Therapeutic Activity of High-Dose Intratumoral IFN-β Requires Direct Effect on the Tumor Vasculature

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Endogenous type I IFN production after innate immune recognition of tumor cells is critical for generating natural adaptive immune responses against tumors in vivo. We recently have reported that targeting low doses of IFN-β to the tumor microenvironment using tumor-specific mAbs can facilitate antitumor immunity, which could be augmented further with PD-L1/PD-1 blockade. However, sustained high doses of type I IFNs in the tumor microenvironment, which are potently therapeutic alone, may function through distinct mechanisms. In the current report, we demonstrate that high-dose intratumoral type I IFNs indeed exerted a profound therapeutic effect in the murine B16 model, which unexpectedly did not increase T cell responses. Moreover, bone marrow chimeras revealed a role for type I IFN signaling on nonhematopoietic cells, and most of the therapeutic effect was retained in mice deficient in T, B, and NK cells. Rather, the tumor vasculature was ablated with high-dose intratumoral IFN-β, and conditional deletion of IFN-α/βR in Tie2-positive vascular endothelial cells eliminated most of the antitumor activity. Therefore, the major component of the antitumor activity of sustained high doses of type I IFNs occurs through a direct antiangiogenic effect. Our data help resolve conditions under which distinct antitumor mechanisms of type I IFNs are operational in vivo. The Journal of Immunology, 2014, 193: 4254–4260.

It has been nearly two decades since type I IFNs were developed as a cancer therapeutic (1). Clinical evaluation ultimately led to the Food and Drug Administration approval of systemically administered IFN-α2b as a postsurgical adjuvant treatment for patients with melanoma (2). We have recently uncovered a critical role for endogenous type I IFN production in the innate immune recognition of tumors, which serves as a bridge to a spontaneous adaptive T cell response (3, 4). Mechanistic experiments revealed that endogenous IFN-β is produced by CD11c+ dendritic cells (DCs) in response to tumor presence, which in turn acts on the CD8α+ DC lineage to promote cross-priming of tumor Ag–specific CD8+ T cells in vivo (5). This type I IFN-dependent innate tumor recognition pathway appears crucial for directing the initial adaptive immune response against several murine tumors but is not always sufficient to enable tumor regression, largely because of the upregulation of immune inhibitory mechanisms that also come into play (6–8). As one strategy to boost this type I IFN production in the tumor microenvironment, we generated conjugates of IFN-β coupled to tumor-targeting mAbs. Systemic administration of these agents could deliver transient low levels of IFN-β to tumor sites, which supported tumor control in a T cell–dependent fashion (9). Conditional type I IFN gene-targeted mice revealed an essential role for type I IFN signaling on CD11c+ host DCs with that approach, consistent with the mechanism by which endogenous type I IFNs promote antitumor immunity.

Whether local provision of high sustained concentrations of IFN-β in the tumor microenvironment would induce tumor eradication by the same or alternative mechanisms is currently unknown. Indeed, multiple other working mechanisms have been proposed for the therapeutic effect of type I IFNs in the cancer context. Type I IFNs in some instances can directly inhibit tumor cell growth (10–12) and can activate NK cells (13). In addition, type I IFNs have been shown to exert an antiangiogenic effect, which could indirectly slow tumor growth (14). In vitro data suggest that this effect may be via suppression of angiogenic factor production or via direct effects on vascular endothelial cells (15–17). A recent report has suggested that, in the complete absence of IFN-β, neutrophils are massively attracted to the tumor site, improving the vasculature and thus enhancing cancer progression (18). IFN-β gene transfer can inhibit in vivo tumor growth in association with lower vessel densities (19–21). However, despite these multiple proposed candidate mechanisms of antitumor efficacy, the requisite target cell(s) that must be signaled by type I IFNs have not been defined, under conditions when these distinct mechanisms of action have been inferred.

