Targeting the Tumor Microenvironment with Interferon-β Bridges Innate and Adaptive Immune Responses

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SUMMARY

Antibodies (Abs) that preferentially target oncogenic receptors have been increasingly used for cancer therapy, but tumors often acquire intrinsic Ab resistance after prolonged and costly treatment. Herein we armed the Ab with IFNβ and observed that it is more potent than the first generation of Ab for controlling Ab-resistant tumors. This strategy controls Ab resistance by rebridging suppressed innate and adaptive immunity in the tumor microenvironment. Mechanistically, Ab-IFNβ therapy primarily and directly targets intratumoral dendritic cells, which reactivate CTL by increasing antigen cross-presentation within the tumor microenvironment. Additionally, blocking PD-L1, which is induced by Ab-IFNβ treatment, overcomes treatment-acquired resistance and completely eradicates established tumors. This study establishes a next-generation Ab-based immunotherapy that targets and eradicates established Ab-resistant tumors.

INTRODUCTION

Antibodies (Abs) targeting oncogenic receptors can directly inhibit tumor cell growth, providing an effective treatment option for cancer therapy (Hynes and Lane, 2005; Li et al., 2005). The major therapeutic effect of such Ab therapies is attributed to direct cytotoxicity to tumor cells by affecting oncogenic signal transduction. More recently, however, Fc receptor (FcR) signaling on immune cells has also been recognized to be important for an Ab-mediated antitumor effect in vivo (Clynes et al., 2000; Musolino et al., 2008). We and others have shown that Ab-mediated tumor regression also depends on adaptive immunity in Ab-sensitive models (Abès et al., 2010; Mortenson et al.,

2013; Park et al., 2010; Stagg et al., 2011; Yang et al., 2013). In Ab-sensitive tumor models, immune-activating molecules released during Ab-dependent cell-mediated cytotoxicity or by stressed tumor cells can effectively activate antigen-presenting cells (APCs), enhancing their ability to cross-prime and induce cytotoxic T lymphocyte (CTL) responses. In recent exciting clinical trials, Abs have been used to block coinhibitory signals on T cells, including CTL antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed cell death ligand 1 (PD-L1), and these trials have demonstrated that reversing T cell suppression is another important way to improve the therapeutic effects against tumors (Brahmer et al., 2012; Sharma et al., 2011; Topalian et al., 2012; Weber, 2007). These results

Significance

Ab resistance is a major challenge for Ab-based cancer therapies. Current strategies to overcome Ab resistance focus on targeting intrinsic resistance and improving direct killing of tumor cells. Because of the heterogeneity of tumor cells and their microenvironment, these direct killing strategies cannot target all the tumor cells; eventually, Ab resistance will develop. We reveal herein that type I IFNs are essential and sufficient to bridge innate and adaptive immune responses for Ab-induced tumor regression. We have designed a next generation of Ab-based biologics (Ab-IFNβ fusion) to control Ab resistance by revitalizing innate and adaptive immune cells inside the tumor. This study open up several avenues for optimizing targeted immune therapy that may eventually eradicate Ab-resistant tumor cells.

raise the possibility that the effect of targeted Ab cancer therapy can be further enhanced by selected immunotherapy.

Both primary and acquired resistance are major challenges for targeted therapy (Bardelli and Siena, 2010; Cobleigh et al., 1999). Most studies have focused on the intrinsic resistance of oncogenic signaling, such as mutations within targeted oncogenes or in genes related to oncogenic pathways that contribute to Ab resistance (Bardelli and Siena, 2010; Misale et al., 2012; Sharma et al., 2007; Wheeler et al., 2008; Yonesaka et al., 2011). Currently, the primary strategy to overcome Ab resistance in the host is to develop drugs targeting mutated oncogenes or oncogenic pathway-related genes inside tumor cells (Bostrom et al., 2009; Fayad et al., 2013; Hurvitz et al., 2013; Krop et al., 2012; Yoon et al., 2011). On the basis of increasing intrinsic resistance after treatment with the first generation of antioncogenic Abs, we propose a tumor-extrinsic strategy to bypass intrinsic Ab resistance by reactivating both innate and adaptive immune cells inside the tumor. To achieve this goal, potent immune molecules that can elicit antitumor responses need to be identified.

Recently, an increase in type I interferons (IFNs) was found to correlate favorably with clinical immune responses against cancer (Fuertes et al., 2011). Furthermore, type I IFN signaling is essential to initiating antitumor T cell responses during spontaneous tumor rejection or various additional antitumor therapies (Burnette et al., 2011; Diamond et al., 2011; Fuertes et al., 2011; Stagg et al., 2011). These data suggest that type I IFNs are essential to initiating specific T cell responses against tumor cells. Type I IFNs have also been reported to activate memory T cells during viral infection (Kohlmeier et al., 2010). Thus far, however, systemic delivery of type I IFNs has been used cautiously in the clinic for cancer therapy because of its limited potency and severe side effects (Trinchieri, 2010). Indeed, the action of this cytokine is poorly understood, because it may function as either an immune-activating or immune-suppressing reagent in different disease models (González-Navajas et al., 2012; Teijaro et al., 2013; Wilson et al., 2013). Timing, duration, and dosing of type I IFNs could be critical for determining its function as an immune-activating or immune-suppressing reagent.

Anti-CD20 coupled with IFNα had a better antitumor effect than anti-CD20 Ab alone by direct and potent killing of type I IFNα receptor (IFNAR)-positive lymphoma (Xuan et al., 2010). The data published by Xuan et al. demonstrate that IFNAR expression on tumor cells is important for the antitumor effect in an Ab-sensitive tumor model. However, the role of IFNAR on host cells has not been well-investigated. In this study, we linked IFNβ to antioncogenic receptor Abs that directly target various carcinomas to test whether it could overcome Ab resistance. We aimed to investigate the detailed mechanism of how Ab-IFNβ changes the immune-suppressive tumor microenvironment to induce antitumor immune responses and design efficient strategies to optimize targeted immune therapy.

RESULTS

Type I IFNs Are Required for Effective Tumor Response to Antibody Therapy In Vivo

Type I IFNs have emerged as potential key danger signals that initiate antitumor T cell responses during spontaneous tumor rejection or after initiation of various antitumor therapies (Burnette et al., 2011; Diamond et al., 2011; Fuertes et al., 2011; Stagg et al., 2011). We hypothesize that antioncogenic receptor Abs induce type I IFN production in tumor tissues to bridge innate and adaptive immunity. To test whether the sensitivity of tumors to antioncogenic receptor Abs in our models correlates with type I IFN levels after treatment, we generated two different tumor cell lines: Ab-sensitive and Ab-resistant. TUBO cells derived from Her2/neu-transgenic (Her2/neu-Tg) mice, in which neu is the dominant signal for cell growth, served as the anti-neu Ab-sensitive tumor cell line (Rovero et al., 2000). The epidermal growth factor receptor (EGFR)-transfected B16 cells, in which EGFR is unable to deliver growth signals, served as the tumor cell line that is completely resistant to anti-EGFR treatment. We treated mice bearing these tumors with their respective Abs and evaluated type I IFN production inside the tumor. We found that IFNα5 (Figure 1A) and IFNβ (data not shown) production increased in the Ab-sensitive tumor model, but not in the Ab-resistant tumor model, suggesting that increased type I IFN production was caused by Ab-induced oncogenic receptor blockade and stress. Also, consistent with these results, we treated EGFR-transfected TUBO cells with anti-EGFR, in which the transfected EGFR can deliver signals and partially contribute to cell growth. The EGFR-transfected TUBO cells served as the tumor cell line that is partially resistant to anti-EGFR Ab treatment, and we observed a similar reduction of type I IFN production (Figure S1 available online). To test whether type I IFNs are required for the Ab-mediated antitumor effect in vivo, we treated mice with anti-IFNAR-blocking Ab during anti-neu treatment in the Ab-sensitive TUBO tumor model. We found that intratumorally blocking type I IFN signaling impaired the therapeutic effect of anti-neu Ab (Figure 1B), suggesting that type I IFNs might be the cytokines essential for Ab-mediated tumor regression. It also raises the possibility that lower levels of type I IFNs may limit immune responses in host bearing Ab-resistant tumors. To further test whether delivering additional type I IFNs directly into tumors is sufficient to control Ab-resistant tumor growth, tumor-bearing mice were treated with adenovirus encoding IFNβ (adenovirus IFNβ). As shown in Figure 1C, adenovirus-IFNβ treatment by itself was able to sufficiently control Ab-resistant tumor growth. Taken together, these data argue that directing type I IFNs into tumors may be sufficient to overcome tumor immune evasion or antibody resistance.

