution of host TLR3 in modulating tumor growth (Fig. 1C). To further investigate the direct role of polyI:C on prostate cancer epithelium, TRAMP C2 cells were stimulated with incremental doses of polyI:C *in vitro* and proliferation was assessed. Statistically insignificant reductions in

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proliferation occurred even at the highest doses of polyI:C, supporting minimal direct effects of polyI:C on prostate epithelial tumor cells in our model (Fig. 1D).

Suppression of autochthonous prostate cancer growth in polyI:C-treated mice. To further assess polyI:C-induced tumor suppression activity *in vivo*, we used the established murine autochthonous prostate cancer TRAMP model (15). When crossed with FVB mice, the TRAMP C57Bl6 × FVB F1 $Tg^{+/-}$ transgenic mice develop a more rapidly progressing phenotype with consistent development of localized adenocarcinoma by 12 weeks. In our studies, 10-week-old mice were subjected to weekly i.p. administration of polyI:C and sacrificed 6 weeks after initiation of treatment. Examination revealed no gross metastases in the liver, spleen, or lungs in all animals (data not shown). When compared with saline-treated animals, the prostate, bladder, and seminal vesicles in the polyI:C-treated group were approximately half the weight at 16 weeks (Fig. 2A). Furthermore, prostate and seminal vesicles from polyI:C-treated mice at 16 weeks appeared similar in size to glands from animals of pretreated age at 10 weeks (Fig. 2B).

Under H&E staining, 10-week-old TRAMP C57Bl6 × FVB prostates contained normal glands juxtaposed with areas of well-differentiated adenocarcinoma. At 16 weeks, prostates from TRAMP C57Bl6 × FVB mice showed progression to moderate and poorly differentiated adenocarcinoma with areas involving sheets of anaplastic cells. Strikingly, following six weekly treatments with polyI:C, minimal progression compared with 10-week-old TRAMP C57Bl6 × FVB prostates was observed (Fig. 2C).

Type I IFN–dependent and type I IFN–independent requirements in polyI:C-mediated tumor inhibition. TLR3 is unique to TLRs in that it transduces its signal exclusively through TRIF to activate both NF-κB and IRF-3 (18). To differentiate between type I IFN–dependent and type I IFN–independent functions, we examined the growth of tumors from s.c. implanted TRAMP cells in $IFNAR1^{-/-}$ mice and $IFNAR1^{+/+}$ counterparts and found increased growth in the absence of IFNAR1 (Fig. 3A). In addition, $IFNAR1^{-/-}$ mice exhibited reduced response to polyI:C-mediated suppression in the growth of subcutaneous TRAMP tumors (Fig. 3B).

To further investigate type I IFN–dependent mechanisms, we next examined the gene expression of a panel of inflammatory genes *in vivo* from $IFNAR1^{+/+}$ and $IFNAR1^{-/-}$ mice with s.c. implanted TRAMP tumors in response to i.p. polyI:C stimulation after 4 hours (19). Categorizing genes based on dependency on type I IFNs may give insight into TLR3-mediated effector functions. We found induction of