On the basis of these ideas, we investigated whether provision of high levels of IFN-β within the tumor microenvironment would promote tumor rejection via the same immune-potentiating mechanism we recently observed to be operational with low levels, using IFN-β–mAb conjugates. We focused on alternative strategies for intratumoral delivery, either transfection to overexpress IFN-β by melanoma cells or direct intratumoral injection of recombinant IFN-β. Indeed, a very potent therapeutic effect of intratumoral type I IFNs against B16 melanoma was observed in vivo. Surprisingly however, the adaptive immune response appeared dispensable for the major component of this antitumor activity. Genetic experiments confirmed that the major mechanism of therapeutic efficacy was via nonhematopoietic cells, with a re-
quirement for IFN-α/βR expression on Tie2-expressing cells, correlating with a potent antiangiogenic effect. Our results suggest that the mechanism of tumor control with sustained high doses of intratumoral IFN-β is distinct from that mediated by transient low doses of IFN-β.

Materials and Methods

Cells
The B16.F10 murine melanoma cell line (originally obtained from ATCC, catalog no. CRL-6475) was cultured in DMEM supplemented with 10% FCS, MOPS, t-arginine, t-glutamine, folic acid, l-asparagine, 2-ME, and antibiotics. The B16-F10-SIY-dsRED (B16.SIY) was designed to express the model Ag SIY, which is presented to CD8+ T cells by Kb (22, 23).

Retroviral vectors and virus production
Retroviral vectors pMX–IFN-α2–IRES–GFP and pMX–IFN-β–IRES–GFP were generated by cloning the respective commercially synthesized murine cDNA sequences (GenScript) into the empty pMX-IRES-GFP vector. Generation of retroviral supernatants and retroviral transductions were performed as described previously (24).

Mice
All animals were used according to protocols approved by the Institutional Animal Use Committee of the University of Chicago and maintained in pathogen-free conditions in a barrier facility at the University of Chicago. Mice on a C57BL/6 background were ordered from Taconic Farms or The Jackson Laboratory. C57BL/6 IFN-α/βR−/− (IFNAR1−/−) and IFN-α/βR−/− mice were a kind gift of Dr. A. Chong (University of Chicago). Bone marrow chimeras were generated by i.v. injections of 10 × 10^6 bone marrow cells into lethally irradiated hosts and an engraftment period of >14 wk.

Tumor challenge
For tumor growth experiments, mice were injected s.c. on the flank with 1 × 10^6–6 × 10^6 cells in 100 μl. In pilot experiments, we confirmed that injection of this range of different numbers of IFN-β–overexpressing B16 cells in wild-type mice showed nearly complete tumor regression after initial growth. Tumor growth was followed by measuring its diameter in two directions generally twice a week. Mice with ulcerating tumors or initial growth. Tumor growth was followed by measuring its diameter in two dimensions (Supplemental Fig. 1D). Indeed, s.c. implantation of this cell line in syngeneic C57BL/6 mice led to complete tumor regression after a short initial establishment (Fig. 1C). During a follow-up period of >4 mo, no tumor recurrence was observed in mice that eliminated the tumor, either at the site of inoculation or as distant metastases.

To determine whether the therapeutic effect required IFN-β signaling on host cells, the IFN-β–secreting B16.F10 tumor cells were implanted into mice lacking the IFN-α/βR. The tumors grew progressively in these mice, illustrating that the IFN-β effect on tumor elimination was dependent on signaling via host cells rather than a direct effect on the tumor cells (Fig. 1D).

Results

Intratumoral IFN-β induces host-mediated elimination of B16 melanoma cells in vivo
To investigate whether intratumoral expression of high doses of type I IFNs could promote improved immune-mediated tumor control in vivo, we used the murine melanoma cell line B16-F10 as a model and introduced the IFN-β gene in these cells by retroviral transduction together with the model Ag SIY to facilitate monitoring of in vivo T cell responses (Fig. 1A). Transduced cells secreted IFN-β at high levels in the culture supernatant, which also upregulated expression of MHC class I molecules (Fig. 1B, Supplemental Fig. 1A). Indeed, s.c. implantation of this cell line in syngeneic C57BL/6 mice led to complete tumor regression after a short initial establishment (Fig. 1C). During a follow-up period of >4 mo, no tumor recurrence was observed in mice that eliminated the tumor, either at the site of inoculation or as distant metastases.

