Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8α+ dendritic cells

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Despite lack of tumor control in many models, spontaneous T cell priming occurs frequently in response to a growing tumor. However, the innate immune mechanisms that promote natural antitumor T cell responses are undefined. In human metastatic melanoma, there was a correlation between a type I interferon (IFN) transcriptional profile and T cell markers in metastatic tumor tissue. In mice, IFN-β was produced by CD11c+ cells after tumor implantation, and tumor-induced T cell priming was defective in mice lacking IFN-α/βR or Stat1. IFN signaling was required in the hematopoietic compartment at the level of host antigen-presenting cells, and selectively for intratumoral accumulation of CD8α+ dendritic cells, which were demonstrated to be essential using Batf3−/− mice. Thus, host type I IFNs are critical for the innate immune recognition of a growing tumor through signaling on CD8α+ DCs.

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Abbreviations used: BMDC, BM-derived DC; dsDNA, double-stranded DNA; mDC, myeloid DC; pDC, plasmacytoid DC; TLR, Toll-like receptor.

Most tumors express antigens that can be recognized by T cells of the host immune system (Huang et al., 1994; Boon and Old, 1997). Despite the expression of antigens, tumors grow progressively and evade immunity. It has generally been assumed that immune evasion is a result of a failure to initiate an antitumor adaptive immune response. However, recent results have indicated that in many instances, spontaneous T cell responses against tumor antigens can be detected in both human cancer patients and in murine models, and that immune escape in those cases appears to occur through dominant inhibition by immunoregulatory pathways (Vesely et al., 2011). For example, high frequencies of CD8+ T cells specific for MelanA/MART-1, MAGE-10, and NY-Eso-1 have been detected in the blood of subsets of patients with metastatic melanoma (Pittet et al., 1999; Valmori et al., 2001; Mortarini et al., 2003; Peterson et al., 2003). Spontaneous antibody responses against a range of tumor-associated antigens have been previously described (Tan and Zhang, 2008). Antibody responses in early stage prostate cancer have been reported to be

detected before PSA becomes detectably elevated (Wang et al., 2005). Moreover, we and others have shown that some human melanoma metastases contain activated CD8+ T cells, including tumor-reactive cells (Anichini et al., 1999; Harlin et al., 2009), suggesting that spontaneous immune responses can be generated all the way through to the step of effector cell migration into tumor sites. Expression of multiple immune evasion mechanisms likely blunts immune function at the effector phase and allows tumor outgrowth in those instances (Rabinovich et al., 2007).

The observation that a T cell response can ever become spontaneously primed against a growing tumor mass raises the question of how this is possible given the tight regulation of innate immune signals that dictate whether a bridge to adaptive immunity can occur. Most malignancies (including melanoma) lack an obvious infectious etiology and therefore would not contain abundant external ligands for Toll-like receptors (TLRs). In this context, studies

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from several groups have revealed that dying cells can release endogenous adjuvants (Kono and Rock, 2008), providing activation signals for DCs and other APCs that lead to up-regulation of co-stimulatory molecules and consequently yield productive T cell activation and differentiation (Kono and Rock, 2008). Although these early results indicate that tumor cells can, under certain conditions, liberate products that can theoretically elicit innate immune signals, how these or other signals may lead to the spontaneous activation of a tumor-specific adaptive T cell response remains unclear.

Type I IFNs have been studied extensively in the context of viral infections (Stetson and Medzhitov, 2006b). During various types of viral infection, type I IFNs induce the expression of an array of genes that act to prevent viral spread, thus creating an antiviral state (Stark et al., 1998). But type I IFNs also regulate antiviral immune effector responses and play an important role in promoting the cross-presentation of viral antigens to CD8$^+$ T cells (Le Bon et al., 2003). Although a role for type I IFNs has been described for immunosurveillance against carcinogen-induced tumors and for rejection of transplanted tumors (Dunn et al., 2005, 2006), the source of type I IFNs and the mechanism of action of this cytokine during the priming phase of an antitumor immune response have not yet been elucidated. We have recently reported that gene expression profiling of human melanoma metastases revealed a subset of tumors that contained infiltrating CD8$^+$ T cells (Harlin et al., 2009). Reasoning that interrogation of those gene array data might provide an indication regarding innate immune signals associated with the presence of a T cell response, we herein report a correlation between the presence of T cell–specific transcripts and a set of genes known to be induced by type I IFNs. Using a series of murine models, we show that shortly after tumor challenge in vivo, type I IFN production was detected by DCs in tumor-draining lymph nodes, and that host type I IFN signaling on CD8α^+ DCs was required for spontaneous cross-priming of tumor antigen–specific CD8$^+$ T cells. Our results suggest a model in which a growing tumor can elicit production of type I IFNs from the host, which leads to accumulation of CD8α^+ DCs that in turn promote CD8$^+$ T cell activation against tumor-derived antigens.

RESULTS

Tumors growing in immunocompetent hosts induce T cell priming and IFN-β production by CD11c$^+$ cells in tumor-draining lymph nodes

Gene expression profiling of human melanoma metastases along with confirmatory assays revealed that a subset of tumors showed evidence of spontaneous inflammation that included the presence of infiltrating CD8$^+$ T cells (Harlin et al., 2009). Parallel studies have indicated that among tumor-associated CD8$^+$ T cells there are cells that recognize human melanoma antigens as reflected by class I MHC/peptide tetramer staining (Speiser et al., 2002; Harlin et al., 2006). This evidence of spontaneous T cell priming against tumor antigens raised the question of what innate immune signals might

mediate sterile immunity against tumors arising from self tissues. Reanalysis of the gene expression profiling data revealed that several transcripts indicative of type I IFN signaling, such as IRF1 and the IFN-induced protein of 30 kD were co-expressed in those tumors that contained T cell transcripts (Fig. 1). This correlation prompted mechanistic experiments to determine whether host type I IFN signals might be necessary for spontaneous priming of CD8$^+$ T cells against tumor antigens when it does occur.

We investigated whether spontaneous priming of antigen-specific CD8$^+$ T cells could be detected in lymphoid organs after subcutaneous implantation of murine cell lines in immunocompetent mice. To provide a defined antigen, tumor cells were transduced to express the model antigenic peptide SIYRYYGL (SIY) that is presented by the class I molecule K^b. The SIY antigen is advantageous because of the set of tools we have assembled for monitoring details of the host immune response, including peptide/MHC tetramers, TCR transgenic T cells, and high-affinity TCR tetramers to monitor processed antigen on APCs (Kline et al., 2008; Zhang et al., 2008). B16 melanoma cells expressing the SIY antigen (B16.SIY) induced a significant frequency of peptide-specific IFN-γ–producing cells in the spleen 6 d after subcutaneous tumor implantation in the flank (Fig. 2 A). This was accompanied by an increase in the frequency of SIY-specific CD8$^+$ T cells detected by SIY/K^b tetramer staining (Fig. 2 B). This T cell activation is completely dependent on cross-presentation by host DCs, as it was ablated in CD11c-DTR transgenic mice treated with diphtheria toxin (Fig. S1). Flow cytometry confirmed that the majority of CD8α^+ and plasmacytoid DCs (pDCs), and a fraction of myeloid DCs (mDCs), were depleted with this approach (Fig. S1). It is interesting to note that these tumors grow progressively over time and are not ultimately rejected by the host, but this failure is not caused by an absence of early T cell priming. Rather, recent observations suggest that the immune response wanes over time because of dominant-negative regulatory mechanisms that eventually lead to tumor outgrowth (Kline et al., 2008). Regardless of those late events, early T cell
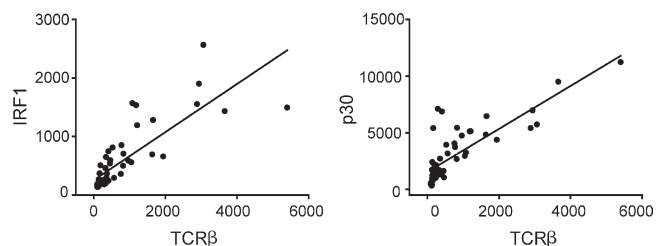