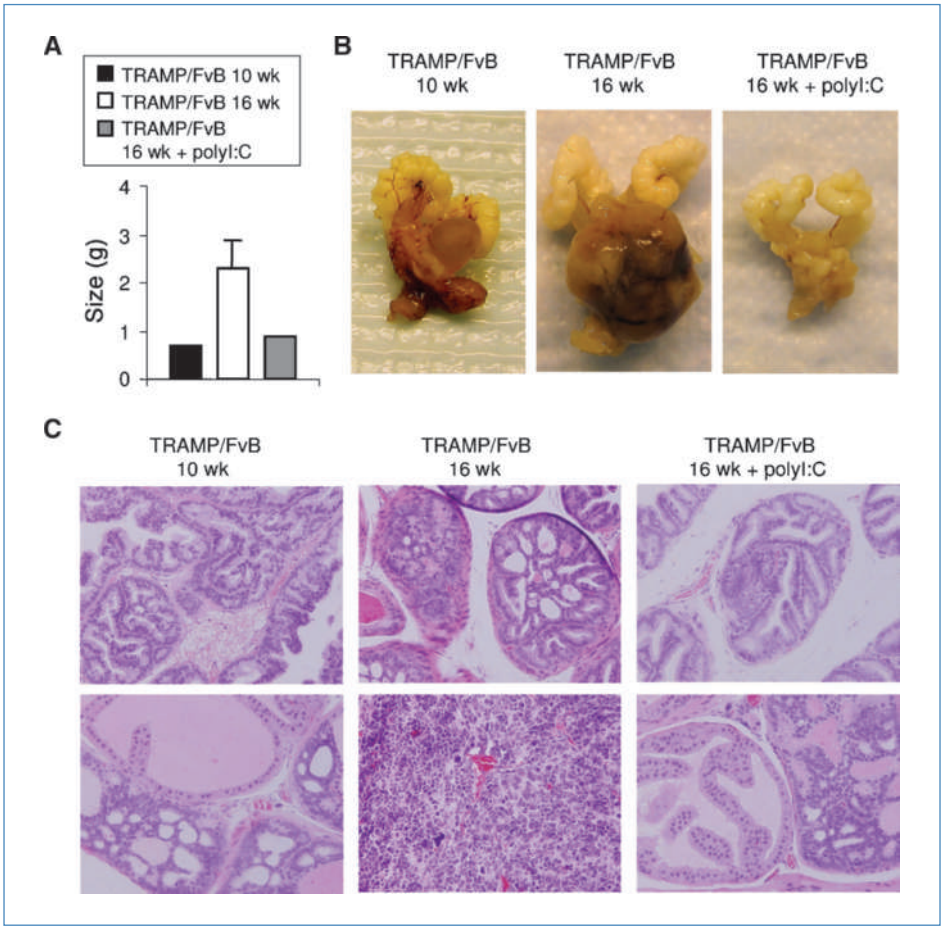

Figure 2. Suppression of autochthonous prostate cancer growth by polyI:C. Prostate and seminal vesicles from 10-wk-old C57Bl6 × FvB F1 $Tg^{+/-}$ transgenic mice and 16-wk-old mice treated with saline or polyI:C weekly for 6 wk were examined for (A) weight, (B) gross examination, and (C) histology with H&E staining at ×20 magnification. Representative examples are shown. Columns, mean of three mice; bars, SD. Data are representative of two independent experiments.

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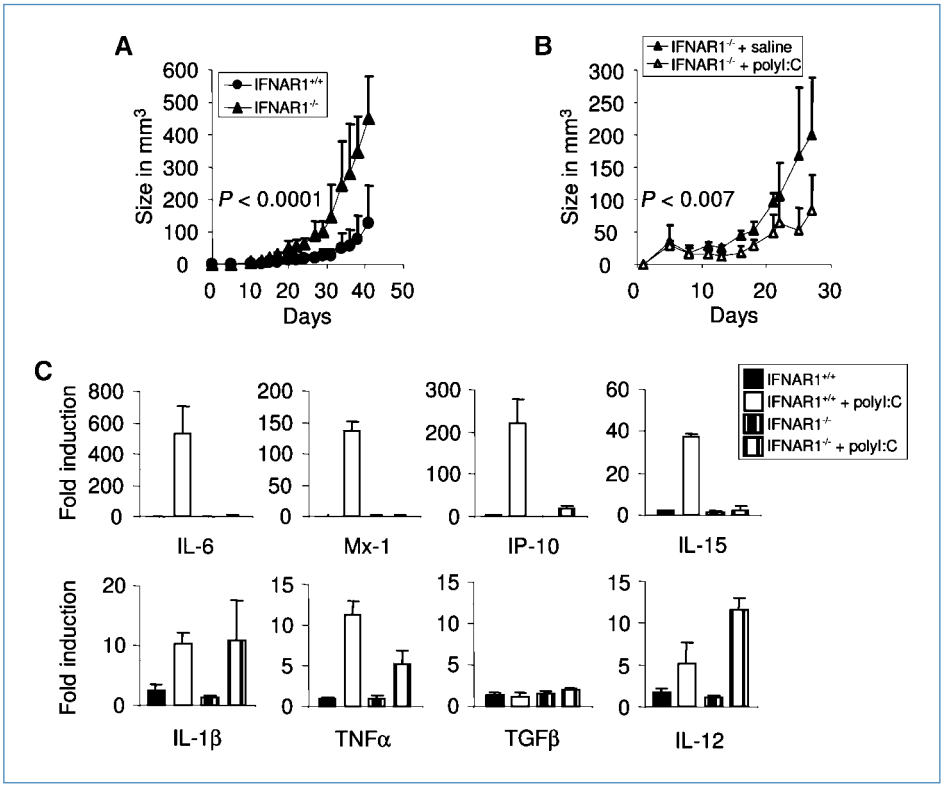