Targeted Delivery of IFNβ Enhances the Antibody-Mediated Therapeutic Effect

Our data suggest that targeting tumors with type I IFNs has a potentially therapeutic effect, even for Ab-resistant tumors. There are two issues to consider when using type I IFNs directly for cancer therapy: (1) Local delivery of type I IFNs might not be feasible for many patients, and (2) in the clinic, systemic type I IFN administration has dose-dependent side effects, and its limited ability to shrink tumors may be associated with failure to achieve a high-enough concentration within tumor tissues. To better increase type I IFN concentrations selectively inside tumors, we generated an anti-EGFR-IFNβ Ab fusion protein to deliver IFNβ directly into EGFR-expressing tumor tissues (Figure S2A). We first checked that the functions of both
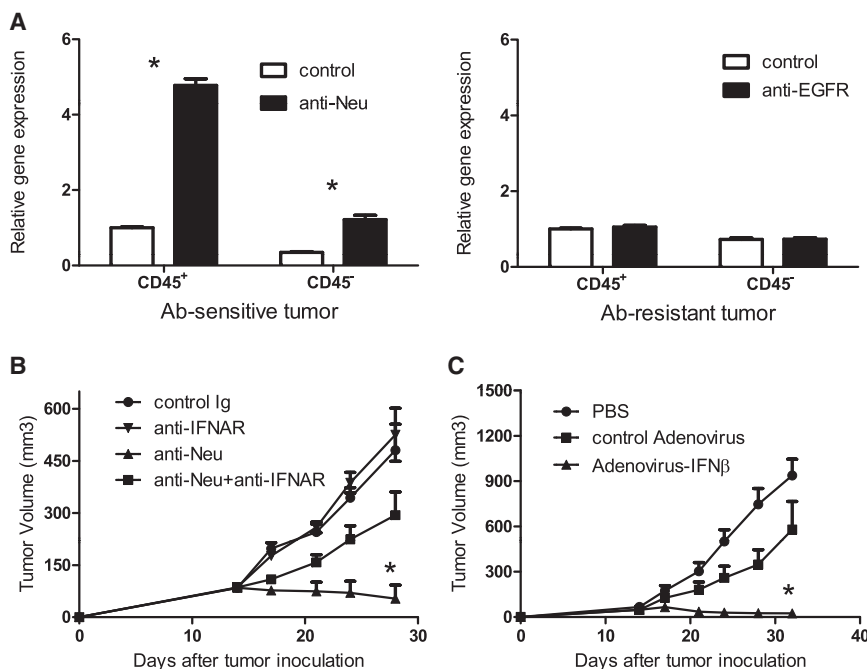
Figure 1. Type I IFNs Are Induced and Necessary during Ab-Mediated Tumor Regression

(A) Wild-Type (WT) BALB/c mice (n = 4/group) were injected subcutaneously with 5×10^5 TUBO cells, then 100 μg of anti-*neu* antibody (Ab) (left graph) or control immunoglobulin G (IgG) was administered on day 14. WT B6 mice (n = 4/group) were injected subcutaneously with 7×10^5 B16-EGFR cells, then 100 μg of anti-epidermal growth factor receptor (anti-EGFR) (right graph) or control IgG was administered on day 14. Four days after the treatment, the tumor was digested and cells were sorted into CD45+ and CD45− populations. Quantitative real-time PCR was performed to detect the expression of mRNA levels of mouse interferon α5. Mean ± SD values are shown. *p < 0.05. Error bars indicate SD.

(B) WT BALB/c mice (n = 5/group) were injected subcutaneously with 5×10^5 TUBO-EGFR cells, then 100 μg of anti-*neu* was administered on days 14 and 21. Anti-type I interferon-α receptor (anti-IFNAR) or control immunoglobulin (Ig) (200 μg of each) was intratumorally administered on the same days. The growth of the tumor was measured and compared twice weekly. Mean ± SEM values are shown. p < 0.05. Error bars indicate SEM.

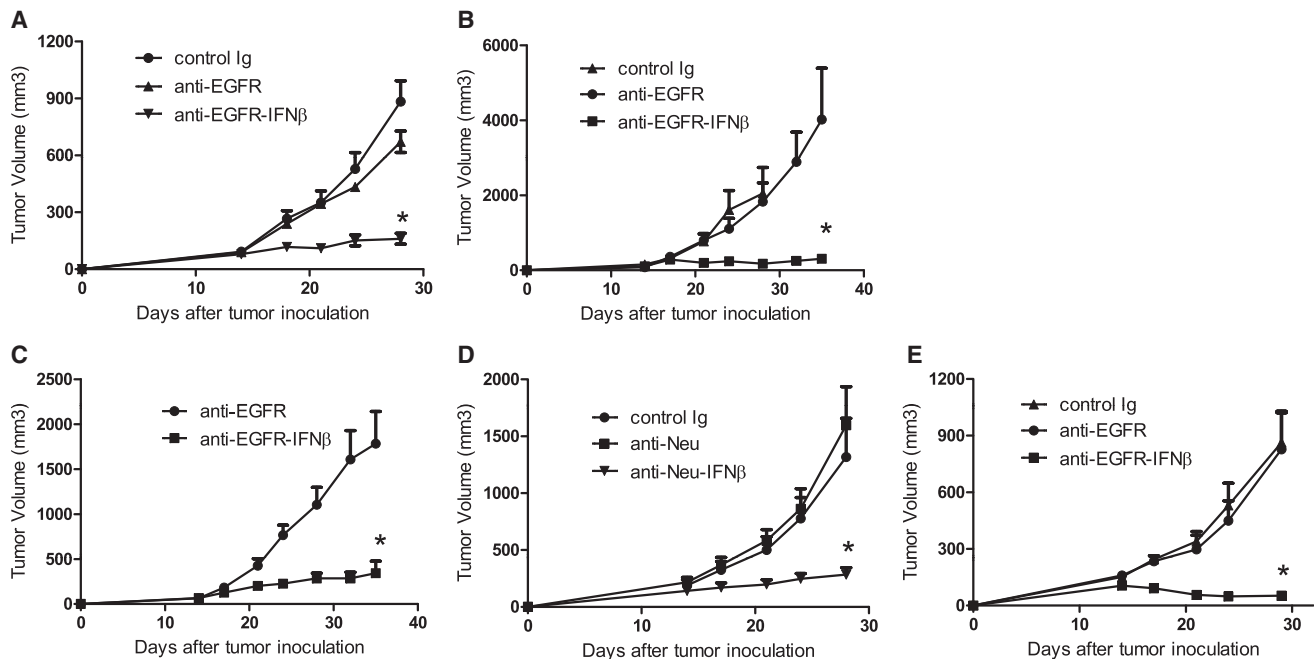(C) WT B6 mice (n = 5/group) were injected subcutaneously with 5×10^5 B16-EGFR cells, then 2×10^9 adenovirus-interferon β (adenovirus-IFNβ) or control virus was intratumorally administrated on days 14 and 21. The growth of the tumor was measured and compared twice weekly. Mean ± SEM values are shown. *p < 0.05 compared with control group. Error bars indicate SEM. One representative experiment of a total of three is depicted. See also Figure S1.

anti-EGFR and IFNβ remained intact in this fusion protein. Both functions were well-maintained in this fusion protein, as evidenced by the protein's ability to bind to EGFR+ cells (Figure S2B) and activate the IFNAR signaling pathway (data not shown). We then examined whether anti-EGFR-IFNβ could specifically deliver IFNβ to the EGFR+ tumor site in vivo. Indeed, the concentration of anti-EGFR-IFNβ in tumor tissues remained high for at least 7 days (Figure S2C), whereas it dramatically decreased in other tissues less than 1 week after initial injection. Furthermore, we investigated the side effects of the anti-EGFR-IFNβ by measuring serum cytokines, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels (biomarkers for tissue injury). Among the cytokines we checked, including tumor necrosis factor, interleukin-12 (IL-12), IFNγ, monocyte chemoattractant protein-1 (MCP-1), IL-6, and IL-10, we observed slightly increased levels of IFNγ and MCP-1 expression at 6 hr and 1 day after injection (Figure S2D). We observed no increase in ALT and AST levels above baseline after treatment (data not shown).