Figure 1. Human melanoma metastases show a positive correlation between T cell markers and IFN-induced transcripts. Tumor samples were obtained by core biopsy or excisional biopsy or obtained from material resected from patients as part of routine clinical management. Total RNA was extracted from tumor samples ($n = 52$) and gene levels were analyzed by Affymetrix. Arbitrary expression units according to Affymetrix gene levels are shown. (IRF1, $R^2 = 0.648$; p30, $R^2 = 0.658$).

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priming can be used in this model as a readout for determining the host innate immune requirements for the initial recognition of tumor.

To determine whether implanted tumors might also induce a type I IFN profile in mice, we inoculated B16 melanoma cells into recombination-activating gene 2-deficient (Rag2$^{-/-}$) mice, to eliminate a contribution of the adaptive immune system. We compared gene expression of the tumors recovered from the mice to the B16 cells grown in vitro, reasoning that the differentially expressed genes would be those induced in the host by the presence of the tumor. Interestingly, this gene expression profiling confirmed the upregulation of multiple transcripts reflective of innate immune activation, including a panel of IFN-inducible genes (Table S1). A repeat of this experiment in type I IFNR$^{-/-}$ mice confirmed that induction of these transcripts required type I IFN signaling on host cells (unpublished data). To assess directly whether type I IFNs are produced early in response to a growing tumor, we compared IFN-β mRNA levels by quantitative real-time PCR in lymph nodes from naive mice and from draining lymph nodes of C57BL/6 mice challenged with B16.SIY melanoma cells. We found that IFN-β was produced in tumor draining lymph nodes as early as 4 d after tumor challenge (Fig. 2 C). We next sorted cells from tumor draining lymph nodes on the basis of their expression of the DC marker CD11c and analyzed IFN-β mRNA levels, which revealed that IFN-β production after tumor challenge was confined to the CD11c$^+$ DC subpopulation (Fig. 2 D).

As an alternative approach to determining whether DCs were the dominant population producing IFN-β, we depleted DCs in vivo using CD11c-DTR transgenic mice treated with diphtheria toxin. After tumor implantation, these mice showed markedly reduced production of IFN-β in the tumor-draining lymph node compared with control mice (Fig. 2 E). Together, these results indicate that DCs are the major source of type I IFNs early after tumor challenge.

To determine whether this ability to induce a rapid host immune response and type I IFN production was a general phenomenon, an additional panel of C57BL/6-derived tumor cells was transduced to express the SIY antigen. All the tumor lines tested (EL4, MC57, and C1498) similarly induced a spontaneous CD8$^+$ T cell response as assessed by ELISPOT (Fig. 2 F) and peptide/MHC tetramer analysis (not depicted), and IFN-β production in the tumor-draining lymph node (Fig. 2 G). Together, these results indicated that it is not uncommon for implanted tumors to trigger type I IFN production and a rapid CD8$^+$ T cell response against tumor-associated antigens in vivo.

Host IFN-α/β signaling is critical for spontaneous tumor-specific T cell priming

Having observed the early production of type I IFNs after tumor inoculation, we sought to determine whether host type I IFN signaling was necessary for spontaneous priming of CD8$^+$ T cells to tumor antigens in vivo. We therefore examined the effect of B16.SIY challenge on the CD8$^+$ T cell
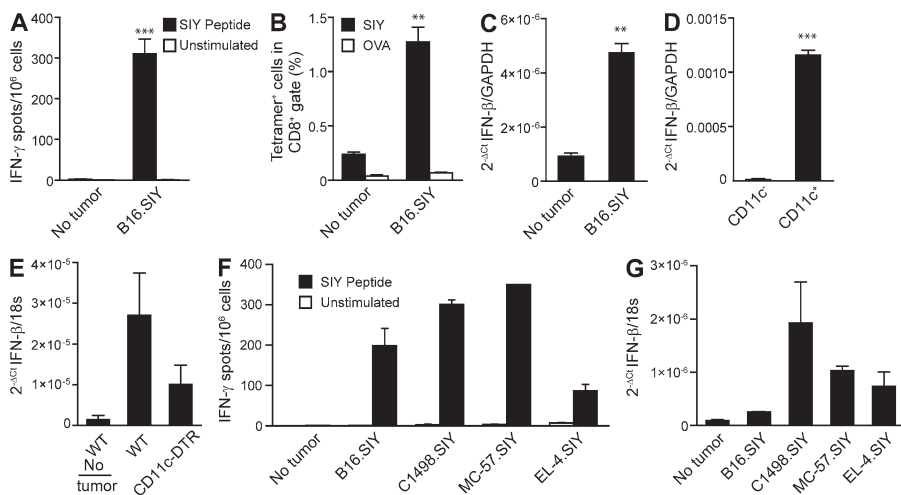

Figure 2. Tumors induce CD8$^+$ T cell priming, which is accompanied by IFN-β production by CD11c$^+$ DCs in the tumor-draining lymph nodes. (A and B) C57BL/6 mice were inoculated or not with 5 × 10^6 B16.SIY tumor cells (s.c.), splenocytes were harvested 6 d later, and restimulated for 16 h in the presence or absence of soluble SIY peptide (A). The frequency of tumor-specific IFN-γ–producing cells was assessed by ELISPOT. ***, P < 0.0001 versus No tumor. (B) Cells were gated on CD8$^+$CD4$^-$B220$^-$, and the frequency of SIY-specific CD8$^+$ T cells was assessed by FACS using specific tetramers. **, P = 0.0063 versus No tumor. (C and D) C57BL/6 mice were inoculated (s.c.) or not with 5 × 10^6 B16.SIY tumor cells, and inguinal lymph nodes were recovered 4–6 d later. IFN-β mRNA expression was assessed by real-time RT-PCR analysis in total lymph nodes, and the results are expressed as 2$^{-\Delta Ct}$ using GAPDH as endogenous control. **, P = 0.0046 versus No tumor (C) or in CD11c$^+$ and CD11c$^-$ cells sorted from lymph nodes. ***, P = 0.0008 versus CD11c$^-$ (D). (E) Wild-type C57BL/6 mice (expressing the congenic marker CD45.1$^+$) were lethally irradiated and reconstituted with either wild-type (CD45.2$^+$) or CD11c-DTR (CD45.2$^+$) BM cells. Mice were allowed to reconstitute for 3 mo, and then were injected i.p. with diphtheria toxin (100 ng in 100 μl of DPBS) once a day for 8 d, starting 2 d before s.c challenge with 5 × 10^6 B16.SIY tumor cells in the left flank (n = 5). Inguinal lymph nodes were recovered 6 d later, and IFN-β expression in total inguinal lymph nodes was measured by real-time PCR. The results are expressed as 2$^{-\Delta Ct}$ using 18s as endogenous control. (F and G) C57BL/6 mice were inoculated s.c. with the indicated tumor cell lines (5 × 10^6; F), and splenocytes were harvested 6 d later and restimulated for 16 h in the presence or absence of soluble SIY peptide. Frequency of tumor-specific IFN-γ–producing cells was assessed by ELISPOT. (G) Real-time RT-PCR analysis of IFN-β mRNA expression in total inguinal lymph nodes. The results are expressed as 2$^{-\Delta Ct}$ using 18s as endogenous control. Data represent mean ± SEM (n = 5) and are representative of four independent experiments (A–C) or two independent experiments (D–G).

