Targeting the Effector Site with IFN-αβ-Inducing TLR Ligands Reactivates Tumor-Resident CD8 T Cell Responses to Eradicate Established Solid Tumors

Andrew J. Currie, Robbert G. van der Most, Steve A. Broomfield, Amy C. Prosser, Michael G. Tovey and Bruce W. S. Robinson

*J Immunol* 2008; 180:1535-1544; doi: 10.4049/jimmunol.180.3.1535

http://www.jimmunol.org/content/180/3/1535

References

This article cites 58 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/180/3/1535.full#ref-list-1

**Why The *JI*** Submit online.

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Targeting the Effector Site with IFN-αβ-Inducing TLR Ligands Reactivates Tumor-Resident CD8 T Cell Responses to Eradicate Established Solid Tumors

Andrew J. Currie,1,2,3* Robbert G. van der Most,2,* Steve A. Broomfield,† Amy C. Prosser,* Michael G. Tovey,‡ and Bruce W. S. Robinson*†

Effective antitumor CD8 T cell responses may be activated by directly targeting the innate immune system within tumors. We investigated this response by injecting a range of TLR agonists into established tumors using a mouse model of malignant mesothelioma stably transduced with the hemagglutinin (HA) gene as a marker Ag (AB1-HA). Persistent delivery of the dsRNA mimetic poly(I:C) into established AB1-HA tumors resulted in complete tumor resolution in 40% of mice, with the remaining mice also showing a significant delay in tumor progression. Experiments in athymic nude mice along with CD8 depletion and IFN-αβ blocking studies revealed that tumor resolution required both CD8 T cells and type I IFN induction, and was associated with local changes in MHC class I expression. Surprisingly, however, tumor resolution was not associated with systemic dissemination or tumor infiltration of effector CD8 T cells. Instead, the antitumor response was critically dependent on the reactivation of tumor-resident CD8 T cell responses. These studies suggest that, once reactivated, pre-existing local CD8 T cell responses are sufficient to resolve established tumors and that in situ type I IFN is a determining factor. The Journal of Immunology, 2008, 180: 1535–1544.

Tumor Ags are efficiently cross-presented to the immune system (1). T cell responses against these Ags are complex and vary from tolerance to (weak) activation (2–4), most likely governed by the status of dendritic cells (DC) in the tumor draining lymph nodes (5). The emerging paradigm suggests that tumors have evolved a wide repertoire of immune-suppressive mechanisms, involving TGF-β, IL-10, regulatory T cells, B7-H1, and possibly many others, which can interfere with the context of Ag presentation and can block the antitumor T cell response at all possible levels (6). Furthermore, tumors or tumor-derived factors may interfere with proper DC maturation. However, it may be possible to overcome such immune-suppressive mechanisms by directly altering the context in which tumor Ag cross-presentation occurs at the effector site, i.e., in the tumor and/or in the tumor draining lymph nodes, potentially leading to therapeutic responses (7).

It is increasingly clear that the immunogenic context of a given Ag is decoded by a system of receptors, among which the TLRs have received the most attention (8). Agonists for TLRs include pathogen-associated molecular structures such as LPS, dsRNA, unmethylated CpG-containing oligodeoxynucleotides, and others (9). TLRs are expressed by DC in which they control functions such as activation, maturation, and migration (10). In the absence of TLR ligation, or other analogous activating stimuli, Ag presentation by DC is likely to induce tolerance. This implies that TLR agonists could provide an immunogenic context to otherwise tolerizing or weakly priming Ags. Indeed, it has been shown that persistent TLR stimulation breaks tolerance against tumor-associated Ags and that these signals can be provided by viruses or synthetic ligands (11). The therapeutic promise of TLR ligands as antitumor agents, based on the premise that TLR stimulation adds an immunogenic context to tumor Ags, is now receiving considerable attention, both in animal models as well as in clinical trials (12).

TLRs are expressed in DC, macrophages, T cells as well as in some tumor cells (10, 13). The complex expression patterns of TLRs suggest that a further understanding of the interaction among tumor Ags, T cells, and TLRs is required to further optimize the therapeutic implementation of TLR ligands (14). It is often assumed that the addition of a proimmunogenic TLR signal to cross-presenting DC triggers a de novo T cell response. However, the precise effects of TLR stimulation on existing and possibly tolerized T cell responses have been less well studied. Yang et al. (11) have clearly shown that persistent TLR ligation breaks immunological tolerance, and these data suggest that TLR ligands might have the potential to rescue T cell responses that have been tolerized or ineffectively activated in the immunosuppressive tumor environment. Also, the finding that the TLR3 ligand polyinosinic-polycytidylc acid (poly[I:C]) “shields” CD8 T cells from regulatory T cell-mediated suppression, through IL-6, implies that TLR ligation could mobilize the existing antitumor T cell repertoire (15). Consistent with this idea is the recognition that intralesional administration of TLR ligands is therapeutically beneficial: local stimulation of tumor-sensitized T cells could...
drive antitumor immunity. The classical example for this is Coley’s toxin, a *Serratia marcescens* and *Streptococcus pyogenes* bacteria mixture, which was strikingly efficacious when administered intravenously in a post-surgery setting (14, 16). The efficacy of Coley’s toxin is now attributed to unmethylated bacterial DNA, stimulating TLR9 (14).