We treated mice bearing established EGFR+ tumors with our next-generation anti EGFR-IFNβ fusion protein or the first-generation anti-EGFR Ab, cetuximab, in order to compare the potency of the fusion protein against its predecessor. Impressively, we observed that the therapeutic effect of anti-EGFR-IFNβ was much more effective than anti-EGFR Ab alone at lower doses and shorter durations in the partially resistant TUBO-EGFR tumor model (Figure 2A). Similarly, elevated antitumor effects were observed in an intramammary fat pad tumor injection model (data not shown). To further test whether Ab-IFNβ could control tumor growth in a completely resistant tumor model,

we tested the efficacy of anti-EGFR-IFNβ in B16-EGFR, an Ab-resistant model. As predicted, though anti-EGFR treatment alone did not inhibit B16-EGFR tumor growth in vivo, anti-EGFR-IFNβ treatment was again able to greatly control tumor growth (Figure 2B). *KRAS* mutations have been reported to be key factors contributing to anti-EGFR resistance in many patients bearing EGFR+ tumors (Misale et al., 2012). To test whether anti-EGFR-IFNβ is effective in a *KRAS* mutation-induced, Ab-resistant tumor model, we treated a *KRAS*-mutated H460 human tumor with anti-EGFR-IFNβ in our previously established adaptive immune-reconstituted *Rag1*−/− mice (Lee et al., 2009; Yang et al., 2013). We adoptively transferred 2 million unpurified lymph node (LN) cells from ovalbumin-specific class I-restricted T cell (OT-I)-Tg mice, which consisted of about 3%–5% non-OT-I-CD8 T cells of the total CD8 T cells, into *Rag1*−/− mice bearing established human H460 tumors. Therefore, only a few hundred T cells will react to human antigens (Ags), which is similar to the number of tumor-reactive T cells observed in human patients. In this model, the presence of 95% of OT-I cells was used to prevent rapid homeostasis of extremely low numbers of CD8+ T cells in *Rag1*−/− mice. Antitumor T cell responses can be initiated for tumor regression if proper activation is triggered by targeted treatment in this tumor model (Lee et al., 2009; Yang et al., 2013). Although murine IFNβ cannot suppress human tumor cells in vitro, anti-EGFR murine IFNβ showed superior therapeutic effects compared to anti-EGFR Ab alone in vivo (Figure 2C). To test whether anti-EGFR-IFNβ could control tumor metastasis, we injected B16-EGFR intravenously into wild-type (WT) mice in order to mimic tumor metastasis. We found that anti-EGFR-IFNβ could control

Figure 2. IFNβ Dramatically Enhances the Antitumor Effect of Antibody

(A) WT BALB/c mice (n = 5/group) were injected subcutaneously with 5×10^5 TUBO-EGFR cells and treated with 25 μg of anti-EGFR, anti-EGFR-IFNβ, or control antibody (Ab) on days 14, 18, and 22. The tumor growth was measured and compared twice weekly.

(B) WT B6 mice (n = 5/group) were injected subcutaneously with 7×10^5 B16-EGFR and treated with 25 μg of anti-EGFR, anti-EGFR-IFNβ or control Ab on days 14, 18, and 22. The tumor growth was measured and compared twice weekly.

(C) $Rag1^{-/-}$ mice (n = 5/group) were injected subcutaneously with 3×10^6 H460 cells, and 2×10^6 ovalbumin-specific class I-restricted T cell (OT-I) LN cells were adoptively transferred on day 13. Anti-EGFR-IFNβ or control Ab (25 μg each) was administered on days 14, 18, and 22. The growth of the tumor was measured and compared twice weekly.

(D) $neu^{OT-I/OT-II}$-transgenic ($neu^{OT-I/OT-II}$-Tg) female mice (n = 5/group) were injected subcutaneously with 1×10^6 NOP23 and treated with 25 μg of anti-neu, anti-neu-IFNβ or control Ab on days 14, 18, and 22. The tumor growth was measured and compared twice weekly.

(E) EGFR-Tg mice (n = 5/group) were injected subcutaneously with 5×10^5 B16-EGFR and treated with 25 μg of anti-EGFR, anti-EGFR-IFNβ or control Ab on days 14, 18, and 22. The tumor growth was measured and compared twice weekly.

*p < 0.05 compared with control group. Mean ± SEM values are shown. Error bars indicate SEM. One representative experiment of a total of three is depicted. See also Figure S2 and Table S1.

metastatic tumors better than anti-EGFR alone and also prolonged the survival of the mice (data not shown).
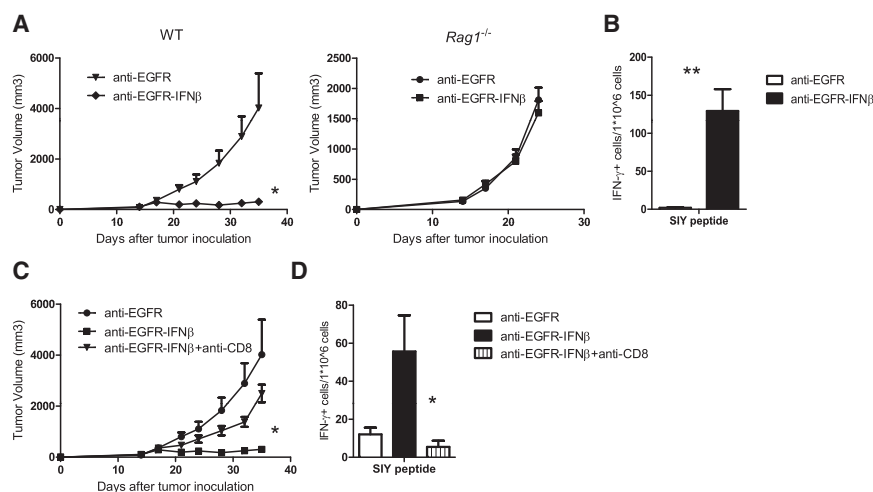
Antitumor immunity mediated by anti-neu Ab treatment is reduced in neu-Tg mice, as high neu expression (as self- and tumor-associated Ags) in all mammary glands tolerizes host immune cells during early life due to the nature of transgene expression. The $neu^{OT-I/OT-II}$-Tg mice model, in which oncogenic rat neu is highly expressed in both mammary tumors and normal mammary glands, is an important model for monitoring the T cell response during spontaneous tumor development and adoptive transfer cancer therapy (Wall et al., 2007; Yang et al., 2009). NOP23 is one of the neu^+ mammary tumor cell lines generated from $neu^{OT-I/OT-II}$-Tg mice (Yang et al., 2009). In this study, we used NOP23 tumor-bearing $neu^{OT-I/OT-II}$-Tg mice as an neu-tolerized host model. Impressively, anti-neu-IFNβ more profoundly inhibited tumor growth than anti-neu Ab alone, even in a tolerized model (Figure 2D). To test whether anti-EGFR-IFNβ could induce similar tumor regression in an EGFR-tolerized host, we crossed EGFR-Tg mice in a mixed background (Politi et al., 2006) to B6 background for ten generations to allow the growth of syngeneic B16-EGFR tumors. We

treated B16-EGFR-bearing EGFR-Tg mice with anti-EGFR-IFNβ. Consistent with anti-neu-IFNβ, anti-EGFR-IFNβ showed a better antitumor effect than anti-EGFR alone (Figure 2E). We also observed a similar increased antitumor effect by arming another anti-EGFR Ab clone (cetuximab, C225) with IFNβ (data not shown). Collectively, these data suggest that Ab-IFNβ fusion protein therapy at low doses and for short durations is superior to first-generation Ab therapy for controlling tumors, even in Ab-resistant tumor models and in tolerized hosts (Table S1).

Therapeutic Effect of Anti-EGFR-IFNβ Fusion Protein Depends on Adaptive Immunity

Because a previous study demonstrated that type I IFNs can directly and potently induce apoptosis of tumor cells for tumor regression (Xuan et al., 2010), we speculated that the anti-EGFR-IFNβ should show similar antitumor effects in $Rag1^{-/-}$ mice compared with WT mice when the major mechanism is the direct killing of tumor cells. However, anti-EGFR-IFNβ was surprisingly unable to inhibit tumor growth in these immune-compromised $Rag1^{-/-}$ mice (Figure 3A). Thus, these

Figure 3. Anti-EGFR-IFNβ Can Induce Anti-Tumor-Specific Cytotoxic T lymphocyte Responses that Are Responsible for Tumor Regression

(A) Wild-Type (WT) and *Rag1*$^{-/-}$ B6 mice (n = 5/group) were injected subcutaneously with 5×10^5 B16-EGFR-SIY cells and treated with 25 μg of anti-EGFR-IFNβ or control Ab on days 14, 18, and 22. The tumor growth was monitored twice weekly. EGFR, epidermal growth factor receptor; IFN, interferon.