Figure 3. Role of type I IFNs in polyI:C-mediated tumor suppression. A, IFNAR1$^{+/+}$ and IFNAR1$^{-/-}$ mice s.c. implanted with TRAMP C2 cells were assessed for tumor growth. Points, mean from three to five mice in each group; bars, SD. This experiment was repeated four times with consistent results. B, IFNAR1$^{-/-}$ mice s.c. implanted with TRAMP C2 cells were challenged with i.p. saline or polyI:C on days 5, 12, 19, and 26 following implantation and assessed for tumor growth. Points, mean from three to five mice in each group and representative of four independent experiments; bars, SD. C, quantitative RT-PCR performed for the indicated genes on splenocytes from TRAMP tumor-bearing IFNAR1$^{+/+}$ and IFNAR1$^{-/-}$ mice treated with saline or polyI:C weekly for 4 wk with the final stimulation 4 h before sacrifice. Columns, mean from three mice in each group and representative of two independent experiments; bars, SD. All *P* values were determined by two-tailed Student's *t* test.

IL-6, myxovirus resistance-1 (Mx-1), IFN-inducible protein-10 (IP-10), and IL-15 by polyI:C to be type I IFN dependent, whereas expression of IL-1β, TNFα, and IL-12 was independent of type I IFNs. We also examined the gene induction of TGFβ, a cytokine important in the development of Treg cells. Minimal gene induction was observed at 4 hours following challenge by polyI:C with and without IFNAR1 (Fig. 3C).

Regulation of CD4$^+$ CD25$^+$ Treg cells by NK cells. Previous studies have implicated NK cell activation in response to TLR3 stimulation to be largely TRIF mediated (8). Consistent with this finding, we found strong type I IFN–dependent induction of IL-15, a potent activator of NK and T cells (20). This seems specific to TLR3, as no splenocytic induction of IL-6 and IL-15 in response to polyI:C was observed in TLR3$^{-/-}$ mice (data not shown). To confirm the ability of polyI:C to induce NK cells *in vivo*, we stimulated C57Bl6 mice with polyI:C and examined levels of splenocytic CD4$^+$, CD8$^+$, NK, and CD4$^+$CD25$^+$Foxp3$^+$ Tregs at 0, 24, and 48 hours after stimulation. Increased levels of splenocytic NK cells were observed at 48 hours after polyI:C stimulation (Fig. 4A). To assess the relevance of NK cells in our model of polyI:C-mediated tumor suppression, we examined the response to polyI:C upon NK cell depletion using monoclonal antibodies. Significantly enhanced tumor growth compared with polyI:C alone was observed with depletion of NK cells (Fig. 4B). This confirmed previous data supporting a major role for NK cells in TLR3-mediated antitumor effects (8).