On this basis, we hypothesized that intratumoral delivery of TLR ligands would be sufficient to break tolerance/suppression in established tumors. By administering TLR ligands directly at the effector site, we predict that tumor-infiltrating DC will be activated, which will in turn lead to T cell activation in the draining lymph nodes to which these DC migrate. Thus, intratumoral TLR activation should stimulate the entire tumor–lymph node loop. We tested this notion using a panel of different TLR agonists in a murine model of malignant mesothelioma. We have used transduced influenza virus hemagglutinin (HA) protein as a neo-tumor Ag, allowing us to visualize Ag presentation and to track T cell responses using TCR transgenic CD8 T cells specific for the dominant H-2Kd restricted epitope in this protein (17). In the present study, we show that a subset of TLR agonists, namely those specific for TLR3, TLR7, and TLR9, stimulate antitumor responses. TLR2 and TLR4 agonists have no effect on tumor growth. The antitumor efficacy of poly(I:C) was critically dependent on CD8 T cells and type I IFN. Our data further indicate that a pre-existing, tumor-sensitized CD8 T cell population was the ultimate target of TLR stimulation. Thus, TLR ligands mobilizes a local tolerized, or ineffective activated T cell repertoire in an IFN-αβ-dependent fashion.

### Materials and Methods

#### Reagents

High purity poly(I:C), LPS from *Escherichia coli*, and loxoribine were purchased from InvivoGen. Pam3CysKKK was from EMC Microcollections. CpG 1668 (5’-TCCATGCAGTTCCTGATGCT-3’) or the non-CpG oligodeoxynucleotide 1720 control (5’-TCCATGACGTTCCTGATGCT-3’) were from TIB-MOLBIOL. The TLR7 agonist 3M-019 was provided by Dr. R. Kedl (3M Pharmaceuticals). CFSE was from Molecular Probes. HA peptide (IYSTVASSL (residues 518-526) of A/PR8/8/34 (H1N1) influenza virus, allowing us to visualize Ag presentation and to track T cell responses using TCR transgenic CD8 T cells specific for the dominant H-2Kd restricted epitope in this protein (17). In the present study, we show that a subset of TLR agonists, namely those specific for TLR3, TLR7, and TLR9, stimulate antitumor responses. TLR2 and TLR4 agonists have no effect on tumor growth. The antitumor efficacy of poly(I:C) was critically dependent on CD8 T cells and type I IFN. Our data further indicate that a pre-existing, tumor-sensitized CD8 T cell population was the ultimate target of TLR stimulation. Thus, TLR ligands mobilizes a local tolerized, or ineffective activated T cell repertoire in an IFN-αβ-dependent fashion.

#### Animals

Hybridoma supernatant containing rat anti-mouse MHC class I (TIB-126) was provided by D. Andrews (Lions Eye Institute, Perth, Western Australia). The anti-mouse mAbs either unconjugated or coupled to FITC, PE, PE-Cy5, or allophycocyanin as required, along with appropriate matching isotype controls, were purchased from BD Pharmingen, eBioscience, and R&D Systems and are as follows: TCR-β (H57-597), CD3ε (145-2C11), CD4 (RM4-5), CD8 (53-6.7), B220 (RA3-6B2), CD11b (M1/70), Gr-1 (RB6-8C5), Ly6A/E (D7), CD11c (N418), and CD49b (DX5). Flow cytometry was performed using a BD FACSCalibur instrument and analyzed using CellQuest software (BD Immunocytometry Systems). Absolute cell numbers were determined in some experiments using BD Trucount tubes, according to the manufacturer’s instructions (BD Immunocytometry Systems).

### Tumor cell culture and inoculation

Generation of the BALB/c-derived mouse mesothelioma cell line AB1 and transfection with the gene encoding influenza HA (AB1-HA) has been previously described (17). Cell lines were maintained in RPMI 1640 (Invitrogen Life Technologies) supplemented with 20 mM HEPES, 0.05 mM 2-ME, 60 µg/ml penicillin (CSL), 50 µg/ml gentamicin (West), and 5% FCS (Invitrogen Life Technologies). AB1-HA transfectants were selected by culture in medium containing the neomycin analog geneticin (Invitrogen Life Technologies) at a final concentration of 400 µg/ml. The level of HA expression on transfected cells was measured by FACS analysis, using the biotinylated HA-specific mAb H18 (18), originally obtained from Dr. W. Gerhard (The Wistar Institute, Philadelphia, PA). Tumor cells (1 × 105 in 100 µl of PBS) were injected s.c. into the shaven right flank of recipient mice on day 0 and subsequent tumor growth monitored by taking two perpendicular diameter measurements using microloupes. Mice were euthanized when tumors reached 100 mm3 (typically fewer than day 28 in untreated animals), which we used as the basis for calculated survival times. For dual tumor experiments, mice received two injections of tumor cells (as described) in the same flank ~2 cm apart. Mice were euthanized in these experiments when either tumor reached 100 mm3.

### Intratumoral therapy

Intratumoral treatment for BALB/c wild-type mice commenced on day 10 for small tumors (~4 mm3) and day 16 for large tumors (~16 mm3). Treatment of tumor-bearing BALB/c nude mice commenced on day 7 (equivalent in size to day 10 in wild-type mice). Mice were lightly restrained and the surface of the tumor swabbed with alcohol before direct injection of various adjuvants diluted in 50 µl of sterile injectable saline (Baxter Healthcare). Care was taken to slowly deliver the bolus and the base of the tumor was clamped firmly to prevent liquid penetrating through the Baxter. Small tumors were observed to swell slightly and blanch upon injection. Various multiple dosing regimens underwent trial as indicated in the study.