(B) Seven days after last treatment, draining lymph node (dLN) cells were collected and an IFNγ ELISPOT assay was performed with SIY peptide restimulation.

(C) WT B6 mice (n = 5/group) were injected subcutaneously with 5×10^5 B16-EGFR-SIY and treated with 25 μg of anti-EGFR-IFNβ or control Ab on days 14, 18, and 22. A CD8-depleting antibody (200 μg/mouse) was administered on the same day as anti-EGFR-IFNβ. The tumor growth was measured and compared twice weekly.

(D) Seven days after the last treatment, dLN cells were collected and IFNγ ELISPOT assay was performed with SIY peptide restimulation.

*p < 0.05 and **p < 0.01 compared with control group. Mean ± SEM values are shown. Error bars indicate SEM. One representative experiment of a total of three is depicted. See also Figure S3.

data support the hypothesis that the anti-EGFR-IFNβ-mediated therapeutic effect requires adaptive immunity, not direct killing of tumor cells.

CD8$^+$ T lymphocytes are the major cell population involved in controlling the growth of many tumors. To determine whether they are also involved in the anti-EGFR-IFNβ-mediated anti-tumor effect, we tracked the antitumor T cell response during the priming phase. We established a B16-EGFR-SIY tumor cell line in which the SIY peptide (SIYRYYGL) was linked to EGFR molecule. This SIY peptide serves as a surrogate marker that can be specifically recognized by endogenous or 2C CD8$^+$ T cells from Tg mice. After anti-EGFR-IFNβ treatment, draining lymph node (dLN) lymphocytes were isolated from tumor-bearing mice and stimulated with SIY peptide, and IFNγ production was measured as an effector function readout of activated T cells. As shown in Figure 3B, anti-EGFR-IFNβ treatment increased IFNγ production from tumor Ag-specific T cells compared with anti-EGFR treatment alone. To address whether CD8$^+$ cells are essential for the therapeutic effect of anti-EGFR-IFNβ, we administered a CD8-depleting Ab during anti-EGFR-IFNβ treatment in B16-EGFR-bearing WT B6 mice and measured tumor growth. CD8$^+$ cell depletion eliminated the therapeutic effect of anti-EGFR-IFNβ (Figure 3C), suggesting that adaptive immune responses are required for controlling tumor growth. Consistent with this finding, we found that the T cell response ex vivo dramatically decreased after anti-CD8 depletion (Figure 3D). The depletion of other cells, including natural killer (NK) and B cells, did not affect the antitumor effect of anti-EGFR-IFNβ (Figure S3). Taken together, these data suggest that anti-EGFR-IFNβ treatment can increase T cell priming for tumor regression.

Therapeutic Effect of Anti-EGFR-IFNβ Requires IFNAR Expression on Host Bone Marrow-Derived Cells

IFNAR is widely expressed on almost all cell types, thus both normal and tumor cells are potential targets of anti-EGFR-IFNβ. The efficacy of anti-CD20-IFNα fusion protein has been reported to require IFNAR expression on tumor cells (Xuan et al., 2010). We therefore hypothesized that host cell-expressed IFNAR may not be required for the therapeutic effect of anti-EGFR-IFNβ. To this end, we compared the antitumor effect in tumor-bearing *Ifnar1*$^{-/-}$ mice, which lack IFNAR expression on host cells but not on tumor cells. To our surprise, the therapeutic effect of anti-EGFR-IFNβ was abolished in *Ifnar1*$^{-/-}$ mice (Figure 4A). To study whether the IFNAR signaling on host cells is associated with adaptive immune responses, we checked the T cell responses after treatment and found that there was no increased CTL response in *Ifnar1*$^{-/-}$ mice (Figure 4B). These results suggest that the therapeutic effect and related CTL responses by anti-EGFR-IFNβ require IFNAR-mediated activation in host cells, but not in tumor cells. Because all host tissues express IFNAR, we constructed IFNAR bone marrow chimeric (BMC) mice to further dissect whether IFNAR-expressing, bone marrow-derived cells or stromal cells of the host are required for the antitumor effect. We found that IFNAR expression on bone marrow-derived cells is required because the antitumor effect of anti-EGFR-IFNβ was dramatically impaired in *Ifnar1*$^{-/-}$ BMC-reconstituted mice (Figure 4C). These data suggest that anti-EGFR-IFNβ mediated its antitumor effect, not by directly inhibiting tumor cell growth but by activating host bone marrow-derived cells to change the tumor microenvironment.

Elevated Dendritic Cell Cross-Presentation Contributes to the Antitumor Effect of Anti-EGFR-IFNβ

Given that CD8$^+$ cells are essential for the antitumor effect of anti-EGFR-IFNβ treatment, we further explored the mechanisms underlying how anti-EGFR-IFNβ enhance CTL responses. We observed a dramatic increase in IFNγ-producing CD8$^+$ cells after anti-EGFR-IFNβ treatment, even without exogenous SIY peptide stimulation, suggesting that cross-presentation of APCs is increased (Figure 5A). Because cross-presentation was proposed to be the dominant priming mechanism to activate CTLs for antitumor immunity (Huang et al., 1994;
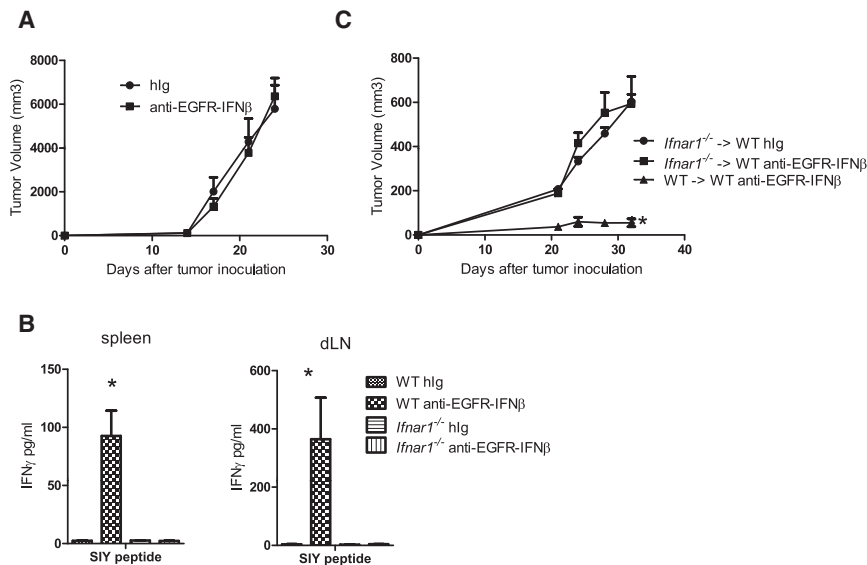
Figure 4. The Antitumor Effect of Anti-EGFR-IFNβ Requires IFNAR Expression on Host Bone Marrow-Derived Cells

(A) WT and *Ifnar1*$^{-/-}$ B6 mice (n = 5/group) were injected subcutaneously with 5×10^5 B16-EGFR-SIY cells and treated with 25 μg of anti-EGFR-IFNβ or control Ab on days 14, 18, and 22. Tumor growth was monitored twice weekly.

(B) Seven days after the last treatment, draining lymph node (dLN) and spleen cells were collected and IFNγ produced in supernatant was detected by cytometric bead array assay.

(C) Thirty days after indicated bone marrow chimera reconstitution, mice were injected subcutaneously with 7×10^5 B16-EGFR-SIY cells and treated with 25 μg of anti-EGFR-IFNβ or control immunoglobulin three times. Tumor growth was measured and compared twice weekly.

*p < 0.05 compared with control group. Mean ± SEM values are shown. Error bars indicate SEM. One of two representative experiments is depicted.