response to the SIY tumor antigen in mice deficient for the IFN-α/βR compared with WT mice. 17 d after tumor inoculation, splenocytes were assayed for the frequency of IFN-γ–producing cells in response to soluble SIY peptide or irradiated B16.SIY cells by ELISPOT. In comparison to the vigorous response observed in the wild-type group, splenocytes from IFN-α/βR$^{-/-}$ mice displayed a dramatically reduced frequency of IFN-γ–producing effector cells after tumor challenge (Fig. 3 A). To determine whether the effect of type I IFNs on T cell priming was at the level of expansion versus differentiation of CD8$^+$ T cells, analysis using SIY/K^b tetramers was performed. Whereas an expanded population of tetramer-reactive CD8$^+$ T cells was detected in the spleens of wild-type mice, IFN-α/βR$^{-/-}$, and IFN-α/βR$^{-/-}$/IFN-γR$^{-/-}$ mice failed to display an increased frequency of tumor reactive CD8$^+$ T cells (Fig. 3 B). The combined elimination of IFN-α/βR and IFN-γR reduced T cell priming completely to background levels, suggesting partial compensation by IFN-γ in the absence of type I IFN signaling. However, mice singly deficient in the IFN-γR generated a normal expanded frequency of SIY-specific CD8$^+$ T cells (Fig. 3 C), suggesting that type I IFNs are dominantly required and that IFN-γ is not necessary for this stage of an antitumor T cell response.

As type I IFNs mediate signaling through Stat1, we additionally analyzed spontaneous CD8$^+$ T cell priming in Stat1$^{-/-}$ mice. Consistent with our previous results, Stat1$^{-/-}$ mice also showed a markedly decreased priming of tumor antigen–specific CD8$^+$ T cells as assessed by ELISPOT and SIY/K^b tetramer analysis (Fig. 3, D and E). Together, these results indicate that host type I IFN signaling through Stat1 is required for optimal expansion of tumor antigen–specific CD8$^+$ T cells after tumor challenge in vivo.

IFN signaling is required in the hematopoietic compartment for spontaneous rejection of tumors in vivo

To determine whether the defect in natural CD8$^+$ T cell priming in the absence of host IFN signaling would be associated with failed tumor rejection, we used a model in which tumors are spontaneously rejected. At the same time, it was of interest to determine the cellular compartment in which IFN signaling must occur. Toward this end, we turned to the immunogenic variant of the P815 mastocytoma, P198, which initially grows but is spontaneously rejected in syngeneic DBA/2 mice (Fallarino et al., 1996). To determine if type I IFN signaling for tumor rejection was required in the hematopoietic or nonhematopoietic compartment, we generated radiation BM chimeras in which Stat1-sufficient versus Stat1$^{-/-}$ DBA/2 mice were irradiated and reconstituted with either wild-type or Stat1$^{-/-}$ DBA/2 BM cells. After a period of at least 3 mo, to allow for recovery of the immune system, mice received a tumor challenge with P198 cells on the flank and were monitored for tumor progression. As expected, P198 cells were rejected by wild-type mice that had been reconstituted with wild-type BM, and tumors grew progressively in Stat1$^{-/-}$ mice reconstituted with Stat1$^{-/-}$ BM (Fig. 4 A). In contrast, Stat1$^{-/-}$ mice

reconstituted with wild-type BM cells rejected the tumor normally, indicating that the expression of Stat1 in hematopoietic cells is sufficient for tumor rejection and that Stat1 expression is not required in non–BM-derived cells for tumor
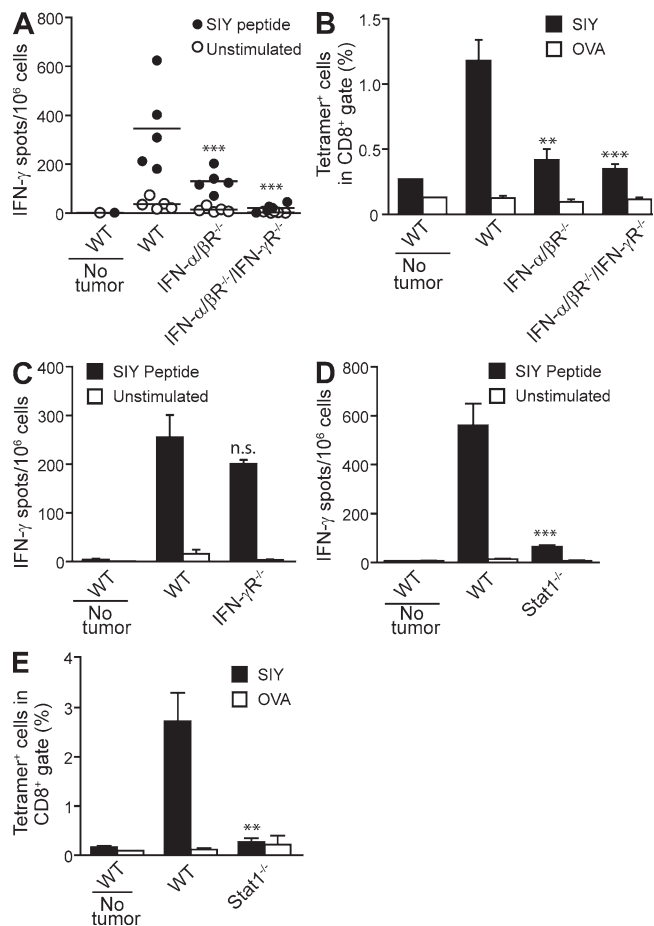