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Abs, FACS, and ELISPOT
All Abs for FACS staining (specified in text) were purchased from BD Biosciences or eBioscience. PE-coupled H-2Kb tetramers were used to stain SIY (SIYRYYGL)– or OVA (SIINFEKL)–specific T cells derived from spleens or tumor-draining lymph nodes (Proimmune). Flow cytometry data were acquired using a FACS Canto (BD Biosciences) and analyzed using FlowJo (TreeStar). High-speed cell sorting was performed using a FACS Aria (BD Biosciences). The number of cytokine-producing T cells was measured by IFN-γ ELISPOT, as described (BD Pharmingen) (3, 25). Cytokine concentrations were determined using standard sandwich ELISAs (R&D Systems) in 100 μl supernatant of an 18-h 200-μl culture of 20,000 cells.

Histologic analysis of the microvasculature
For angiogenesis staining, mice were i.v. injected with 100 μl biotinylated tomato lectin (1 μg/μl in PBS, Vector Labs). Five minutes after injection, mice were sacrificed and tumors were dissected. Cryofrozen tumor samples were stained using AF594-conjugated streptavidin (Molecular Probes). Images were taken on an Axiosver200 microscope and quantitatively analyzed using ImageJ and statistically analyzed using GraphPad Prism software.
Established B16-F10 can be effectively treated with local IFN-β

For therapeutic consideration, it was of interest to determine whether pre-established tumors could be treated with a type I IFN–based strategy. We first explored whether a bystander effect was tenable, by coimplanting several ratios of IFN-β–expressing B16-F10 with empty vector–expressing B16-F10. Remarkably, even when only 10% of IFN-β–secreting cells were present within the tumor cell mix, the overall tumors regressed. Thus, intratumoral IFN-β can indeed have a potent bystander effect (Fig. 2A). To further examine potential effects on pre-established tumors, wild-type B16-F10 tumors were allowed to grow for 8 d. At that time, IFN-β–expressing B16-F10 cells were injected into the tumor microenvironment. In this case as well, a strong antitumor effect was observed, with complete tumor elimination being achieved in 40% of mice (Fig. 2B). As a more clinically relevant approach, we also investigated injection of a high concentration of recombinant IFN-β into the tumor microenvironment, which also showed a potent antitumor effect and completely eliminated established B16 tumors in >40% of mice (Fig. 2C). These results together illustrate that local application of type I IFNs can effectively cause rejection of established melanoma tumors in vivo.

The major component of the IFN-β–mediated antitumor effect is independent of adaptive immunity

Our working hypothesis was that the therapeutic effect of these effective high doses of intratumoral type I IFNs would occur through enhancement of host immunity. To determine whether antitumor T cell responses were augmented by IFN-β, we used engineered expression of the model Ag SIY in the B16 melanoma cells (Fig. 1A). No increase in the frequency of SIY-specific CD8+ T cells was observed in tumor-draining lymph nodes of mice receiving IFN-β–secreting cells, as assessed by IFN-γ ELISPOT (Fig. 3A). Notably, a modest increase was observed in the spleen, but this was reflected by a higher background production of IFN-γ. Furthermore, the frequency of SIY/Kb tetramer–positive cells was not increased in the spleens or the tumor-draining lymph nodes of mice implanted with IFN-β–expressing tumor cells (Supplemental Fig. 2). Thus, these data suggest that the potent therapeutic effect of high doses of intratumoral IFN-β might not be mediated through augmentation of host T cell responses.