Kurts et al., 2010), we speculated that an increase in cross-priming by fusion protein might be essential to restore dendritic cell (DC) function to reactivate CTLs inside tumors and dLNs. To address the possibility that anti-EGFR-IFNβ could increase the cross-presentation of APCs, we used an Ag-specific system to track the priming and activation of tumor Ag-specific T cells. DCs from the dLNs of anti-EGFR-IFNβ-treated B16-EGFR-SIY-bearing mice were assessed for their ability to enhance the specific antitumor CTL response by incubating them with SIY-reactive 2C T cells in an ex vivo assay. Indeed, DCs from anti-EGFR-IFNβ-treated mice induced more IFNγ production from 2C T cells (~33 times) compared to anti-EGFR treatment, even without restimulation by exogenous SIY peptide (Figure 5B). Cumulatively, the data suggest that anti-EGFR-IFNβ-activated DCs enhance CD8$^+$ T cell activation through an increased cross-priming function. Furthermore, these results indicate that the IFNβ component of the fusion protein is responsible for activating the DC cross-presentation pathway, as anti-EGFR Ab alone did not induce strong DC activation.

Additionally, exogenous antigenic peptide (SIY) was added to the culture to illicit an increased SIY-specific 2C T cell response for better analysis of the overall direct priming function of DCs. DCs from the anti-EGFR-IFNβ-treated mice induced approximately two times more IFNγ production from the 2C T cells than anti-EGFR Ab treatment alone upon exogenous SIY peptide restimulation (Figure 5B). These results suggest that DCs from the dLNs of anti-EGFR-IFNβ-treated hosts are likely to be more activated than DCs from mice treated with anti-EGFR Ab alone. Indeed, these DCs have high expression of activation markers, including CD86, as assessed by flow cytometry (Figure 5C). To further discern whether anti-EGFR-IFNβ-induced DC activation is responsible for the enhanced T cell activation, tumor-bearing CD11c-diphtheria toxin receptor (CD11c-DTR) BMC mice were treated with diphtheria toxin to deplete DCs during anti-EGFR-IFNβ treatment. We found that the anti-EGFR-IFNβ-mediated therapeutic effect is impaired when DCs are not present (Figure 5D). Taken

together, these data suggest that improved CD8$^+$ CTL priming and function by increased DC cross-presentation might be the major mechanism underlying the therapeutic effect of anti-EGFR-IFNβ.

Anti-EGFR-IFNβ Directly Targets DCs to Reverse the Tolerized Tumor Microenvironment

Our data reveal that increased cross-priming is important for the improved antitumor effect of anti-EGFR-IFNβ treatment. However, the IFNAR-expressing cells directly responsible for the therapeutic effect of anti-EGFR-IFNβ need to be identified. To address this issue, *Ifnar1*$^{flox/flox}$ mice were bred to various Cre-Tg mice. When IFNAR was selectively absent in CD11c$^+$ cells in CD11c-Cre$^+$*Ifnar1*$^{flox/flox}$ mice, the antitumor effect of anti-EGFR-IFNβ disappeared (Figure 6A), suggesting that direct DC activation by anti-EGFR-IFNβ may be the major contributor to its therapeutic effect. When IFNAR was selectively absent in T cells in CD4-Cre$^+$*Ifnar1*$^{flox/flox}$ mice, the antitumor effect of anti-EGFR-IFNβ was slightly impaired (Figure 6B), suggesting that direct targeting of IFNAR on T cells may further activate T cells for an improved antitumor effect. To further test this idea, we evaluated the effect of anti-EGFR-IFNβ stimulation in both DC and T cell activation in an in vitro assay. Indeed, we found that anti-EGFR-IFNβ increased the activation of both DCs and T cells (Figure S5). These data collectively suggest that direct activation of IFNAR-expressing DCs plays a major role in the anti-EGFR-IFNβ-mediated therapeutic effect, which can be further enhanced by engaging IFNARs expressed on T cells.

Antagonizing Anti-EGFR-IFNβ-Induced PD-L1 Expression Achieves Tumor-free Outcome

Although anti-EGFR-IFNβ fusion achieved a more effective antitumor effect than anti-EGFR Ab alone, residual tumor eventually relapsed. We wondered whether anti-EGFR-IFNβ treatment might induce inhibitory molecule expression on tumor cells to prevent tissue-damaging immune responses,
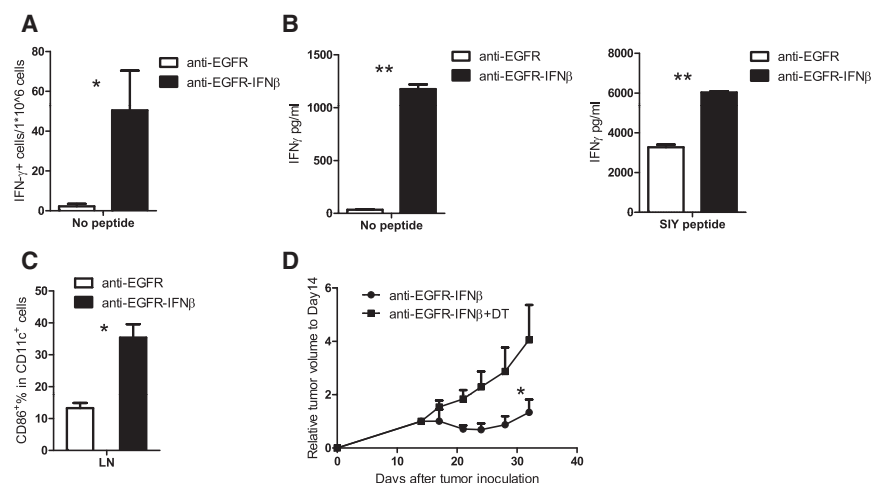
Figure 5. Anti-EGFR-IFNβ Restores the Cross-Presentation Ability of DCs

(A) WT B6 mice (n = 5/group) were injected subcutaneously with 5 × 10⁵ B16-EGFR-SIY cells and treated with 25 μg of anti-EGFR-IFNβ or control Ab on days 14 and 18. Seven days after the last treatment, draining lymph node (dLN) cells were collected and an interferon-γ (IFNγ) ELISPOT assay was performed without SIY peptide restimulation. EGFR, epidermal growth factor receptor.

(B) WT B6 mice (n = 5/group) were injected subcutaneously with 5 × 10⁵ B16-EGFR-SIY cells and treated with 25 μg of anti-EGFR-IFNβ or control Ab on days 14 and 17. Four days after the last treatment, dendritic cells (DCs) from dLNs were purified by CD11c⁺ selection and incubated with purified naive 2C T cells with or without SIY peptide restimulation. IFNγ production was measured 2 days later.

(C) DC activation markers were measured by flow cytometry.

(D) About 40 days after the indicated bone marrow chimera reconstitution, mice were injected subcutaneously with 5 × 10⁵ B16-EGFR-SIY cells and treated with 25 μg of anti-EGFR-IFNβ or control immunoglobulin on days 14, 18, and 22. Diphtheria toxin or PBS was administrated on the same day as treatment. Tumor growth was measured and compared twice weekly.

*p < 0.05 and **p < 0.01 compared with control group. Mean ± SEM values are shown. Error bars indicate SEM. One representative experiment of two or three is depicted. See also Figure S4.

ultimately weakening the antitumor effect over time. Although IFNγ is known to be the major inducer of PD-L1 on tumor cells (Blank et al., 2004), it raises the possibility that type I IFNs have a similar effect. We accordingly evaluated PD-L1 expression after anti-EGFR-IFNβ treatment and clearly observed increased PD-L1 expression on tumor cells, both in vivo and in vitro (Figures 7A and 7B). The data confirm that type I IFNs could induce the expression of this inhibitory molecule directly. To test whether blocking PD-L1 expression could potentiate IFN-mediated tumor rejection, we next combined anti-PD-L1 with anti-EGFR-IFNβ treatment and observed that anti-PD-L1 blockade further enhanced the long-term efficacy of anti-EGFR-IFNβ; indeed, mice remained tumor-free for at least 60 days after treatment (Figure 7C). To test whether such treatment might have a prolonged protective effect in preventing the growth of dormant residual cancer, mice without detectable tumors were rechallenged with a lethal tumor dose. Impressively, all of them were completely resistant to tumor rechallenge (data not shown). Moreover, blocking the PD-L1 pathway by anti-PD-L1 treatment further enhanced the specific antitumor T cell response (Figure 7D). To test whether IFN-induced PD-L1 is a dominant mechanism for immune evasion, we tested whether blockade of two major inhibitory pathways on T cells, CTLA-4 and B- and T-lymphocyte attenuator (BTLA), could act synergistically with anti-EGFR-IFNβ treatment. In contrast to anti-PD-L1 Ab, we did not observe a similar synergistic effect when we combined anti-EGFR-IFNβ with anti-CTLA-4 or anti-BTLA Ab (Figure S6). Collectively, our data indicate that antagonizing anti-EGFR-IFNβ-induced PD-L1 expression can maximize the antitumor effect of anti-EGFR-IFNβ and bring about an impressive tumor-free outcome, even for Ab-resistant tumors. This combination-based strategy will likely increase the overall response and cure rates of Ab-resistant hosts, even in hosts that initially fail to respond to anti-PD-1 or anti-PD-L1 Abs (Figure 8).