Figure 3. **IFN-α/β, and not IFN-γ signaling, is critical for spontaneous CD8$^+$ T cell priming to tumor–associated antigens.** (A and B) Wild-type, IFN-α/βR$^{-/-}$, or IFN-α/βR$^{-/-}$/IFN-γR$^{-/-}$ mice were inoculated s.c. with 10^6 B16.SIY tumor cells. Splenocytes were harvested 17 d later and restimulated for 16 h in the presence or absence of or soluble SIY peptide (A). The frequency of tumor-specific IFN-γ–producing cells was assessed by ELISPOT. ***, $P < 0.0001$ versus WT. (B) cells were gated on CD8$^+$CD4$^-$B220$^-$ and the frequency of SIY-specific CD8$^+$ T cells was assessed by FACS using specific tetramers. ***, $P < 0.0009$; **, $P < 0.0027$ versus WT. (C) Wild-type and IFN-γR$^{-/-}$ mice were inoculated s.c. with 10^6 B16.SIY tumor cells, and splenocytes were harvested 17 d later and restimulated for 16 h in the presence or absence of soluble SIY peptide, and then the frequency of tumor-specific IFN-γ–producing cells was assessed by ELISPOT. $P = 0.268$ versus WT. (D and E) Wild-type and Stat1$^{-/-}$ mice were inoculated s.c. with 10^6 B16.SIY tumor cells, and splenocytes were harvested 17 d later and restimulated for 16 h in the presence or absence of soluble SIY peptide (D). The frequency of tumor-specific IFN-γ–producing cells was assessed by ELISPOT. ***, $P < 0.0001$ versus WT. (E) cells were gated on CD8$^+$CD4$^-$B220$^-$, and the frequency of SIY-specific CD8$^+$ T cells was assessed by FACS using specific tetramers. **, $P = 0.0029$ versus WT. Data represent mean ± SEM ($n = 5$), and are representative of three independent experiments.

immunity (Fig. 4 A). In addition, the failure of wild-type mice reconstituted with Stat1$^{-/-}$ BM to reject the P198 tumor demonstrates that expression of Stat1 in the hematopoietic

compartment is necessary for spontaneous tumor rejection in vivo and that Stat1 in non–BM-derived cells is not sufficient for tumor rejection (Fig. 4 A).

IFN signaling is required in non–T cell BM-derived cells for tumor antigen–specific T cell priming

Among the cells of the hematopoietic compartment, IFN signaling could be playing a T cell–intrinsic role in the generation of an antitumor immune response and/or be important in non–T cells, perhaps at the level of APCs. To address whether T cell–intrinsic IFN signaling was required for the acquisition of an effector phenotype, we compared wild-type and Stat1$^{-/-}$ T cells primed to become effector CTL in a mixed lymphocyte culture in vitro. However, Stat1$^{-/-}$ and wild-type T cells were equally able to lyse allogeneic target cells (Fig. 4 B), suggesting that T cell–intrinsic Stat1 is not absolutely required for the development and execution of lytic effector function by cytotoxic T lymphocytes. IFN-γ production by Stat1$^{-/-}$ CTL was also preserved (unpublished data). To investigate more directly whether IFN signaling on non–T cells was required for activation of tumor antigen–specific CD8$^+$ T cells, we CFSE labeled wild-type CD8$^+$ T cells purified from 2C TCR Tg/Rag2$^{-/-}$ mice (Sha et al., 1988) specific for the SIY octameric peptide in the context of K^b (Udaka et al., 1996) and adoptively transferred them into wild-type or Stat1$^{-/-}$ syngeneic mice. We then challenged the mice with B16.SIY tumors. 7 d after tumor challenge with B16.SIY, we analyzed CFSE dilution of SIY-specific CD8$^+$ T cells in the spleen. A large percentage of the 2C CD8$^+$ T cells transferred into wild-type mice displayed a decreased intensity of CFSE fluorescence, consistent with antigen-specific T cell proliferation and successful cross-presentation of the SIY antigen. In contrast, CFSE dilution was markedly reduced upon transfer into Stat1$^{-/-}$ hosts (Fig. 4, C and D). Finally, to determine whether T cell priming could occur in the absence of T cell–intrinsic IFN signaling in vivo, we vaccinated Stat1$^{-/-}$ versus wild-type mice with wild-type BM-derived DCs (BMDCs) loaded with SIY peptide. When assayed 20 d later for T cell priming by IFN-γ ELISPOT, comparable induction of SIY-specific T cells was observed in both wild-type and Stat1$^{-/-}$ hosts (Fig. 4 E). This result demonstrates that T cell–intrinsic IFN signaling is not required for T cell priming in vivo, and suggests that the defect in T cell priming lies upstream, likely at the level of host APCs.

Analysis of DC subsets from mice deficient in IFN signaling

In a search for potential mechanisms responsible for the lack of tumor antigen–specific CD8$^+$ T cell priming in the absence of host IFN signaling, we analyzed multiple phenotypic characteristics of DCs from wild-type versus Stat1$^{-/-}$ or IFN-α/βR$^{-/-}$ mice. We first compared frequencies and absolute numbers of the different DC subpopulations (mDCs, CD11C$^+$B220$^-$CD8α$^-$CD11b$^+$; CD8α$^+$DCs, CD11C$^+$ B220$^-$CD8α$^+$CD11b$^-$; and pDCs, CD11C^{int}B220$^+$PDCA$^+$) in the spleen and lymph nodes of unmanipulated or tumor-bearing mice but found no difference between Stat1$^{-/-}$ or
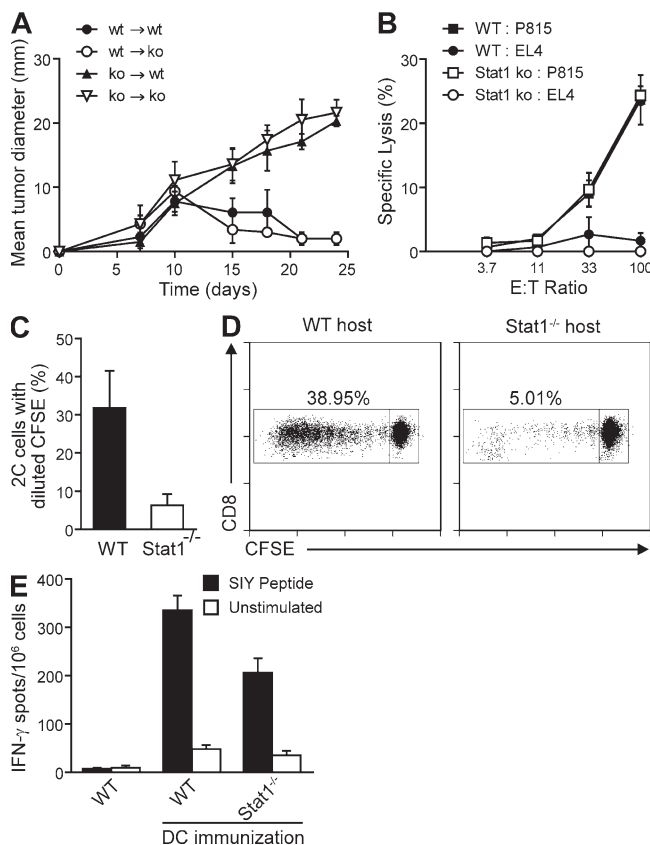