The fact that introduction of high doses of IFN-β in the tumor microenvironment did not substantially improve T cell priming led us to consider whether the IFN-β–mediated elimination of B16 tumors might be independent of T cells. To investigate this possibility in vivo, we implanted IFN-β–secreting tumors into Rag1–/– mice, which are deficient in T and B cells. In fact, although IFN-β–expressing B16 cells were not completely eliminated, they underwent substantial regression and were potently controlled for >2 mo, arguing for only a minor contribution of the adaptive immune system (Fig. 3B). Although complete regression of IFN-β–secreting tumors was not always observed even in wild-type mice, this observation also may support the notion that induction of an adaptive immune response is not a major component of the therapeutic effect of type I IFNs in the tumor microenvironment. Inasmuch as NK cells are also capable of responding to type I IFNs and contributing to tumor control, we also investigated tumor growth in wild-type or Rag1–/– mice depleted of NK cells starting prior to implantation of B16 cells. In both mouse strains, the tumors were controlled similarly, compared with treatment with an isotype Ab, indicating that NK cells are not required for control of IFN-β–secreting B16 tumors (data not shown). We also examined therapeutic effects in Rag2–/– or γc–/– mice that are deficient in T, B, and NK cells, and in this case, as well, most of the antitumor effect of IFN-β was preserved (Fig. 3C). Collectively, these data indicate that classical immune effector cells are not required for the major component of the antitumor effect of intratumoral IFN-β.

IFN-γ and inducible NO synthase are not required for IFN-β–mediated tumor control in vivo

In further pursuit of a potential mechanism by which intratumoral IFN-β might effectively mediate tumor control in the absence of adaptive immune cells, we examined a potential requirement for...
host factors that can be produced by innate immune cells, namely, IFN-γ and inducible NO synthase (iNOS). To test this notion, IFN-β–expressing tumors were implanted into IFN-γ−/− and iNOS−/− mice. However, in these mice, as well, the local expression of IFN-β induced regression of B16-F10 cells, showing that the effector proteins IFN-γ and iNOS are not involved in high-dose IFN-β–mediated tumor control (Supplemental Fig. 3A, 3B).

**IFN-β signaling on nonhematopoietic host cells is crucial for the antitumor effect**

Because interrogation of the classical immune effector mechanisms did not reveal the major mechanism for IFN-β–mediated tumor elimination, we wondered whether this effect was caused by hematopoietic-derived cells at all. To investigate this possibility, we generated chimeric mice by transfer of wild-type or IFN-α/βR−/− bone marrow into lethally irradiated wild-type and IFN-α/βR−/− host mice and implanted IFN-β–expressing B16-F10 after 14 wk. Surprisingly, the tumors were still controlled in mice with IFN-α/βR−/− bone marrow–derived cells, whereas they grew progressively in mice specifically lacking IFN-α/βR−/− in the non–bone marrow–derived compartment (Fig. 4A, 4B). Thus, bone marrow–derived cells can be excluded as the main recipients of type I IFN signals in this model. Moreover, intratumoral IFN-β must act on non–bone marrow–derived cells in order for the majority of the therapeutic effect to be observed. Because we were enriching this cytokine directly within the tumor site, these results point to a potential effect on sessile stromal cells within the tumor microenvironment.

Angiogenesis is directly inhibited in the presence of intratumoral IFN-β

Because non–bone marrow–derived cells within the tumor microenvironment were directly targeted by IFN-β for the antitumor effect to occur, we turned to an analysis of angiogenesis. We therefore examined microvessel density within tumors, comparing empty vector– and IFN-β–expressing B16-F10 tumors shortly after implantation, prior to elimination of the latter. In fact, a marked diminution of blood vessel density was observed in the IFN-β–secreting tumors, illustrating that the therapeutic effect of IFN-β is associated with impaired angiogenesis (Fig. 5A). To investigate whether the IFN-β effect was directly occurring on endothelial cells, we interbred conditional IFN-α/βR−/− mice (IFN-α/βRβdRfl/fl) with Tie2-Cre transgenic mice (26). Although the Tie2 promoter can also be expressed in hematopoietic cells in addition to endothelial cells, we had already demonstrated that type I IFN signaling within the hematopoietic compartment was largely dispensable. Indeed, implantation of the IFN-β–expressing B16 cells into IFN-α/βRβdRfl×Tie2-Cre transgenic mice resulted in loss of tumor control, arguing for the requirement of a direct effect of IFN-β on vascular endothelial cells (Fig. 5B, 5C). Moreover, the decreased functional vasculature of IFN-β–expressing tumors was partially restored in IFN-α/βRβdRfl×Tie2-Cre transgenic mice, supporting the role of endothelial cell–expressed IFN-α/βR in mediating the antiangiogenic effect of IFN-β (Fig. 5D, 5E). Taken together, these results demonstrate that the major therapeutic effect of high doses of intratumoral type I IFNs occurs through signals on nonhematopoietic Tie2+ cells, which is associated with profound inhibition of angiogenesis.