### Cell depletion studies

NK cell depletion was performed using anti-asialo-GM1 Ab (Wako Fine Chemicals). A total of 20 µl of anti-asialo-GM1 Ab diluted with 180 µl of sterile saline was injected i.p. 1 day before poly(I:C) treatment and every 3 days thereafter for a total of 4 doses. NK depletion (>90%) was verified during treatment by FACS analysis of peripheral blood using Abs specific for TCR-β and CD49b. CD4 and CD8αa T cell depletion was performed using the purified GSK1.1 and YTS.169 mAbs, respectively, prepared by K. Davern, Monoclonal Antibody Facility (Western Australian Institute for Medical Research, Perth, Australia). Mice received an initial dose of 200 µg i.v., 1 day before poly(I:C) treatment, followed by a second dose of 150 µg administered i.p. on the day of treatment and then 150 µg i.p. every second day thereafter for a total of six doses. CD4 and CD8 depletion (>95%) was verified during treatment by FACS analysis of peripheral blood using Abs specific for CD4 and CD8. To assess in vivo injection, to remove CD8 T cells during the early phase of tumor growth only, mice received 150 µg of CD8 depleting Ab i.p. on days −1, 0, +2, and 50 µg on day +4, with respect to AB1-HA implantation.

### Preparation and staining of tissues for flow cytometry

For flow cytometry analysis, tumors, spleens, and lymph nodes were removed from mice and placed into ice-cold PBS containing 1% FCS (v/v). The axillary and inguinal nodes were pooled for the tumor flank (draining lymph nodes) and for the contralateral flank (nondraining lymph nodes) in all cases. Tissues were homogenized into cell suspensions using frosted glass slides, washed twice with cold PBS with 1% FCS (v/v) and 0.01% NaN3 (v/v; wash buffer), before being resuspended in staining buffer (PBS with 1% BSA (w/v), 5% FCS (v/v), and 0.01% NaN3 (v/v)) along with appropriate dilutions of Abs or isotype controls. After 30 min, cells were washed thrice in wash buffer and resuspended in 2% formalin/PBS (v/v) (Sigma-Aldrich) before FACS analysis.

### Lyons-Parish analysis of Ag presentation

CFSE labeling was performed as previously described (19). Briefly, lymph node cells from TCR-transgenic CL4 mice were resuspended in 20 ml of RPMI 1640 at 107 cells/ml and incubated with 2.5 µM CFSE for 10 min at room temperature. Cells were centrifuged through a FCS cushion twice, followed by PBS washes. A total of 1 × 106 cells were injected i.v. into recipient mice. CFSE-labeled cells were recovered from secondary lymphoid organs and tumors 3 days after adoptive transfer, counterstained with Cy5 and CD8 and analyzed by FACS.

### In vivo CTL assay

Detection of HA-specific in vivo CTL was performed as previously described (20). Erythrocytes were removed from BALB/c spleens by resuspending cells...
In vivo IFN-αβ blockade

The origin, purification, and assay of IFN-αβ neutralizing sheep Ig (anti-IFN-αβ) and matching normal sheep Ig have been previously described in detail (21, 22). Control sheep IgG or anti-IFN-αβ was delivered as described (23). In brief, mice were i.v. injected with 0.2 ml of Ig on day −1, +2, and +4 with respect to poly(I:C) administration. Neutralization was confirmed by staining for Ly6A/E expression on peripheral blood CD4 and CD8 T cells 24 h after the first dose of poly(I:C).

CD8 T cell transfer studies

CD8 T cells for adoptive transfer were prepared by negative selection from whole spleens of naive or poly(I:C)-cured mice using the Miltenyi Biotec CD8 T cell isolation kit according to manufacturer’s instructions. Purified CD8 T cells (>95% purity) from either naive or Ag-experienced donors were i.v. injected (1 × 10^7/300 μl of saline/mouse) into nude mice on day 6–9 of tumor growth, 1 day before commencement of poly(I:C)-treatment.

Immunohistochemistry

Surface Ags were detected using the streptavidin-biotin labeling immunoperoxidase staining technique. Tumors were removed, placed in compound embedding medium (OCT; Miles), snap-frozen using dry ice, and stored at −80°C. Sections (10 μm) were cut, collected on poly-L-lysine-coated slides, and allowed to air dry. Slides were stored at −20°C over desiccant before staining. Before immunostaining, sections were fixed with cold ethanol (15 min) and blocked with 1% H2O2 (v/v) (5 min) followed by avidin/biotin block (10 min each). Sections were incubated with the appropriate dilutions of primary rat anti-mouse Abs against MHC class I, CD8, CD4, GR1, or isotype controls for 1 h followed by incubation with a biotinylated secondary Ab for 30 min (mouse anti-rat IgG F(ab')2; Jackson Immunoresearch Laboratories). Immunostaining was detected by incubating with streptavidin-HRP (DakoCytomation) for 30 min and with diaminobenzidine-H2O2 (Sigma-Aldrich) for 5–10 min. Slides were washed three times for 5 min each time in PBS between each incubation step, counterstained with hematoxylin, and mounted in aqueous mounting medium.