Prior reports have implicated cross-regulation between NK cells and Tregs. Tregs have been shown to inhibit the dendritic cell–mediated activation of NK cells, whereas others have linked a direct inhibitory effect on NK cells to a TGFβ-dependent mechanism (21, 22). Although IFNγ has been implicated in inhibiting Treg development, little is known about how activation or depletion of NK cells influences Tregs (23). When we examined the serum in mice depleted of NK cells in our tumor suppression model, we found elevated levels of TGFβ and lower levels of IL-6 from mice depleted of NK cells and stimulated with polyI:C compared with mice stimulated with polyI:C alone (Fig. 4C). Consistent with prior gene induction results, no significant elevation of TGFβ levels were observed with polyI:C treatment alone. This suggested that following depletion of NK cells, the systemic environment favors the development of Tregs. To investigate further, we examined the levels of CD4$^+$CD25$^+$Foxp3$^+$ cells in the splenocytes of mice with and without NK depletion in our polyI:C-induced tumor suppression model. As expected, no statistically significant increase in Tregs was observed with polyI:C stimulation alone, but elevated Treg levels were detected with the combination of polyI:C stimulation and NK cell depletion compared with no treatment (Fig. 4D).

Autochthonous prostate cancer inflammatory infiltrate in polyI:C-treated mice. To correlate the systemic effects with the tumor microenvironment, we examined the autochthonous TRAMP tumors for infiltration of inflammatory cells by immunohistochemistry. We observed that at 10 weeks, prostates from TRAMP C57Bl6 × FvB F1 Tg$^{+/-}$ mice exhibited small numbers of infiltrating CD3$^+$ and NK cells, with no detectable CD45R- and Foxp3-staining cells. As the tumors

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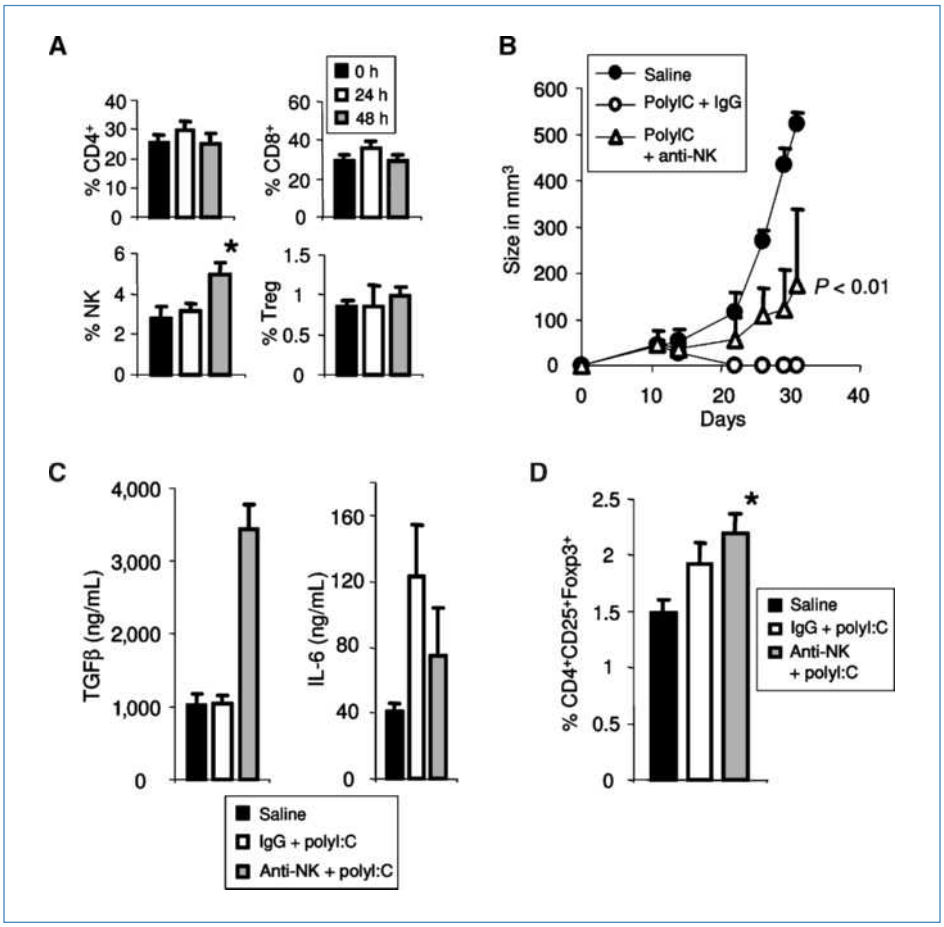