**Discussion**

Our results support the idea that intratumoral type I IFNs have therapeutic applicability for cancer. Although systemic administration of IFN-α2 has been used clinically for multiple solid tumors, including melanoma and kidney cancer (27–30), local intratumoral administration can achieve much higher cytokine concentrations and directly affect the relevant target cell populations. Our previous work had indicated that transient, low doses of IFN-β delivered to the tumor microenvironment using targeting mAb functioned by acting on host DCS to boost antitumor T cell responses. Our data presented in this article argue that sustained high doses of intratumoral IFN-β have therapeutic activity via direct action on endothelial cells or on another uncharacterized nonhematopoietic Tie2+ cell population. An antiangiogenic effect of type I IFNs has been suspected in the clinical treatment of kidney cancer, which led to combination studies with avastin and sorafenib for synergy in this pathway (29–33). Taken together, these observations suggest that the dose and/or duration of type I IFN exposure in the tumor microenvironment likely dictate the dominant mechanism of the antitumor effect in vivo.
Intratumoral therapies are challenging to consider for clinical development, but several efforts have revived this area of investigation. Recent positive clinical trial data utilizing intratumoral injection of an oncolytic virus encoding GM-CSF in patients with melanoma have generated renewed interest in such approaches clinically (34). Levy and colleagues (35) have developed a clinical

**FIGURE 4.** The IFN-β therapeutic effect depends on type I IFN signaling on nonhematopoietic cells. Chimeras were generated by i.v. transfer of $10^6$ bone marrow cells derived from wild-type (WT) or IFN-α/βR$^{-/-}$ C57BL/6 mice into lethally irradiated wild-type or IFN-α/βR$^{-/-}$ C57BL/6 mice ($n = 4$ for WT→IFN-α/βR$^{-/-}$ and IFN-α/βR$^{-/-}$→WT; $n = 3$ for WT→WT and IFN-α/βR$^{-/-}$→IFN-α/βR$^{-/-}$). At 14 wk later, the mice were inoculated s.c. with $2 \times 10^6$ IFN-β-secreting B16 cells and followed up over time for tumor development. (A) Depicted are the mean tumor diameter and SEM. $p < 0.001$ for WT→IFN-α/βR$^{-/-}$ versus WT→WT; $p < 0.05$ for IFN-α/βR$^{-/-}$→WT versus WT→WT. (B) The Kaplan–Meier curves for the different groups are shown. The Mantel–Cox test with Bonferroni correction was performed for (B): $p < 0.025$ for WT→IFN-α/βR$^{-/-}$ versus WT→WT; $p = ns$ for IFN-α/βR$^{-/-}$→WT versus WT→WT.