Statistics

Data was statistically evaluated using Prism software (GraphPad). Survival responses were analyzed by Kaplan-Meyer using log-rank test. Growth curves were compared using a two-tailed paired t test, with pairs defined by time point. All other variables were compared using a two-tailed Mann-Whitney U test. Significance was defined as p < 0.05.

Results

Intratumoral delivery of nucleic acid TLR ligands inhibits tumor growth

To assess the therapeutic efficacy of local TLR stimulation, we screened a panel of different TLR agonists in our murine model of mesothelioma (AB1-HA). Ligands for TLR2, TLR3, TLR4, TLR7, and TLR9 (Pam3Cys, poly(I:C), LPS, 3M-019, and CpG 1668, respectively) were analyzed for their ability to interfere with growth of established (>16 mm²) AB1-HA tumors, by delivering three intratumoral doses with a 3-day interval starting at day 16 postinoculation (Fig. 1A). We found that the ligands for TLR3, TLR7, and TLR9 caused significant delay in the growth of AB1-HA, resulting in a >25% increased survival time (p < 0.05). This effect was not due to a nonspecific effect of nucleic acid-like substances because the non-CpG oligodeoxynucleotide (1720) control had no impact on tumor growth. The TLR2 and TLR4 agonists had no effect on tumor growth. Treating tumors with pairwise combinations of poly(I:C), 3M-019, and CpG 1668 showed no additional benefit over monotherapy (data not shown), suggesting a common mode of action.

To further investigate the efficacy of local TLR agonist administration, we focused on the TLR3 ligand poly(I:C). We first studied the dosage kinetics of poly(I:C) in more detail. Increasing or decreasing the dose of poly(I:C) by a log-fold from that used to screen ligands (10 μg) had no impact on its efficacy (data not shown). Furthermore, increasing the number of doses from 3 to 6, without changing the frequency of delivery (six intratumoral doses with a 3-day interval), did not further delay tumor growth when treatment was started at day 16 (Fig. 1B). In contrast, increasing the frequency of delivery to a once-daily interval (six intratumoral doses with a 1-day interval) significantly improved the delay in tumor growth with an almost doubling in survival time. Importantly, treatment of smaller, yet established tumors (day 10, ~4 mm²) with this regimen resulted in complete cure in 40% of mice (>100 days tumor-free) (Fig. 1B). All cured mice (n = 14) were able to resist rechallenge with the original tumor line. Similar responses to poly(I:C) treatment were observed in BALB/c mice bearing non-HA-transfected AB1 tumors or unrelated AB2 tumors.

FIGURE 1. Persistent intratumoral delivery of viral nucleic acid mimetics inhibits tumor progression. A. Various TLR ligands were screened for their ability to interfere with growth of established (day 16) AB1-HA tumors by injection of three doses at 3-day intervals (q3dx3). Mice were culled when tumor sizes reached 100 mm². *, p < 0.05, comparing all ligands to saline treatment survival curve. Data shown are pooled from three repeat experiments (n = 10 mice). B. Two different poly(I:C) (pI:C) regimens were tested on either day-10 or day-16 tumors and survival responses were monitored. *, †, p < 0.05, comparing six intratumoral doses on a once-daily interval (q1dx6) (day10) or six intratumoral doses on a 3-day interval (q3dx6) (day 16), respectively, to untreated control. †, p < 0.05, comparing q1dx6 (day 16) to q3dx6 (day 16). Data shown are pooled from several experiments (n = 28 mice) in the untreated group, (n = 35) in the q1dx6 (day 10) treatment group, (n = 5) in the q1dx6 (day 16) treatment group, and (n = 9) in the q3dx3 (day 16) treatment group.
not observed when mice were depleted of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells using PC61 Ab (Fig. 2B). In contrast to NK and CD4 depletion, CD8 T cell depletion completely abrogated poly(I:C)-induced tumor resolution and delay in tumor growth. Similar to the innate effect observed in nude mice, there was a significant delay in tumor growth progression during treatment in CD8 T cell-depleted mice. However this effect was not sustained once treatment stopped, with tumors growing more rapidly after therapy in these mice.

Role of poly(I:C)-induced IFN-αβ in antitumor responses

Viral-derived TLR ligands, such as the TLR3, TLR7, and TLR9 agonists that we have used, are potent activators of systemic type I IFN responses. In tumors, type I IFNs are associated with increased CD8 T cell responses to cross-presented Ags (25). However, intratumoral delivery of TLR ligands did not result in detectable levels of either IFN-α or IFN-β by ELISA in the serum, despite significant responses after systemic (i.p.) delivery (data not shown). Therefore, we analyzed type I IFN production after local TLR ligand treatment by measuring the expression of the IFN-αβ inducible surface marker Ly6A/E on peripheral blood CD8 and CD4 T cells (23). Indeed, intratumoral delivery of poly(I:C) induced a marked up-regulation of Ly6A/E on both circulating CD4 and CD8 T cells (Fig. 3A). This was dependent on type I IFN because the response was completely blocked in mice that also received IFN-αβ-neutralizing Abs throughout treatment (Fig. 3B). Ly6A/E induction on circulating T cells was also observed after intratumoral treatment with CpG or 3M-019 ligands. In contrast, intratumoral treatment with LPS induced Ly6A/E expression on <20% of circulating CD8 T cells and not at all on CD4 T cells (Fig. 3A), despite the fact that LPS can stimulate IFN-β production (data not shown). To test whether the level of type I IFN produced was sufficient to affect the T cell phenotype in the secondary lymphatics, we examined the expression of Ly6A/E on CD8 T cells in the spleen and inguinal and axillary lymph nodes (draining and nondraining) after intratumoral poly(I:C) treatment. Ly6A/E expression was observed on the majority of CD8 T cells in the secondary lymphoid organs of poly(I:C)-treated mice (>50% Ly6A/E<sup>+</sup>) (Fig. 3C). However, the proportion of positive CD8 T cells and the level of expression was significantly higher in the nodes draining the tumor site, suggesting that the effects of type I IFN may be exerted in a local field/gradient in the tumor or draining lymph nodes.