Figure 4. IFN signaling is required in non–T cell BMDCs for tumor-specific T cell priming and for spontaneous rejection of immunogenic tumors in vivo. (A) Wild-type and Stat1$^{-/-}$ DBA/2 mice were lethally irradiated and reconstituted with either wild-type or Stat1$^{-/-}$ DBA/2 BM cells, and 3 mo later they were challenged s.c. with 10^6 P198 cells in the left flank (n = 5). Tumor size was measured at different time points. Results are shown as mean tumor diameter ± SEM. Data are representative of two independent experiments. (B) T cells from wild-type and Stat1$^{-/-}$ (H-2^b) mice were stimulated with T cell–depleted irradiated splenocytes from DBA/2 (H-2^d) mice for 5 d in an allogeneic MLR. Cytotoxic activity was measured by standard ^{51}Cr-release assay against P815 (H-2^d, cognate targets) and EL4 cells (H-2^b, control targets). (C and D) CD8$^+$ T cells were purified from the spleens of 2C Tg/RAG2$^{-/-}$ (Stat1-sufficient) mice, CFSE labeled, and transferred by retroorbital injection to wild-type or Stat1$^{-/-}$ mice. The next day, those mice were inoculated s.c. with 10^6 B16.SIY cells in the flank, and 7 d later splenocytes were harvested and CFSE dilution of 2C CD8$^+$ T cells was assessed by FACS. (C) Percent of cells with decreased CFSE intensity within the DAPI$^-$CD8$^+$1B2$^+$ gate. Data show mean ± SEM of individual mice in each group. Data are representative of two independent experiments. (D) Representative dot plots of CFSE dilution. Numbers indicate percent of cells in the indicated gate. (E) 10^6 SIY-pulsed wild-type BMDCs were s.c. inoculated in the flank of wild-type or Stat1$^{-/-}$ mice, and 20 d later splenocytes were harvested and restimulated in vitro for 16 h in the presence of culture medium or soluble SIY peptide. The frequency of tumor-specific IFN-γ–producing cells was assessed by ELISPOT. Data are representative of two independent experiments.

IFN-α/βR$^{-/-}$ and wild-type mice (unpublished data). In addition, the surface expression of CD80, CD86, CD40, and class I and class II MHCs in CD11c$^+$ tumor-draining lymph node cells was comparable (Fig. 5 A and Table S2), and LPS–induced IL-12 production was still detected in Stat1$^{-/-}$ CD11c$^+$ cells (not depicted). Moreover, adherent splenocytes from wild-type and Stat1$^{-/-}$ mice did not differ in their ability to stimulate 2C TCR Tg CD8$^+$ T cells to produce IL-2 upon loading with the SIY peptide in vitro (Fig. 5 B).

It was conceivable that host IFN signaling was generally important for the migration of DCs into the draining lymph node compartment in vivo. To address this possibility, the skin of wild-type and Stat1$^{-/-}$ mice was painted with FITC and the draining lymph nodes were analyzed for green fluorescence on CD11c$^+$ cells. However, comparable appearance of FITC$^+$ DCs was observed in both sets of mice (unpublished data). We also considered the possibility that type I
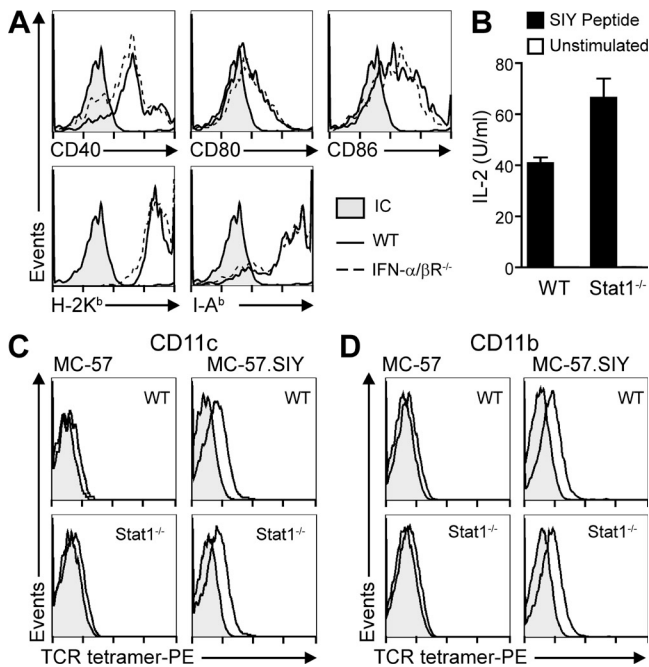
IFNs would induce chemokines by the DCs that would aid in the recruitment of T cells to the lymph node for subsequent activation. Analysis of DCs stimulated with IFNα/β revealed substantial production of CXCL9 and CXCL10, which could potentially mediate recruitment of activated CD8$^+$ T cells via CXCR3 (unpublished data). However, there was no defect in priming of SIY-specific CD8$^+$ T cells in response to B16.SIY tumors in CXCR3$^{-/-}$ mice (unpublished data), arguing for a lack of importance of this pathway at the early stages of T cell priming in vivo. Finally, it was reasoned that type I IFN signaling might be necessary for DCs to be able to take up, process, and present tumor antigen in the context of class I MHC molecules. This possibility was addressed through the use of tetramers generated from a high-affinity variant of the 2C TCR, which can bind to SIY–K^b complexes with sufficient avidity to allow analysis by flow cytometry. Analysis of the APC compartments was performed in wild-type versus Stat1$^{-/-}$ mice crossed to a RAG2$^{-/-}$ background, to eliminate the possibility that host T cells might eliminate antigen-expressing APCs. In fact, when tumor–derived CD11c$^+$ or CD11b$^+$ cells were analyzed from wild-type versus Stat1$^{-/-}$ RAG2$^{-/-}$ mice bearing MC57.SIY tumors, comparable binding of the 2C TCR tetramer was observed (Fig. 5, C and D). Absence of binding to APCs derived from wild-type MC57 tumors confirmed specificity of tetramer binding only when the SIY antigen was present. These results suggest that the APC subsets that accumulate in the tumor site process and present the SIY tumor–derived antigen normally, even in the absence of host type I IFN signaling.

In vivo, endogenous type I IFN signaling is required for intratumoral accumulation of the CD8$\alpha$$^+$ DC subset

Given the comparable properties of DC subsets in the lymphoid organs of wild-type and Stat1$^{-/-}$ mice, and the intact expression of SIY–K^b peptide–MHC complexes on the total APC populations that are present within the tumor site, we next addressed whether there was a defect at the level of the specific DC subsets that accumulate within the tumor microenvironment. We therefore challenged wild-type and Stat1$^{-/-}$ mice with B16.SIY cells and evaluated the frequency and absolute number of the three major DC subpopulations accumulating in the tumor at day 16. We found that the mDC and pDC subpopulations accumulated in the tumors of both groups of mice. However, the CD8$\alpha$$^+$ DC population, which accounted for up to 20% of the DCs infiltrating the tumors in wild-type mice, was almost completely absent in the tumors grown in Stat1$^{-/-}$ mice (Fig. 6, A and B; and Fig. S2). A similar defect in CD8$\alpha$$^+$ DC accumulation was observed in IFN-α/βR$^{-/-}$ mice (Fig. 6 C). As an alternative quantitative approach to evaluate the presence of CD8$\alpha$$^+$ DCs, molecular markers were used. XCR1 is a chemokine receptor exclusively expressed by CD8$\alpha$$^+$ DCs (Dorner et al., 2009) and Batf 3, a transcription factor preferentially expressed by CD8$\alpha$$^+$ DCs and absolutely required for their development. Using quantitative RT-PCR to analyze