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**FIGURE 5.** IFN-β signals via IFN-α/βR on Tie2$^+$ cells, linking the antitumor effect to an angiogenesis defect. (A) Mice were implanted s.c. with $2 \times 10^6$ B16-empty vector or B16–IFN-β cells ($n = 5$ per group). After 5 d, mice were injected i.v. with biotinylated tomato lectin and sacrificed. Dissected tumors were stained with AF594-coupled streptavidin (green) for blood vessels and DAPI (red) for the nuclei. Representative images are shown. Wild-type (WT), IFN-α/βRfl/fl, or IFN-α/βRfl/fl×Tie2-Cre mice were implanted s.c. with $10^6$ B16 empty vector or IFN-β-secreting cells ($n = 5$ for WT and IFN-α/βRfl/fl×Tie2-Cre mice injected with IFN-β cells and $n = 4$ for the other groups). Mice were followed up for tumor growth over time. The mean diameter with SEM (B) and the Kaplan–Meier survival curve (C) are shown. (B) $p < 0.001$ for WT versus IFN-α/βRfl/fl×Tie2-Cre at day 17. The Mantel–Cox test was used for (C): $p < 0.01$ for WT versus IFN-α/βRfl/fl×Tie2-Cre. After >20 d, tumors from WT and IFN-α/βRfl/fl×Tie2-Cre mice injected with B16 empty vector or IFN-β were analyzed as described in (A). Representative images were taken (D) (DAPI, blue; AF594-coupled streptavidin, red) and quantified (E) as the percentage of blood vessel area of the total stained area. Scale bar in (A) and (D), 100 μm. Statistical significance was determined by one-way ANOVA with Bonferroni post hoc test. **$p < 0.01$, ***$p < 0.001$. 

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**FIGURE**

- **A** Mice
- **B** Bone Marrow
- **C** Mice
- **D** B16 Empty Vector
- **E** B16 IFN-β
strategy involving radiation of one site of disease in lymphoma patients, combined with intratumoral administration of innate immune activators such as CpG oligonucleotides. This approach is designed to enhance DC-mediated cross-presentation of tumor-associated Ags derived from dying tumor cells in the treated lesion (36). Intratumoral delivery of type I IFNs also could be considered for therapeutic testing in patients. This use could be investigated either by direct injection or via systemic administration of a tumor-targeting mAb carrying IFN-β as a payload (9). The recent Food and Drug Administration approval of the anti-Her2 Ab–drug conjugate TDM1 has revived interest in linking payloads to mAbs as a therapeutic strategy.

Type I IFNs show complex biological effects on T cell responses in vivo. Low levels of transiently produced type I IFNs are associated with productive T cell priming, including activation of T cells in the tumor context (3, 4). However, high and/or persistent levels of type I IFNs have been associated with strong effector T cell induction but poor generation of immunological memory (37, 38). In the chronic lymphocytic choriomeningitis virus model, blockade of the type I IFN receptor has been reported to restore functional immunity in vivo (37). Thus, the dose, schedule, and timing of intratumoral delivery of IFN-β have been considered carefully when the goal is augmentation of antitumor immune responses.

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Disclosures

The authors have no financial conflicts of interest.

References


SUPPLEMENTAL FIGURE 1.  

A, The B16.SIY IFN-β (line) and empty vector (filled) transduced cells were analyzed by FACS for cell surface expression of MHC class I.  
B, C57BL/6 mice were implanted with $10^6$ B16 IFN-β, IFN-α2 or empty vector cells (n=5 per group).  Error bars represent the standard error of the mean.  p<0.001 for either B16 IFN-β or IFN-α2 versus empty vector group at day 21.
SUPPLEMENTAL FIGURE 2. Single cells derived as described in Fig. 3A were stained with tetramers and assessed by FACS. Depicted are the frequencies of SIY-specific or control OVA-specific CD8+ T cells (gated on CD8+CD4–B220–). Error bars represent the standard error of the mean. p=ns comparing splenic populations; p<0.05 comparing the lymph node derived populations (student t-test).
SUPPLEMENTAL FIGURE 3. Host IFN-γ and iNOS are not necessary for IFN-β-mediated tumor control. Wildtype, IFN-γ−/− (A) and iNOS−/− (B) mice were s.c. implanted with 10⁶ B16.SIY cells expressing empty vector or secreting IFN-β (n=5 per group). Mean tumor diameter and standard error of the mean are plotted. p<0.001 at day 24 for the comparison of B16.SIY empty vector with IFN-β in IFN-γ−/− as well as iNOS−/− mice.