Because type I IFNs have recently been shown to directly promote CD8 T cell responses to cross-presented Ags (26), we next examined the effect of poly(I:C) therapy on HA-specific CTL responses in the lymphatics and in the tumor. We have previously shown in the AB1-HA model that the dominant H-2K<sup>d</sup>-restricted CD8 T cell epitope in the HA neo-tumor Ag (IYSTVASSL<sub>res 518–526</sub>) is constitutively cross-presented in the tumor draining lymph nodes (17). Indeed, we observed robust cross-presentation of the HA<sub>518–526</sub> epitope in AB1-HA tumor-draining lymph nodes, as measured by the in vivo proliferation of HA<sub>518–526</sub>-specific TCR-transgenic CD8 T cells (data not shown). However, HA<sub>518–526</sub> cross-presentation was associated with a weak HA-specific in vivo CTL response (<5% killing of HA<sub>518–526</sub> peptide-pulsed targets) (Fig. 4B). No significant CTL responses were detected in the spleen or the nondraining nodes. Poly(I:C) treatment resulted in a small but significant decrease in HA<sub>518–526</sub>-specific presentation in the draining lymph nodes but a concomitant increase within the tumor itself (data not shown). However, poly(I:C) treatment resulted in a significant 5-fold increase in HA<sub>518–526</sub>-specific killing, with four animals (of 13) showing responses of >30%
Intratumoral poly(I:C) induces systemic type I IFN-induced T cell responses. A, Ly6A/E expression on peripheral blood CD8 (left) and CD4 (right) T cells from mice treated with intratumoral saline, poly(I:C), or LPS. Data shown are representative histograms from peripheral blood taken 24 h after dose six of treatment from one of three mice per treatment group. Saline (dashed line histogram), poly(I:C) (black line histogram), and LPS (gray line histogram) are represented. B, Ly6A/E expression on peripheral blood CD8 (left) and CD4 (right) T cells from mice treated with intratumoral poly(I:C) with or without systemic delivery of IFN-α-β-neutralizing sheep serum (anti-IFN-α-β). Data are representative histograms from peripheral blood taken 24 h after dose one of poly(I:C) from one of three mice per treatment group. Untreated (dashed line histogram), poly(I:C) plus normal sheep IgG serum treated (black line histogram), and poly(I:C) plus anti-IFN-α-β treated (gray line histogram) mice are represented. C, The draining lymph nodes (DLNs), nondraining lymph nodes (nDLNs), and spleen of AB1-HA-bearing mice were removed for analysis of CD8 T cell Ly6A/E expression, 24 h after a sixth and final dose of poly(I:C) (six intratumoral doses with a 1-day interval, starting day 10). Responses in untreated and saline-treated mice are shown for comparison. Data shown are mean ± SEM (n = 4). *p < 0.05, comparing responses in draining lymph nodes to nondraining lymph nodes.

FIGURE 3. Intratumoral poly(I:C) promotes CTL activity confined to the draining lymph nodes. A, Representative histograms showing unpulsed (M1) and CL4 peptide-pulsed (M2) targets recovered from naïve and CL4 TCR-transgenic recipients 18 h after transfer. B, HA-specific killing response in the draining lymph nodes, nondraining lymph nodes, and spleen 18 h after target transfer into tumor-bearing mice treated with three doses of saline or poly(I:C). Responses of individual mice from three separate experiments are shown along with group mean (horizontal scale bar) in saline-treated (n = 11) and poly(I:C)-treated (n = 13) groups.

To evaluate the impact of potentially local IFN-αβ-dependent responses on tumor resolution, AB1-HA tumor-bearing mice were treated with poly(I:C) in the presence of the polyclonal IFN-αβ-neutralizing antiserum previously used to assess Ly6A/E responses (Fig. 3B). Importantly, IFN-αβ neutralization completely inhibited poly(I:C)-driven tumor resolution, although mice still displayed a delay in tumor progression compared with untreated controls (Fig. 5A). Treatment of mice with control sheep serum had no effect on poly(I:C) treatment, with 40% of mice resolving their tumors and the remaining mice showing significant delay in tumor growth.

Poly(I:C) induced antitumor responses are confined locally

The confinement of in vivo CTL responses to the draining lymph nodes, even after poly(I:C) treatment, suggested that antitumor T cell responses were generated, and possibly acted, locally. Type I IFNs have also been shown to affect MHC class I expression levels in mice, resulting in increased CD8 T cell-dependent killing at the effector site (27). This prompted us to examine the effect of intratumoral poly(I:C) on MHC class I expression within the tumor. Indeed, injection of poly(I:C) significantly up-regulated the expression of MHC class I across the entire tumor (Fig. 5B). When expression was studied in more detail ex vivo, we observed that the increase in MHC class I expression was strongest in the CD11b+ (macrophage) fraction of the tumor (>2-fold increase), although expression in the remaining CD11b− fraction (containing tumor cells themselves) was still significantly up-regulated (Fig. 5C).