DISCUSSION

A first generation of Abs to oncogenic receptors has been increasingly used, but intrinsic and extrinsic resistance have become major clinical challenges. In our present study, we have revealed that type I IFNs play an essential and sufficient role to bridge innate and adaptive antitumor immune responses during Ab-based antitumor therapy. We have developed Ab-IFNβ as a representative, clinically relevant next generation of Ab-based therapy that is superior to first-generation Ab therapy, even in Ab-resistant tumor cells. Our study demonstrates the following essential and sufficient role of type I IFNs for Ab-mediated tumor control. (1) Type I IFN production is elevated after Ab-based antitumor treatment. Blocking type I IFN signaling impairs Ab-mediated tumor regression. (2) Targeted delivery of IFNβ inside the tumor site by Ab-IFNβ fusion protein greatly amplifies the therapeutic effect of Abs. (3) Such antitumor effects depend on DCs and CTLs. (4) Ab-IFNβ increases DC cross-presentation and antitumor CTLs by directly targeting IFNAR on DCs, but not on tumor cells or T cells (Figure 8).

Most current strategies to improve the therapeutic effect of Abs from a tumor-intrinsic angle focus on how to increase tumor cell cytotoxicity by Ab and cytotoxic drug conjugates (ADCs) (Fayad et al., 2013; Hurvitz et al., 2013; Krop et al., 2012). Similar to the first generation of Abs, host resistance can develop after prolonged ADC treatment. Indeed, many patients still undergo relapse or develop metastasis despite initial regression by the potent cytotoxic effects of ADCs. We propose that this resistance develops because ADC relies on directly killing specific tumor cells and thus cannot target all tumor cells, causing some to be selected or to acquire the ability to escape direct killing. The next generation of Ab-based treatment we describe herein, however, can overcome these types of resistance by revitalizing innate and adaptive immune cells inside the tumor and generating specific T cell
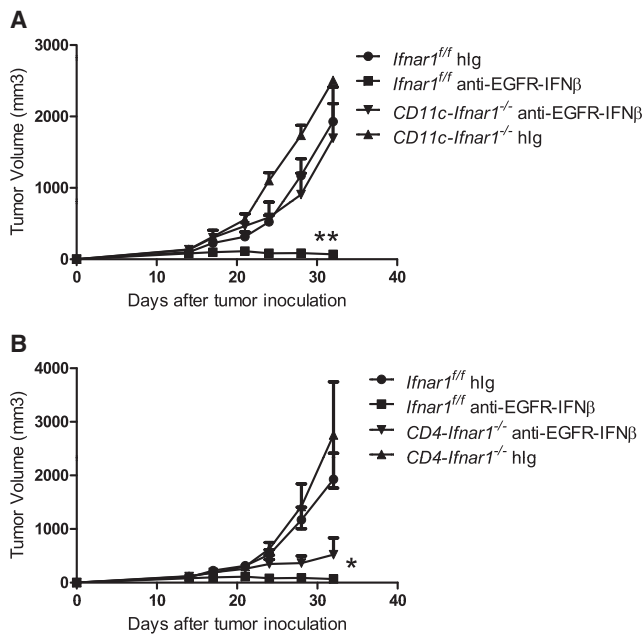
Figure 6. Dendritic Cells Are the Major Cell Type Directly Responding to Anti-EGFR-IFNβ Treatment

(A) $Ifnar1^{flox/flox}$ and CD11c-Cre $Ifnar1^{flox/flox}$ mice (n = 5/group) were injected subcutaneously with 5×10^5 B16-EGFR-SIY cells and treated with 25 μg of anti-EGFR-IFNβ or control Ab on days 14, 18, and 22. Tumor growth was measured and compared twice weekly.

(B) $Ifnar1^{flox/flox}$ and CD4-Cre $Ifnar1^{flox/flox}$ mice (n = 5/group) were injected subcutaneously with 5×10^5 B16-EGFR-SIY cells and treated with 25 μg of anti-EGFR-IFNβ or control Ab on days 14, 18, and 22. Tumor growth was measured and compared twice weekly.

*p < 0.05 and **p < 0.01 compared with control group. Mean ± SEM values are shown. Error bars indicate SEM. One representative experiment of two is depicted. See also Figure S5.

responses that target multiple shared tumor-mutated Ags. Thus, use of this strategy might be able to eradicate residual tumor cells that Abs cannot directly target. Our study further supports the argument that improving the effectiveness of antitumor effector and memory T cell responses is important for preventing relapse.

Type I IFNs have multiple potential effects on tumor growth, including inhibiting proliferation, inhibiting angiogenesis, activating innate cells, bridging innate and adaptive immunity, and directly activating adaptive immune responses. The reported role of type I IFNs on tumor cells and immune cells is complex and sometimes controversial. In the pioneer study by Xuan et al. (2010), use of anti-CD20-IFNα clearly showed that type I IFNs can have direct cytotoxic effects in lymphoma. Indeed, lymphomas, including human lymphoma, are very sensitive to type I IFN-mediated direct killing, not only in vitro but also in vivo. Unexpectedly, we have shown that our Ab-IFNβ can control various carcinomas by the following different modes of action. (1) $Rag1^{-/-}$ mice bearing carcinomas failed to respond to Ab-IFNβ, suggesting the major role of adaptive immune cells. (2) CD8-depleted mice showed early relapse, suggesting an essential role of CTLs. (3) IFNAR+ carcinomas in $Ifnar1^{-/-}$ mice failed to respond to Ab-IFNβ, suggesting that host IFNAR

signaling is essential. (4) We have further pinpointed that IFNARs on DCs is essential for tumor regression. Since most carcinoma cell lines are less sensitive to type I IFNs compared with lymphoma cells, Ab-IFNβ cannot kill them directly. Therefore, the two studies, ours and that of Xuan et al., are complementary and cover two different modes of action for IFN-mediated tumor regression, depending on the sensitivity of the targeted tumor to type I IFNs.

Endogenous type I IFNs have been shown to be required for rejection of highly immunogenic tumor cells, which cannot grow in WT mice (Diamond et al., 2011; Fuertes et al., 2011). Those two studies also showed that type I IFNs are required for only the first 6 days after tumor inoculation before the tumor is established. Once the tumor is established after the first 6 days, the role of endogenous type I IFNs is diminished. Complementarily, our study shows that endogenous type I IFNs can still regain their essential role after blockade of oncogenic addiction on established tumors that subsequently triggers innate and adaptive immune responses. It is worthwhile to mention that the therapeutic effect of Ab-IFNβ in our study was dependent on DCs, but not on CD8α+ DCs as shown in Diamond et al.'s study (Diamond et al., 2011), because our Ab-IFNβ was effective in $Batf3^{-/-}$ mice (Figure S4), which might imply different mechanisms of enhancing the efficacy of Ab-based therapeutics.

It is currently unclear whether the more effective strategy to reactivate the antitumor T cell response within the tumor is to target the activation of T cells or APCs. We recently observed that, though single-chain variable fragments (neu)-IL-2 and -IL-15 could expand T cells, even inside tumor tissues, these T cells then failed to suppress tumor growth (data not shown). Thus, directly targeting T cells without increasing APC function may not initiate tumor-specific immune responses and instead may nonselectively amplify all preexisting T cell responses. Investigators in recent clinical trials who tested Abs that block coinhibitory signals in T cells (CTLA-4, PD-1, and PD-L1) have demonstrated that reversing T cell suppression is important for effective cancer immunotherapy, as it impressively controlled tumor growth in 15%–25% of cancer patients who failed to respond to conventional treatment (Brahmer et al., 2012; Sharma et al., 2011; Topalian et al., 2012; Weber, 2007). However, because these anti-inhibitory receptor Abs target all T cells within the body rather than targeting T cells within tumor tissues directly, they may cause unwanted tissue damage or even autoimmune disease. Our current study demonstrates that selective combination of Ab-IFNβ and anti-PD-L1 can have a synergistic effect on tumor regression, raising interest in developing other combination therapies in the future.