Figure 5. **Analysis of DCs from WT, Stat1$^{-/-}$, and IFN–α/βR$^{-/-}$ mice.** (A) Wild-type and IFN–α/βR$^{-/-}$ mice were inoculated s.c. with 10^6 B16.SIY cells. 6 d later, surface expression of CD80, CD86, CD40, and class I and II MHC was assessed by FACS in tumor-draining lymph node cells gated on CD11c$^+$ cells. Filled histograms correspond to an isotype control (IC), continuous line corresponds to wild-type, and dashed line corresponds to IFN–α/βR$^{-/-}$ mice. (B) Adherent splenocytes from wild-type and Stat1$^{-/-}$ mice were loaded with SIY peptide or left untreated and used to stimulate 2C CD8$^+$ T cells. IL-2 production was assessed by ELISA. (C and D) Wild-type Rag1$^{-/-}$ mice (top) or Rag1$^{-/-}$Stat1$^{-/-}$ mice (bottom) were inoculated with 10^6 MC57 or MC57.SIY tumor cells. 14 d later SIY/K^b expression was assessed by FACS using high-affinity 2C TCR tetramers gated on the tumor-infiltrating CD11c$^+$ population (C) and CD11b$^+$ population (D). Filled histograms correspond to staining with streptavidin-phycoerythrin alone. Data are representative of two independent experiments (n = 4).

transcript abundance in tumors analyzed ex vivo, we found that both transcripts were highly expressed in tumors grown in wild-type mice, yet severely reduced in tumors grown in IFN-α/βR$^{-/-}$ mice (Fig. 6, D and E). Therefore, host IFN signaling appeared to be required for intratumoral accumulation of CD8α^+ DCs within the tumor microenvironment.

CD8α^+ DCs have been shown to be the most important DC population for cross-presentation of antigens to CD8$^+$ T cells in the setting of viral infection (Belz et al., 2004). Recent work has indicated that mice lacking the transcriptional regulator, Batf3, have a specific deficiency in the development of the CD8α^+ DC lineage (which includes a CD103$^+$ DC subset; Hildner et al., 2008). These mice also show defective cross-priming of CD8$^+$ T cells in response to viruses, and were defective in control of immunogenic tumors in vivo (Hildner et al., 2008). We therefore investigated whether these DC subpopulations were required at the level of priming of CD8$^+$ T cells to tumor-derived antigens. Wild-type and Batf3$^{-/-}$ mice were challenged with B16.SIY melanoma cells, and splenocytes were assayed 6 d later for the frequency of IFN-γ–producing cells by IFN-γ ELISPOT and SIY/K^b tetramer staining. Indeed, Batf3$^{-/-}$ mice showed a dramatically reduced frequency of IFN-γ–producing effector cells as compared with wild-type mice (Fig. 7, A and B). The poor T cell priming was comparable to the level of deficiency observed in IFN-α/βR$^{-/-}$ mice (Fig. 3 A), as was the tumor growth rate, which was accelerated
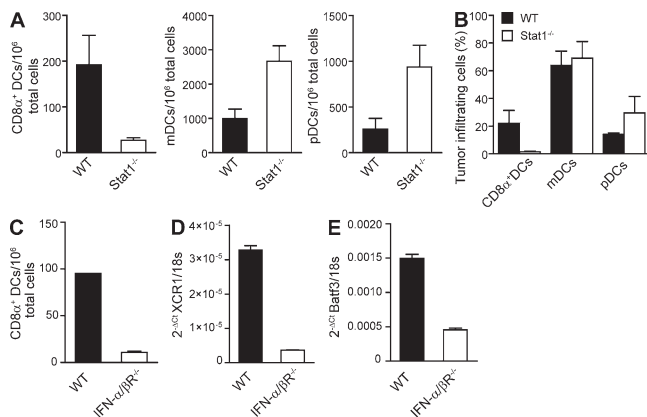

Figure 6. Endogenous type I IFN signaling is required for intratumoral accumulation of CD8α^+ DCs. (A and B) Wild-type and Stat1$^{-/-}$ mice were inoculated s.c. with 10^6 B16.SIY cells, and 15 d later tumors were harvested and frequency (A) and percentages (B) of CD8α^+ DCs, mDCs, and pDCs infiltrating tumors were analyzed by FACS. GFP$^+$DAPI$^+$CD3$^+$ cells were gated out, and the different DCs subpopulations were identified as follows: mDCs, CD11C$^+$B220$^-$CD8α^-CD11b$^+$; CD8αDCs, CD11C$^+$B220$^-$CD8α^+CD11b$^-$; and pDCs, CD11C^{int}B220$^+$PDCA$^+$. Results are shown as mean $\pm$ SEM of 3 independent experiments (n = 4). (C–E) Wild-type and IFN-α/βR$^{-/-}$ mice were inoculated s.c. with 10^6 B16.SIY cells, and 15 d later tumors were harvested and frequency of intratumoral CD8α^+ DCs was assessed by FACS (C). (D and E) XCR1 mRNA expression (D) and Batf3 mRNA expression (E) were assessed by real-time RT-PCR analysis on tumor homogenates. The results are expressed as 2$^{-\Delta Ct}$ using 18s as endogenous control. Results are shown as mean $\pm$ SEM of 2 independent experiments (n = 5).

in Batf3$^{-/-}$ and IFN-α/βR$^{-/-}$ mice (Fig. S3, A and B). Moreover, parental B16 melanoma cells, without the SIY antigen, also grew faster in Batf3$^{-/-}$ mice compared with wild-type (Fig. S3 C), indicating that this is a critical pathway for cross-presentation of natural endogenous tumor antigens. To determine whether CD8α^+ DCs were themselves required for type I IFN production in response to tumor implantation, induction of IFN-β mRNA was assessed in tumor-draining lymph nodes in wild-type versus Batf3$^{-/-}$ mice. However, IFN-β induction was comparable in both sets of mice (Fig. 7 C). Collectively, these findings demonstrate that the CD8α^+ DC subpopulation is critical for the spontaneous priming of tumor antigen–specific CD8$^+$ T cells in response to a growing tumor, downstream from host type I IFN production.

To determine whether the CD8α^+ DC lineage itself must respond to type I IFNs, wild-type mice were irradiated and reconstituted with wild-type, Batf3$^{-/-}$ or IFN-α/βR$^{-/-}$ BM cells, or a mix of wild-type and IFN-α/βR$^{-/-}$ or Batf3$^{-/-}$ and IFN-α/βR$^{-/-}$ in a 50/50 ratio. 3 mo later, those chimeric mice were challenged with B16.SIY melanoma cells, and splenocytes were assayed 6 d later for T cell priming by SIY/K^b tetramer analysis and tumor size was measured. As expected, mice reconstituted with Batf3$^{-/-}$ or IFN-α/βR$^{-/-}$ BM cells showed a dramatically reduced frequency of SIY-specific CD8$^+$ T cells as compared with mice reconstituted with wild-type BM cells (Fig. 8 A). Mice reconstituted with a mix of wild-type and IFN-α/βR$^{-/-}$ cells showed restored T cell priming to the level seen in wild-type mice. However, mice reconstituted with a mix of Batf3$^{-/-}$ and IFN-α/βR$^{-/-}$ BM cells continued to show reduced priming of SIY-specific T cells (Fig. 8 A). This reduced T cell priming was associated with poor tumor growth control (Fig. 8 B). These results demonstrate that type I IFNs must signal on the CD8α^+ DC lineage for optimal priming of tumor antigen–specific CD8$^+$ T cells after tumor challenge in vivo.
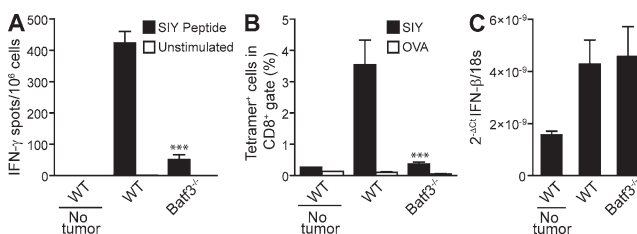