To further verify whether local changes in MHC class I expression could explain the requirement for locally delivered TLR treatment, we investigated the systemic effectiveness of intratumorally
delivered poly(I:C) in vivo in a dual tumor model (Fig. 6A). For these experiments, mice were inoculated concurrently with two tumors on the same flank (i.e., sharing the same draining lymph nodes) and treated in either one or both tumors with poly(I:C), or alternatively, with poly(I:C) in one and LPS in the other tumor. The latter scenario allowed us to induce distal inflammation in the absence of an effective antitumor type I IFN response.

In the poly(I:C)-treated tumor, we observed a significant delay in growth, and in some cases, resolution (Fig. 6B). However, consistent with a local confinement of CD8 T cell effector responses, we found that the second tumor in the same animal did not respond at all (Fig. 6B, right). This finding was also the case even if the second tumor was receiving intratumoral LPS during poly(I:C) treatment of the first tumor (Fig. 6B). This occurred despite the fact that both poly(I:C) and LPS treatments were associated with equally robust GR1⁺CD11b⁺ neutrophilic infiltrates confirmed by H&E staining (data not shown) in treated tumors (Fig. 7A). Tumor growth kinetics of adjacent tumors treated with six intratumoral doses at once-daily intervals from day 10, either singly or dually with saline, poly(I:C), or LPS. Data shown are mean ± SEM (n = 5 mice in each group) and representative of two experiments.

**FIGURE 5.** Poly(I:C)-induced tumor resolution is type I IFN-dependent and associated with increased tumor MHC class I expression. A, Survival response after intratumoral poly(I:C) treatment in the presence of anti-IFN-αβ or control sheep serum (Sh-IgG). Abs were delivered on days -1, +2, +4, with respect to poly(I:C) treatment of six doses at once-daily intervals, which commenced on day 10. Data shown are pooled from two separate experiments (n = 10 mice). *, p < 0.05, comparing poly(I:C) plus control sheep IgG to poly(I:C) plus anti-IFN-αβ treated mice. †, comparing poly(I:C) plus control sheep serum to untreated plus control sheep serum treated mice. B, Mice with day 16 tumors received three consecutive doses of poly(I:C) before sacrifice and processing of tumor section for immunohistochemistry. Representative sections at original magnification of ×20 are shown from three saline- or poly(I:C)-treated tumors stained with anti-MHC class I Ab. C, Day 16 tumors were treated as in B and processed for FACS analysis of MHC class I expression levels on CD11b⁺ and CD11b⁻ populations. Data shown are mean ± SEM (n = 3 mice). *, p < 0.05, comparing poly(I:C) treatment to saline.

**FIGURE 6.** Poly(I:C)-induced antitumor responses are confined locally. A, Mice were implanted concurrently with two adjacent AB1-HA tumors and received treatment into neither, one, or both tumors. An animal with two untreated day 18 tumors is shown (left); note the tumor closest to the hind leg (Tumor 1). An animal on day 18 in which Tumor 1 has resolved after six doses of poly(I:C), whereas Tumor 2 was left untreated (right). B, Tumor growth kinetics of adjacent tumors treated with six intratumoral doses at once-daily intervals from day 10, either singly or dually with saline, poly(I:C), or LPS. Data shown are mean ± SEM (n = 5 mice in each group) and representative of two experiments.
response was not due to exclusion of T cells by the tumor, as they already contained a small (<1% of total tumor) but significant population of tumor-infiltrating lymphocytes before treatment (Fig. 7C). Additionally, HA_{518-526}-specific TCR-transgenic CD8 T cells were clearly capable of entering AB1-HA tumors, even without treatment (data not shown).

**Pre-existing CD8 responses facilitate antitumor responses**

The fact that tumor resolution was absolutely dependent on local administration of TLR ligands and was not associated with an obvious influx of CD8 T cells, implied that poly(I:C) locally reactivated a pool of tumor-resident CD8 T cells. To examine this activity further, we depleted mice of CD8 T cells for the first 4 days of tumor implantation, thereby preventing any early antitumor responses, and allowed them to recover before commencing therapy. With this regimen, peripheral blood CD8 levels had re-covered to at least 15% of normal by day 10. Strikingly, poly(I:C) treatment was completely ineffective under these conditions (Fig. 8A). Again, a direct innate effect of poly(I:C) on tumor growth was observed and, as in the earlier experiments in which CD8 T cells were depleted during therapy (Fig. 2B), tumors appeared to grow faster once poly(I:C) treatment was halted. The same result was observed when mice received only a single dose of depleting Ab (2 days before tumor implantation) even when adoptively transferred with 4 × 10^6 CD8 T cells from spleens of normal mice 1 day before therapy (data not shown).