Cross-presentation is the dominant priming mechanism to activate CTL responses in antitumor immunity (Huang et al., 1994; Kurts et al., 2010). Because DCs are the most important APCs for cross-presentation (Steinman, 2012), targeting DCs with Ab-IFNβ confers several advantages. First, Ab-IFNβ does not directly kill tumor cells, which reduces side effects from Ab-IFNβ targeting normal cells outside tumor tissues. If DCs in normal tissues do happen to become activated by this immunotherapy, they cannot activate Ag-specific T cells due to the lack of mutated neoantigens and preexisting Ag-specific T cells. Second, Ab-IFNβ mobilizes DCs to present a variety of
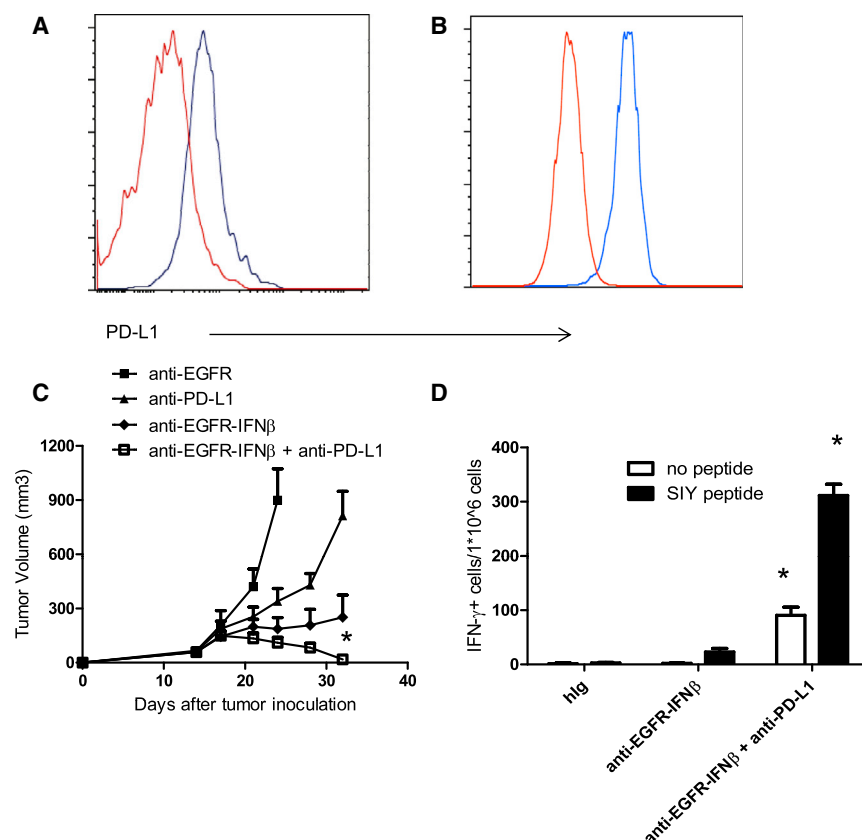
Figure 7. Antagonizing PD-L1 Expression Induced by Anti-EGFR-IFNβ Achieves Tumor-free Outcome

(A) WT B6 mice were injected subcutaneously with 5×10^5 B16-EGFR-SIY cells and treated with 25 μg of anti-EGFR-IFNβ or control Ab on day 14. Two days later, tumor cells were collected and programmed cell death ligand 1 (PD-L1) expression was analyzed by flow cytometry. Red line indicates control immunoglobulin (Ig)-treated group, and blue line indicates anti-EGFR-IFNβ-treated group.

(B) B16-EGFR-SIY cells and treated with 0.02 μg/ml of anti-EGFR-IFNβ during in vitro cell culture. One day later, tumor cells were collected and PD-L1 expression was analyzed by flow cytometry. Red line indicates control Ig-stimulated cells and blue line indicates anti-EGFR-IFNβ-stimulated cells.

(C) WT B6 mice (n = 5/group) were injected subcutaneously with 5×10^5 B16-EGFR-SIY cells and treated with 25 μg of anti-EGFR-IFNβ or control Ab on days 14 and 18. A PD-L1-blocking Ab (400 μg/mouse) was administered on the same day as anti-EGFR-IFNβ. Tumor growth was measured and compared twice weekly.

(D) Fourteen days after the last treatment, splenocytes were collected and an IFNγ ELISPOT assay was performed.

*p < 0.05 compared with control group. Mean ± SEM values are shown. Error bars indicate SEM. One representative experiment of three is depicted. See also Figure S6.

tumor-derived Ags to T cells, initiating T cell responses against multiple mutated neo-tumor Ags from all tumor cells, even those not originally targeted by Ab therapy. This will ultimately help prevent the appearance of resistant clones. Third, Ab-IFNβ therapy is a rather short-term and low-dose treatment, unlike the prolonged high-dose treatment with Abs alone, and should have lower toxicity than Ab-cytotoxic drug conjugates.

Overall, this study has several important implications for the cancer immunotherapy field. First, it establishes a way to create the next generation of Ab-based treatment, such as the Ab-IFNβ fusion protein, which elicits the adaptive arm of the immune response to deal more effectively with Ab resistance and relapse. Enhancing the CTL response can then in turn kill more tumor cells to create a positive feedback loop. Second, our study provides strong evidence that type I IFNs, which link innate and adaptive antitumor immunity, are key players in Ab-mediated tumor regression and therefore provide an important target for cancer immunotherapy. Third, this study reveals that DCs are the major tolerized cell type in tumors, implying that they play a major role in determining the immunosuppressive tumor microenvironment. Therefore, targeting DCs will be another important strategy to improve the efficacy of cancer immunotherapy. Fourth, we found that blocking inhibitory PD-L1 upregulated by Ab-IFNβ treatment further enhanced the antitumor effect, which puts forth the concept that antagonizing immunotherapy-induced adaptive resistance will maximize the therapeutic effect of immune therapy and could guide future clinical treatment. Collectively, the strategies used in this study open

up several avenues for optimizing targeted immune therapy that may have great impact in antitumor drug discovery and clinical cancer therapy.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6J and BALB/c mice were purchased from Harlan. $Rag1^{-/-}$, 2C CD8$^+$ T cell receptor (TCR)-Tg, CD11c-DTR-Tg, $Batf3^{-/-}$, CD11c-Cre, and CD4-Cre-Tg mice were purchased from The Jackson Laboratory. $Ifnar1^{-/-}$ mice were kindly provided by Dr. Anita Chong of the University of Chicago. $Ifnar1^{flox/flox}$ mice were kindly provided by Dr. Ulrich Kalinke of the Institute for Experimental Infection Research, Hanover, Germany (Kamphuis et al., 2006). $neu^{OT-I/OT-II}$-Tg mice in a B6 background were kindly provided by Dr. Brad H. Nelson of the Trev & Joyce Deeley Research Centre, Victoria, BC, Canada (Wall, et al., 2007). Tet-on-EGFR-Tg mice were kindly provided by Harold Varmus (National Cancer Institute) in 2009 and crossed to a B6 background up to ten generations (Politi et al., 2006). All mice were maintained under specific pathogen-free conditions. Animal care and use were carried out in accordance with institutional and National Institutes of Health protocols and guidelines, and all studies were approved by the Animal Care and Use Committee of the University of Chicago.

Cell Lines and Reagents

H460 was purchased from the American Type Culture Collection (ATCC). TUBO cells were cloned from a spontaneous mammary tumor in a BALB/c neu-Tg mouse (Rovero et al., 2000). TUBO-EGFR was selected after transfection of pSEB-EGFR (L858R) plasmid with 2 μg/ml Blasticidin (InvivoGen). B16-EGFR and B16-EGFR-SIY were selected for a single clone after being transduced by lentivirus expressing human EGFR (L858R) or EGFR (L858R)-SIY. NOP23 was cloned from a spontaneous mammary tumor in a
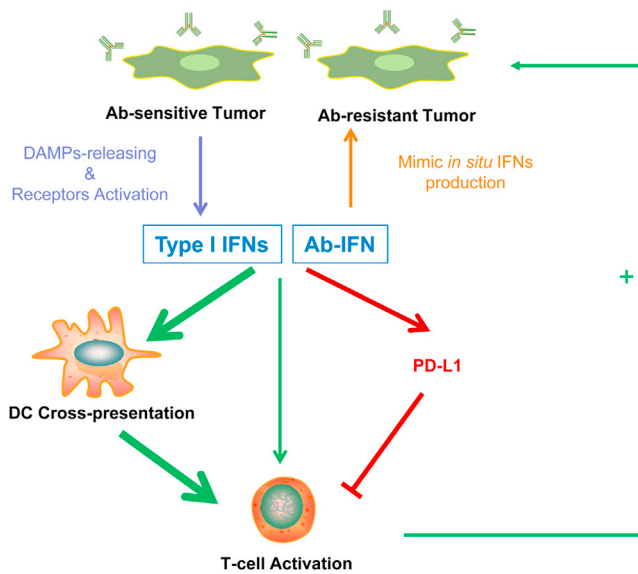
Figure 8. Proposed Model of How Type I IFNs Link Innate and Adaptive Antitumor Immunity

Damage-associated molecular pattern peptides released from Ab-sensitive tumors, but not from Ab-resistant tumors, after Ab therapy will induce type I interferon (IFN) production. Ab-IFN mimics in situ type I IFN production in Ab-resistant tumors. Type I IFNs simultaneously increase dendritic cell (DC) cross-presentation for better cytotoxic T lymphocyte responses, directly activate T cells, and induce programmed cell death ligand 1 (PD-L1) expression to weaken antitumor immunity. Ab-IFN combined with PD-L1 blockade maximizes the antitumor immune responses.