Figure 4. Contributions of NK cells in polyI:C-mediated tumor suppression. A, groups of four C57Bl6 male mice were treated with polyI:C for 0, 24, and 48 h and assessed for splenocytic induction of $CD4^+$, $CD8^+$, NK, and $CD4^+CD25^+Foxp3^+$ (Treg) cells by flow cytometry. *, $P < 0.01$ for NK levels at 0 h compared with 48 h. B to D, groups of three to four C57Bl6 male mice with s.c. implanted TRAMP cells were treated with saline or polyI:C on days 5, 12, 19, and 26 following implantation. Additionally, groups of polyI:C-treated mice were given control IgG or NK-depleting antibodies. B, mice were assessed for tumor growth. *P* value represents data between polyI:C and control IgG versus polyI:C and anti-NK antibody. C, serum was used to determine levels of cytokines by ELISA as indicated. D, percentage of $CD4^+CD25^+Foxp3^+$ cells in total splenocytes was assessed by flow cytometry. *, $P < 0.02$ for NK levels treated with saline versus polyI:C and anti-NK antibodies. Columns, mean of three mice; bars, SD. Data are representative of four independent experiments. *P* values were determined by two-tailed Student's *t* test.

progressed at 16 weeks, similar numbers of inflammatory infiltrates were present. Notably, with weekly polyI:C stimulation, a marked influx of $CD3^+$ cells within both the prostate glands as well as the stroma, and an increase in NK cells in the prostatic stroma, were observed, with no detectable CD45R- and rare Foxp3-staining cells (Fig. 5).

Discussion

Originally described as receptors on immune cells responsible for sensing pathogens, TLRs have now been implicated in broader biological functions. Using the TRAMP orthotopic and subcutaneous tumor models, we provide evidence for TLR3 as a sensor in tumor immune surveillance, with the absence of TLR3 leading to accelerated tumor growth without exogenous stimulation of the receptor. Consistently, activation of TLR3 with exogenous ligand suppressed the growth of TRAMP tumors. In orthotopic prostate tumors, serial polyI:C treatments lead to the recruitment of T lymphocytes and NK cells within the tumor microenvironment, resulting in a striking suppression of tumor growth both in gross size as well as histologic progression of adenocarcinoma. We showed that polyI:C contributes to tumor suppression through a type I IFN–dependent activation of NK cells, as well as suggests a cross-regulation of Treg lymphocytes by NK cells.

In our studies, increased growth of wild-type TRAMP tumors in $TLR3^{-/-}$ compared with $TLR3^{+/+}$ mice implicates TLR3 expressed by immune cells in mediating tumor surveillance. The question of how TLRs sense tumor cells is the subject of ongoing investigations, potentially involving danger-associated molecular patterns released from tumor cells, such as components of tumor mRNA (24, 25). Although we did not observe significant differences in TRAMP cell growth upon direct stimulation with polyI:C, the expression of multiple TLRs has been shown on prostate epithelial cells, and recent *in vitro* studies suggested the potential for direct apoptotic effects of TLR3 stimulation (26). PolyI:C may induce autophagy in tumor cells by expression of inflammatory cytokines or apoptosis through a RIP/FADD/caspase-8–dependent pathway (27). Furthermore, reports have shown protumor effects of TLR activation on tumor cells by enhancing immune evasion or promoting tumor growth through tissue repair mechanisms (3, 10, 28). In humans, a sequence variant in a 3′-untranslated region of TLR4 as well as polymorphisms in the TLR gene cluster encoding TLR1, TLR6, and TLR10, and the downstream signaling mediators IRAK1 and IRAK4 confer increased prostate cancer risk (29–31). How these polymorphisms affect TLR signaling within the tumor microenvironment have yet to be determined. Whether these mutations affect tumor initiation

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or progression of cancers will also be the subject of future studies.