To further support the concept that poly(I:C) efficacy depends on the rescue of pre-existing CD8 T cells, we performed adoptive transfer experiments in athymic nude mice. Thus, we adoptively transferred 10^7 purified CD8 T cells, isolated from spleens of poly(I:C)-cured mice (>100 days posttreatment), into tumor-bearing nude mice before therapy (Fig. 8B). As before, treatment of tumors in nude mice without T cell transfer resulted in a modest, but significant delay in tumor progression. Adoptive transfer of antitumor CD8 T cells alone had no impact on tumor growth. In contrast, the transfer of CD8 T cells coupled with intratumoral poly(I:C) significantly increased survival time and even led to tumor resolution, indicating that poly(I:C) mobilized tumor-sensitized CD8 T cells. Transfer of naive CD8 T cells did not result in any added benefit over poly(I:C) treatment alone (data not shown), further supporting the requirement for pre-existing tumor-sensitized cells. Control mice that received naive CD8 T cells or splenocytes I...
day before tumor implantation and were treated with poly(I:C) 10 days later displayed a significant delay (11 days) in tumor growth compared with mice that received single treatment (data not shown).

To demonstrate that pre-existing cells were only reactivated locally, we repeated the memory CD8 T cell transfer experiments in nude mice in a dual tumor setting. Thus, the inguinal tumors were treated with poly(I:C), whereas the axillary tumors were left untreated (Fig. 9). Without T cell transfer, we found that poly(I:C) had lost all of its efficacy (Fig. 9A, left). In contrast, poly(I:C) treatment significantly delayed tumor growth in mice receiving memory CD8 T cells, but only in the treated tumor (Fig. 9A, right), even though both tumors share the same draining lymph nodes. Thus, antitumor CD8 T cells are only active when locally reactivated. This result was not due to differences in tumor accessibility, as there was no significant difference in the absolute number of infiltrating CD8 T cells in treated and untreated tumors (Fig. 9B, left), despite clear differences in tumor size (Fig. 9B, right). Nor was there any apparent difference in the ability of T cells to respond to local Ag as evidenced by equivalent CD69 expression in the tumor (Fig. 9C).

Discussion

In this study, we demonstrate that direct activation of the innate immune system within a tumor is sufficient to arrest the growth of established tumors and even promote tumor resolution. This effect was specific for those TLR ligands that mimic viral nucleic acids, specifically poly(I:C) (TLR3), 3′-0′19′- and CpG-containing oligodeoxynucleotides (TLR9) (28, 29). Tumor resolution was found to require 1) persistent local TLR signaling, 2) CD8 T cells, and 3) activation of type I IFNs. Importantly, the integration of these three factors appeared to occur locally, possibly through increases in tumor and tumor stroma MHC class I expression.

Requirement for persistent TLR signaling

Optimal antitumor responses required persistent poly(I:C) delivery. Persistent TLR stimulation may function by breaking tumor-induced CD8 T cell tolerance. In support of this possibility, Yang et al. (11) demonstrated that a persistent TLR signal was required to break tolerance in C3-HA mice (in which HA is expressed as a self Ag) and was essential for the protective efficacy of an HA-transduced DC vaccine against HA-expressing A20 lymphoma cells. In both cases, persistent TLR signaling was required to override the suppressive activity of CD4+CD25+ regulatory T cells. Notably, the same response was achieved by vaccinating with a recombinant vaccinia virus bearing HA, suggesting that viruses can provide sufficient TLR signals to overcome regulatory T cell-induced CD8 tolerance. A similar effect was recently demonstrated in a therapeutic model by Kurooka and Kaneda (30), who found that intratumoral delivery of inactivated Sendai virus particles resulted in blocking of regulatory T cell function and resolution of established CT26 colon carcinoma tumors. Our data suggests that dsRNA may provide such a signal, as the efficacy of persistent intratumoral poly(I:C) in our model was not enhanced by concurrent depletion of CD4+CD25+ regulatory T cells using PC61 Ab. Notably, tumor progression was further delayed when mice received poly(I:C) and CD4 depletion, potentially implicating a role for CD4+CD25low− regulatory cells such as IL-10-producing Tr1 cells (31). This would suggest that effective antitumor responses are regulated by a network of Tr1 cells and in keeping with the proposed synergistic activity for these cells (32).

Requirement for local CD8 T cell responses

Our data support a model in which local poly(I:C) administration mobilized the pool of tumor-resident CD8 T cells. Firstly, intratumoral injection of poly(I:C) was essential for efficacy. Secondly, poly(I:C)-driven tumor resolution was not associated with an influx of CD8 T cells into the tumor. In fact, treated and untreated tumors harbored very similar numbers of CD8 T cells with virtually identical CD69+ activation levels. Thirdly, although a poly(I:C)-induced CTL response was clearly detectable in the tumor draining lymph nodes, this response was confined to the lymph nodes draining the tumor, and there was a complete absence of systemic HA-specific effector T cells. Our finding that concurrently growing tumors responded independently to therapy, even when sharing the same draining lymph node confirms the notion that the antitumor response is not driven by the lymph nodes. Combined, our data imply that tumor resolution was mediated by the reactivation of CD8 T cells that were already present in the tumor at the time of therapy. Thus, we propose that poly(I:C) works by
reactivating existing responses rather than through priming of a de novo response. Indeed, poly(I:C) treatment failed to resolve established tumors when CD8 T cells were depleted during the first 4 days of tumor implantation, whereas adoptive transfer of tumor-sensitive but not naive CD8 T cells before therapy could confer responsiveness in nude mice.