B6-*neu*$^{OT-I/OT-II}$-Tg mouse kindly provided by Dr. Brad H. Nelson of the Trev & Joyce Deeley Research Centre. H460, TUBO, and B16 and its derivatives were cultured in 5% CO_2 and maintained in vitro in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 2 mmol/l L-glutamine, 0.1 mmol/l Minimum Essential Medium nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. The anti-EGFR monoclonal Ab (mAb) cetuximab was purchased from ImClone Systems. Anti-CD8 (YTS 169.4.2), anti-NK1.1 (PK136), and anti-*neu* (7.16.4) Abs were produced in-house. Anti-PD-L1 (10F.9G2) and anti-Ly-6G (1A8) Abs were purchased from Bio X Cell. Anti-CD20 (5D2) Ab was kindly provided by Ouyang Wenjun (Genentech). Anti-IFNAR (MAR-5A3) Ab was kindly provided by Dr. Robert Schreiber (Washington University in St. Louis). Adenovirus expressing murine IFNβ was produced as previously described (Burnette et al., 2011).

Production of Ab-IFNβ Fusion Protein

The V region of anti-EGFR was cloned from an anti-EGFR (LA22) hybridoma (ATCC). The V region of heavy chain and light chain were cloned into AbVec-immunoglobulin G1 and AbVec-κ, respectively (Smith et al., 2009). Next, mouse IFNβ was inserted into the C terminal of heavy chain as a fusion protein with an SGGGGSGGGGSGGGGSGGGG linker. The whole heavy chain with IFNβ and the light chain were cloned to pEE6.4 and pEE12.4 (Lonza), respectively. Next, both vectors were digested by NotI and BamHI restriction enzymes. The complete human cytomegalovirus major immediate-early heavy chain/simian virus 40 transcription unit form-digested pEE6.4 plasmid was ligated into the large NotI-BamHI fragment from the digested pEE12.4 plasmid containing the light-chain expression cassette. The plasmid containing both heavy and light chains was transfected into Chinese hamster ovary cells, and stable clones were established according to the manufacturer's manual (Lonza). The fusion protein anti-EGFR-IFNβ was purified by using a protein A column according to the manufacturer's manual (Repligen). Anti-*neu*-IFNβ was produced in the same way, except that the cDNA of the V region was from anti-*neu* 7.16.4 hybridoma cells.

Tumor Growth and Treatments

Approximately 6×10^5 TUBO, TUBO-EGFR, B16-EGFR, B16-EGFR-SIY, or NOP23 cells were injected subcutaneously on the right flank into 6- to 12-week-old mice. Tumor volumes were measured along three orthogonal axes (a, b, and c) and calculated as tumor volume = (a × b × c)/2. After the tumor was established (~14 days), mice were treated with three intratumoral injections of 25 μg of anti-EGFR-IFNβ or control Ab every 4 days. For CD8 depletion experiments, 200 μg of anti-CD8 Ab was injected intraperitoneally at the same time as the anti-EGFR-IFNβ treatment. Approximately 3×10^6 H460 cells were injected subcutaneously on the right flank into 6- to 12-week-old *Rag1*$^{-/-}$ mice. After the tumor was established (~13 days), 2×10^6 LN cells from OT-I TCR-Tg mice were adoptively transferred to mice by intravenous injection. One day later, mice were treated with three intratumoral injections of 25 μg of anti-EGFR-IFNβ or control Ab on days 14, 18, and 22 after tumor inoculation.

Type I IFN mRNA Expression after Antibody Treatment

Approximately 6×10^5 TUBO, TUBO-EGFR, or B16-EGFR cells were injected subcutaneously into the right flank of 6- to 12-week-old mice. Mice were treated with 100 μg of anti-*neu*, anti-EGFR, or control Ab on day 14 after tumor inoculation. Four days later, the tumor was digested with 1 mg/ml collagenase VIII (Sigma-Aldrich) and 200 μg/ml DNaseI (Sigma-Aldrich) at 37°C for 30 min. Live CD45$^+$ and CD45$^-$ cell population were sorted with the BD FACSAria II cell sorter (BD Biosciences). Total RNA was isolated by using the RNeasy Mini Kit (QIAGEN) and reverse-transcribed to cDNA with the Sensiscript RT Kit (QIAGEN). The expression level of type I IFN mRNA was analyzed by quantitative real-time PCR. The following primers were used for the assay.

Murine IFNα5: forward, 5′-ATGAAGTCCATCAGCAGCTC-3′; reverse, 5′-AGGGGCTGTGTTTCTTCTCT-3′
β-actin: forward, 5′-ACACCCGCCACCAGTTCGC-3′, reverse, 5′-ATGGGGTACTTCAGGGTCAGGATA-3′

Measurement of IFNγ-Secreting T Cells by Enzyme-Linked Immunosorbent Spot Assay or Cytometric Bead Array Assay

SIY peptide-reactive T cells were measured by enzyme-linked immunosorbent spot (ELISPOT) assay. Spleen or LN cells were resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. A total of 1 to 4×10^5 spleen or LN cells were used for the assay. SIY peptide was added at a concentration of 5 μg/ml. After 48 hr of incubation, IFNγ production was determined with an IFNγ ELISPOT assay kit according to the manufacturer's manual (BD Biosciences) or by cytometric bead array assay (BD Biosciences). The visualized cytokine spots were enumerated with the ImmunoSpot Analyzer (CTL).

Ex Vivo Dendritic Cell Cross-Presentation Assay

B16-EGFR-SIY-bearing mice were treated with 25 μg of anti-EGFR, or anti-EGFR-IFNβ intratumoral injection on days 14 and 17. Four days later, dLN cells were digested with 1 mg/ml collagenase VIII (Sigma-Aldrich) and 200 μg/ml DNaseI (Sigma-Aldrich) at 37°C for 15 min. DCs were purified by EasySep Mouse CD11c Positive Selection Kit (STEMCELL Technologies). Approximately 1×10^5 DCs were mixed together with purified 2×10^5 2C T cells with or without 5 μg/ml SIY peptide to restimulate the T cells. Two days later, the supernatants were collected and IFNγ was measured by cytometric bead array assay (BD Biosciences).

Generation of Bone Marrow Chimeras

WT mice were lethally irradiated with a single dose of 1,000 rads. The next day, irradiated mice were adoptively transferred with 2 to 3×10^6 WT, *Ifnar1*$^{-/-}$, or CD11c-DTR-Tg donor bone marrow cells. Mice were maintained on sulfamethoxazole and trimethoprim antibiotics (Bactrim) diluted in drinking water for 4 weeks after reconstitution. Mice were injected with tumor cells 5 to 6 weeks after reconstitution.

Detection of Endotoxin in mAb and Fusion Protein Preparation

Endotoxin was measured by carrying out the limulus amebocyte lysate assay (Cambrex). For all mAb preparations, the amount of endotoxin was determined to be less than 0.2 endotoxin units (EU)/mg mAb.

Flow Cytometric Analysis

Single-cell suspensions of cells were incubated with anti-CD16/32 (anti-FcγIII/II receptor, clone 2.4G2) for 10 min, then stained with conjugated Abs. All fluorescently labeled mAbs were purchased from BioLegend or eBioscience. Samples were analyzed on a FACSCanto flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (TreeStar).

Statistical Analysis

Mean values were compared using an unpaired Student's two-tailed t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.12.004.

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