Although classically involved in antiviral responses, type I IFNs have been implicated in immune surveillance of tumors with increased tumor formation in methylcholanthrene-induced sarcomas and growth of syngenic tumor cells in IFNAR1$^{-/-}$ compared with IFNAR1$^{+/+}$ mice (14, 32). Endogenous IFN responsiveness in hematopoietic cells has been shown to be important for anticancer immune responses (33). The lack of type I IFN responsiveness in IFNAR1$^{-/-}$ mice encompasses both MyD88-independent type I IFN production by TLR3 and TLR4 through TRIF as well as MyD88-dependent type I IFN production by TLR7 and TLR9 through IRF-7. However, the partial tumor suppression by polyI:C in IFNAR1$^{-/-}$ mice compared with IFNAR1$^{+/+}$ mice still implicates the importance of IFN-independent pathways. We also show a major contribution of NK cells in polyI:C-mediated tumor suppression, which may also potentiate the effects of antigen-specific CD8^{+} cells (34).

To further discriminate the potential mechanisms of IFN-dependent and IFN-independent effector functions, we surveyed the expression of various genes in our tumor model in IFNAR1$^{+/+}$ and IFNAR1$^{-/-}$ mice. These genes can be categorized into families that are dependent and independent on type I IFNs. We showed IFNAR1-dependent gene induction of IL-6, IL-15, IP-10, and Mx-1, supporting NK cell proliferation, and recruitment of activated T and NK cells to areas of inflammation, by IL-15 and IP-10, respectively. In contrast, gene expression of IL-1β, TNFα, and IL-12 was induced by polyI:C independent of type I IFNs, suggesting a role for these cytokines in mediating tumor toxicity in the type I IFN–independent pathways.

We showed that depletion of NK cells led to an increase in splenic Tregs, showing a balance between immune activation and immune suppression following acute activation of NK cells. This suggests that either inhibition of Tregs or direct activation of NK cells may enhance immune-based tumor therapies. Suppression of Tregs may be sufficient to break tolerance or induce anergy to self-antigens and points to the importance of type I IFNs in the development of immune-based cancer therapy (35). In patients with systemic lupus erythematosus, IFNα secreted by antigen-presenting cells may potentially inhibit Treg activity (36). This is in accordance with clinical observations of elevated levels of Treg cells in patients with prostate cancer and suppression of human prostate tumor–infiltrating CD8^{+} Treg cells by TLR8 *in vitro*