These findings would seem to contrast with other reports (33, 34), demonstrating that local TLR ligand therapy induces expansion of systemic effectors followed by tumor infiltration and subsequent tumor growth inhibition and/or complete resolution. This observation may reflect the biology of our tumor model, in particular the observation that expanded and armed effectors appear to remain in the tumor draining lymph nodes (35). A similar phenomenon has been described for Ad-E1A-transformed tumors (36), which also regress in response to intratumoral CpG therapy (7). However, unlike our tumor model, this response is associated with a dissemination of effector T cells into the periphery and even into tumors. This lack of effector CD8 T cell dissemination in our model allowed us to specifically evaluate the contribution of pre-existing, tumor-resident CD8 responses to tumor resolution.

Our data suggest that AB1-HA tumors initially prime a CD8 T cell response, that antitumor CD8 T cells can enter the emerging tumor but that these T cells are either inactivated or prevented from acquiring full effector functions in the suppressive environment of the tumor. We now show that this pool of suboptimally activated antitumor T cells is reactivated by local TLR stimulation. In this vein, Kilinc et al. (37) demonstrated that established murine lung carcinomas were infiltrated with a population of memory-phenotype CD8+ T cells that were functionally impaired, possibly due to the presence of Foxp3+ regulatory T cells within the tumor environment. Importantly, a single dose of intratumoral IL-12 and GM-CSF in microparticles, which is sufficient to cause primary tumor resolution in this model (38), restored the function of these resident memory cells, increasing their IFN-γ and granzyme expression. Importantly, the reactivation of the resident memory/effector cells in this model was short-lived, with T cells undergoing apoptosis at 4 days posttherapy, but was followed by further CD8 T cell infiltration.

Our observation that larger AB1-HA tumors were affected by therapy but did not undergo regression would suggest that the pool of pre-existing CD8 T cells is sufficient and essential to initiate tumor resolution but is also limiting. The initial response is not sustainable without replacement of frontline effectors as tumor burden increases. We are currently investigating whether exogenous transfer of HA518–526-specific CD8 T cell after initial local TLR activation will augment and sustain regression of larger AB1-HA tumors. It should be pointed out that the merits of such a strategy have already been noted in other models, using either DC vaccines (39, 40) or adoptive cell transfer (41) to enhance systemic antitumor CD8 T cell numbers.

Tumor resolution and type I IFN
Tumor resolution induced by poly(I:C) was critically dependent on production of type I IFNs. Type I IFNs have been shown to influence antitumor responses on several levels (42). Janssen and coworkers (43) recently showed that cell-associated dsRNA, but not soluble dsRNA, enhanced antitumor immunity in a vaccine setting, through a type I IFN and DC dependent mechanism. Type I IFNs also have an important role in promoting cross-priming of CD8 T cells (44, 45), with IFN-α directly enhancing CD8 effector expansion, survival and memory transition (26, 46). This function has also been ascribed to IFN-inducing TLR3 and TLR7 ligands (25, 47), translating with varying success in promoting therapeutic tumor vaccine responses (43, 48). However, because CTL activity was confined to the tumor draining nodes and because tumor resolution was not mediated by de novo priming of CD8 T cells, our data imply that IFN-α enhanced cross-priming of naive CD8 T cells is not involved in resolution of AB1-HA tumors. Rather, the requirement for a tumor-resident CD8 T cell response in our system suggests that the action of IFN-αβ is also mediated locally at the effector site, consistent with positive antitumor effects of locally delivered IFN-α or IFN-β in human solid tumors, including mesothelioma (49–52). The responsiveness of AB1-HA tumors to local treatment correlated directly with increased MHC class I expression in these tumors. Indeed, type I IFNs and their inducers have been shown to be potent enhancers of MHC class I levels in normal and cancerous tissues, increasing their sensitivity to CTL-mediated killing (42, 53). It was recently demonstrated that up-regulation of MHC class I on pancreatic islets was a critical step in converting T cell reactivity into autoimmune mediated destruction, and that this was mediated by the release of IFN-αβ induced by virus or viral TLR ligands (27). Notably, pancreatic destruction was not induced without this IFN-α signal, even when fully armed effectors were specifically recruited into the tissue. Thus, local release of IFN-αβ induced by poly(I:C) may simply lower the threshold for killing by pre-existing CD8 T cells within a tumor.

Our dual tumor experiments were consistent with this. Adjacent untreated tumors did not display increased MHC class I expression and did not respond to therapy despite the presence of CD8 T cells and systemic exposure to IFN-αβ. This may simply reflect a local dose effect, with tumors being less sensitive to the systemic effects of IFNs. Indeed, several studies, including our own (50, 54), have noted potential antitumor benefits for high-dose systemic IFN therapy with responses correlating to increases in MHC class I expression in at least one study (55).

An alternate hypothesis suggests that type I IFNs may be necessary for reactivating existing responses, but are not sufficient with some other locally derived signal required to promote tumor resolution. Because there was a weak but significant effect in nude mice, it is possible that this response is provided by direct activation of the tumor stroma (56) or even activation of TLRs on tumor cells themselves (13, 57), although the role of the latter in tumor biology is still unclear (14, 58). Regardless of the specific mechanism involved in our study, it is clear that a greater understanding of the actions of the TLR system within the complex environment of a tumor is needed to fully harness its therapeutic potential.

Conclusions
Activation of pre-existing tumor-sensitive CD8s by local delivery of TLR adjuvants is sufficient to promote significant antitumor responses. This response was mediated by viral TLR ligand-induced type I IFN, suggesting that a nexus exists between successful antiviral and antitumor responses.

Disclosures
The authors have no financial conflict of interest.

References