Figure 7. CD8α^+ DCs are critical for antitumor CD8$^+$ T cell priming. Wild-type and Batf3$^{-/-}$ mice were inoculated s.c. with 10^6 B16.SIY cells. 6 d later, splenocytes were harvested and restimulated for 16 h in the presence of culture medium or soluble SIY peptide (A). The frequency of tumor-specific IFN-γ–producing cells was assessed by ELISPOT. ***, P < 0.0001 versus WT. (B) the frequency of SIY-specific CD8$^+$ T cells was assessed by FACS using specific anti–K^b-SIY tetramers, cells were gated on the CD8$^+$CD4$^-$B220$^-$ population. ***, P < 0.001 versus WT. (C) IFN-β mRNA expression was assessed by real-time RT-PCR analysis in total lymph nodes. The results are expressed as 2$^{-\Delta Ct}$ using 18s as endogenous control. Results are shown as mean $\pm$ SEM (n = 5) and are representative of at least two experiments.
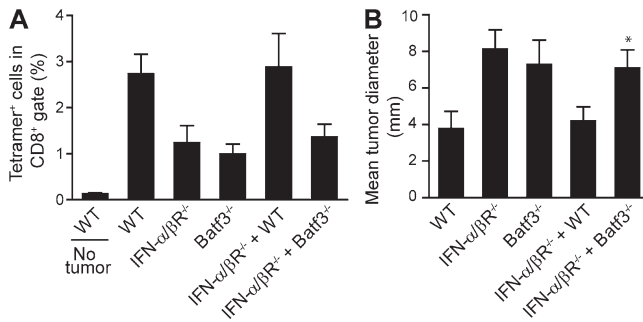
Figure 8.   Type I IFN signaling must occur on the CD8α⁺ DC lineage for antitumor CD8⁺ T cell priming to occur. (A) Wild-type mice were lethally irradiated and reconstituted with wild-type, Batf3⁻/⁻, or IFN-α/βR⁻/⁻ BM cells, or a mix of wild-type and IFN-α/βR⁻/⁻ or Batf3⁻/⁻ and IFN-α/βR⁻/⁻ in a 50/50 proportion. Mice were allowed to reconstitute for 3 mo, and were then inoculated s.c. with 10^6 B16.SIY cells. (A) splenocytes were harvested 6 d later, and the frequency of SIY-specific CD8⁺ T cells was assessed by FACS using specific tetramers. Cells were gated on CD8⁺CD4⁻B220⁻. (B) Tumor size was measured at the end of the experiment. *, P < 0.05 versus IFN-α/βR⁻/⁻ + WT. Results are shown as mean ± SEM of 2 independent experiments ($n = 4$ each).

DISCUSSION

Our results identify type I IFNs as critical mediators in the spontaneous priming of an antitumor CD8⁺ T cell response. Our data show that IFN-β is produced shortly after tumor challenge and, through signaling at the level of CD8α⁺ DCs, promotes tumor antigen–specific T cell priming and tumor rejection. In vivo, endogenous type I IFNs induced the intra-tumoral accumulation of CD8α⁺ DCs, which were essential for spontaneous antitumor CD8⁺ T cell priming and which could explain the critical role of this cell lineage in spontaneous cross-priming of tumor antigen–specific CD8⁺ T cells.

Given the lack of external TLR ligands for most malignancies, the molecular mechanism by which a tumor can provide the right environment to stimulate immune activation remains poorly understood. Emerging evidence indicates that dying cells can release endogenous adjuvants capable of activating APCs (Kono and Rock, 2008). Among these molecules are heat-shock proteins (Basu et al., 2000), uric acid (Shi et al., 2003), HMGB1 (high-mobility-group box 1; Scaffidi et al., 2002), ATP (Haag et al., 2007), and genomic double-stranded (ds) DNA (Ishii et al., 2001). It has been recently demonstrated that after radiotherapy or chemotherapy, dying tumor cells can release HMGB1 that binds to TLR4 (Apetoh et al., 2007) and ATP that activates the NALP3 inflammasome (Ghiringhelli et al., 2009). Presumably there is some degree of spontaneous tumor cell death either upon implantation of transplantable tumor cell lines or, as tumor growth exceeds the available blood supply during attempted neoangiogenesis. However, the massive tumor cell death occurring with chemotherapy or radiation is unlikely to be present in our system of spontaneous T cell priming, so other mechanisms could be operational. Recently, it has been shown that dsDNA can be released from necrotic cells, reach the cytoplasm of APCs, and be recognized by a TLR-independent

pathway leading to IRF3 activation and to type I IFN production (Stetson and Medzhitov, 2006a). It is interesting to speculate that a TLR-independent cytosolic DNA recognition pathway might be involved in innate tumor recognition, IFN-β production, and spontaneous priming of tumor antigen–specific T cells.

Our data have demonstrated that, shortly after tumor challenge, IFN-β is produced by CD11c⁺ DCs in tumor-draining lymph nodes. However, the identity of the specific subset of DCs producing type I IFNs in response to tumor growth remains unclear. The fact that IFN-β production is still observed in Batf3⁻/⁻ mice strongly suggest that the CD8α⁺ DC subpopulation is not required for type I IFN production. Preliminary studies of depletion of pDCs using the anti-PDCA (Krug et al., 2004) mAb have revealed that T cell priming and IFN-β production appear to be intact (unpublished data). Thus, it may be that conventional mDCs are capable of this function. Nonetheless, our data are consistent with a model in which at least two different DC subpopulations collaborate for the induction of spontaneous antitumor T cell priming. In this model, one DC subpopulation (likely either mDCs or pDCs) would sense the presence of the tumor and produce type I IFNs, which through signaling on CD8α⁺ DCs would promote effective cross-priming of CD8⁺ T cells. Consistent with this model, it has been recently shown using quantitative proteomics that the CD8α⁺ DC subpopulation selectively lacks the receptors and signaling molecules (such as DAI [Takaoka et al., 2007] and Sting [Ishikawa et al., 2009]) required for the detection of nucleotides in the cytoplasm (Luber et al., 2010), so if this is indeed the pathway involved in type I IFN production to tumor, a non-CD8α⁺ DC subpopulation would need to be involved.

Although several studies have suggested that CD8α⁺ DC distribution is restricted to lymphoid organs (Randolph et al., 2008) our results clearly indicate that CD8α⁺ DCs (defined as CD3⁻CD11c⁺B220⁻CD11b⁻CD8α⁺ cells) can infiltrate tumors. In agreement with our findings, it has been reported that CD8α⁺ DCs can infiltrate transplantable and spontaneous melanomas in B6 mice (Preynat-Seauve et al., 2006) and sarcomas in BALB/c mice treated with Flt3L and GM-CSF (Berhanu et al., 2006), and that such recruitment is associated with tumor rejection. Even though we found an augmented expression of XCR1 transcripts in tumors growing in wild-type hosts compared with type I IFN receptor–deficient mice, this difference could not be explained by a differential expression of its ligand, the chemokine XCL1, which was present in the tumor microenvironment in both hosts (unpublished data). The detailed mechanism by which type I IFNs induce the intratumoral accumulation of CD8α⁺ DCs will be a crucial area for future investigation.