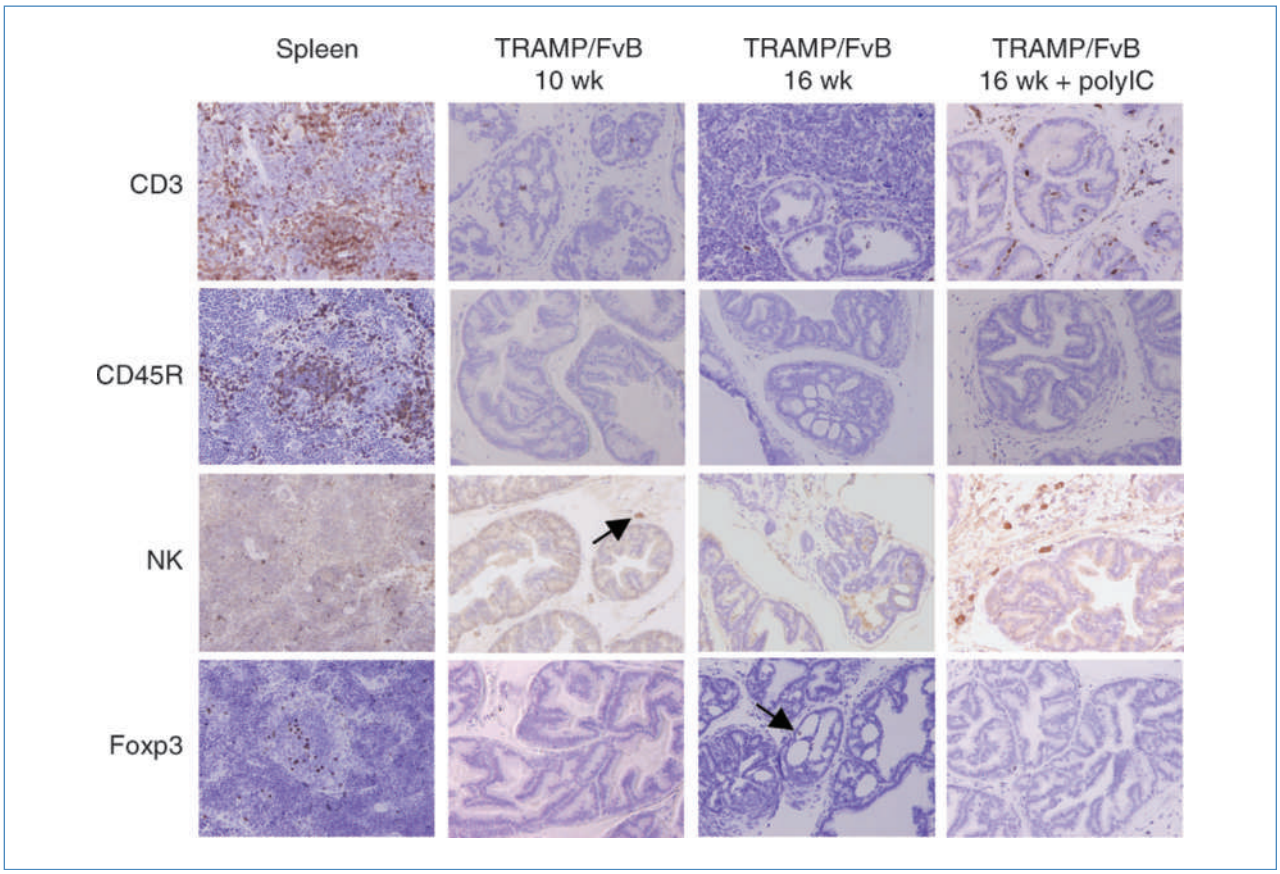

Figure 5. Inflammatory infiltrate in polyI:C-treated autochthonous prostate cancer. Infiltration of CD3, CD45R, NK, and Foxp3 expression cells as indicated were performed by immunohistochemistry in prostates from 10-wk-old and 16-wk-old C57Bl6 × FvB F1 Tg$^{+/-}$ transgenic mice treated with saline or polyI:C weekly for 6 wk. Spleens were used for positive controls. Arrows, rare positive staining cells for the respective antibodies. Representative examples from each group of three mice are shown at ×20 magnification.

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(37, 38). Alternatively, our findings also suggest that persistent activation of NK cells may suppress the development of Tregs. However, following pathogen challenge, induction of Treg cells may play a critical role in protecting against tissue injury and inhibiting autoimmunity (6). Thus, activation of TLRs in cancer therapy may potentially interfere with normal tissue homeostasis and will need to be carefully monitored.

Our future studies will also focus on the tumor microenvironment, which may differ from systemic activation of cytokines and splenocyte effector cells. Although in response to polyI:C, we did not observe a decrease in circulating TGFβ levels or Treg levels, Tregs may still be influenced at the tumor microenvironment. We have shown recruitment of T lymphocytes and NK cells within the glands and stroma of the prostate upon serial polyI:C therapy. Studies will need to define the kinetics of such infiltration to the tumor microenvironment and use more sensitive approaches to follow the activation of Tregs and local cytokine levels.

Emerging studies indicate that TLR activation of multiple inflammatory pathways through the recognition of both pathogen-associated molecular patterns and endogenous ligands may modulate the development and progression of cancer. We have provided evidence supporting a role of TLR3 in promoting tumor immune surveillance and the use of TLR3 agonists as immunotherapy in prostate cancer. Although other TLRs may contribute to tumor progression by overly enhancing inflammation through MyD88-dependent signaling pathways, TLR3 may provide an optimal balance of type I IFN induction and inflammatory cytokine activity. Thus, comparison of type I IFN and proinflammatory effects of different TLRs in tumor settings deserves further study. With the caveats of direct stimulation and activation of cancer cells by TLRs and of disrupting systemic regulation of immune functions, we believe that defining the TLR-dependent pathways responsible for immune surveillance will contribute to developing novel therapeutics. At the same time, use of TLR3 agonists may be promising in suppressing progression of prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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