It is noteworthy that, under conditions in which spontaneous priming of antitumor CD8⁺ T cells was not occurring, we detected expression of processed SIY peptide–Kᵇ complexes on the surface of several subsets of APCs. These results suggest that APC subtypes other than CD8α⁺ DCs are capable of processing antigen into the class I compartment.

Similar results have been reported by others. Hans Schreiber's laboratory has observed expression of processed tumor-derived antigen in tumor-infiltrating macrophages (Zhang et al., 2007). In addition, in the TRAMP model, tumor-infiltrating DCs have been suggested to express processed antigen and behave in a tolerogenic rather than an activating fashion (Anderson et al., 2007). Thus, although the CD8α+ DC subset is quantitatively superior at cross-presenting exogenous antigen into the class I compartment, it is likely that additional qualitative differences explain their ability to better initiate CD8+ T cell priming. Although it would have been ideal to characterize the DCs that had successfully processed antigen and then trafficked to the tumor-draining lymph node, we were unable to detect such cells with the TCR tetramer in the lymph node compartment, arguing that the presumably small number of cells is below the threshold of detection. It also should be pointed out that we studied DC subsets in the tumor microenvironment at relatively late time points, because in small tumors it simply wasn't technically possible to detect them reliably. So we can only infer that a similar defect in CD8α+ DC accumulation is occurring at early times after tumor implantation. Nonetheless, our subsequent experiments solidified a requirement for CD8α+ DCs, and for type I IFN signaling on these cells, in order to attain spontaneous CD8+ T cell priming.

It is currently unknown what dictates why tumors in some patients are capable of inducing spontaneous tumor-antigen specific T cell priming whereas others are not. Single nucleotide polymorphisms (SNPs) in different genes involved in the type I IFN pathway have been reported, including IFNAR (Muldoon et al., 2001) and Stat1 (Fortunato et al., 2008), that could affect levels of expression of the mature proteins, leading to variation in the response to type I IFNs. Alternatively, activation of distinct combinations of oncogenic pathways in individual tumors could lead to expression of distinct sets of genes that facilitate innate immune recognition in vivo.

The involvement of type I IFNs in antitumor immune responses has been appreciated for a number of years. Although early clinical trials of systemic administration of type I IFNs showed encouraging results for the treatment of a broad range of tumors (Neidhart et al., 1984; Motzer et al., 2002), the mechanism by which exogenously administered type I IFNs induces antitumor activity has remained elusive. In addition, injection of mice with blocking antibody to IFN-α/β has been reported to enhance tumor growth, suggesting the importance of endogenous type I IFNs after tumor challenge and a role in inhibiting tumor growth in immunocompetent mice (Gresser et al., 1983). Our findings now describe a link between spontaneous IFN-β production and signaling on CD8α+ DC which is essential for tumor antigen–specific CD8+ T cell priming. In addition, preliminary data have revealed potent rejection of B16 melanoma when transduced to express IFN-β (unpublished data). Collectively, our results have implications for human cancer therapy, providing a strong rationale for the intratumoral administration of type I IFNs, which are already FDA approved for other indications, in order to promote improved activation of tumor antigen–specific CD8+ T cells using the tumor itself as a source of antigen.

MATERIALS AND METHODS

Human samples and gene array analysis. Biopsy processing and gene array analysis were described previously (Harlin et al., 2009). Data were interrogated for expression of IFN-regulated genes and referenced to TCR transcripts in individual tumors.

Mice. C57BL/6 mice, 129 mice, and Stat1$^{-/-}$ mice were purchased from Taconic. For the indicated experiments, Stat1$^{-/-}$ mice were backcrossed for six generations onto DBA/2 mice (Jackson ImmunoResearch Laboratories). IFN-γR$^{-/-}$ mice were obtained from The Jackson Laboratory. IFN-αβR$^{-/-}$ mice and IFN-α/βR$^{-/-}$IFN-γR$^{-/-}$ mice were purchased from B&K Universal. Batf3$^{-/-}$ mice (Hildner et al., 2008) were obtained from K. Murphy (Washington University School of Medicine, St. Louis, MO). Experiments in these strains were done either on the 129 or the C57BL/6 background (at least 5 generations) with similar results. 2C/RAG2$^{-/-}$ mice (Sha et al., 1988) were obtained from D. Loh (Washington University School of Medicine, St. Louis, MO). CD11c-DTR mice (Jung et al., 2002) were provided by D. Littman (New York University School of Medicine, New York, NY). All mice were used between 6 and 10 wk of age and were maintained in specific pathogen–free conditions in a barrier facility at the University of Chicago (Chicago, Illinois). All animal experiments were performed in accordance with protocol approved by the University of Chicago Institutional Animal Care and Use Committee.

Tumor cell lines. The mutagenized DBA/2-derived mastocytoma cell line P198 and the C57BL/6 derived melanoma cell lines B16.F10 and B16.F10. SIY (henceforth referred to as B16.SIY), thymoma cell lines EL4 and EL4. SIY, fibrosarcoma cell lines MC-57 and MC-57.SIY, and the leukemia cell line C1498.SIY were used for experiments. All cells were maintained at 37°C with 7.5% CO$_2$ in DME supplemented with 10% heat-inactivated FCS, MOPS, 2-mercaptoethanol, penicillin, streptomycin, L-arginine, L-glutamine, folic acid, and L-asparagine.

In vivo tumor experiments. Cultured tumor cells were washed three times with Dulbecco's PBS (DPBS), and 10^6 living cells were injected s.c. in 100 µl DPBS on the flank. For tumor growth experiments, the longest and shortest diameters were measured twice per week using calipers, and a mean and SD were calculated. For ELISPOT, tetramer staining, and CFSE dilution splenocytes were analyzed at the indicated time points after tumor challenge. For RT-PCR analysis of IFN-β, tumor-draining inguinal lymph nodes were collected and analyzed 4 to 6 d after tumor challenge. For analysis of tumor-infiltrating DC subpopulations, tumors were recovered 15 d after tumor challenge and were disrupted in complete DME medium containing 1 mg/ml collagenase IV (Sigma-Aldrich) for 30 min at 37°C. Data from groups of three to seven mice were analyzed.

IFN-γ ELISPOT. The enzyme-linked Immunospot assay (ELISPOT) was conducted with the BD mouse IFN-γ kit according to the manufacturer's protocol. Splenocytes were plated at 10^6 cells/well and stimulated overnight with irradiated (10,000 rad) B16.SIY cells (5 × 10^4/well), SIY peptide (80 nM), or PMA (50 ng/ml) and ionomycin (0.5 µM). In experiments analyzing priming by BMDCs, the restimulation was performed in FCS-free culture medium and plates were not blocked. IFN-γ spots were detected using biotinylated antibody and avidin-peroxidase and developed using AEC substrate (Sigma-Aldrich). Plates were read in an Immunospot Series 3 Analyzer and analyzed with ImmunoSpot software (Cellular Technology Ltd).

Flow cytometry. Cells were incubated for 15 min at 4°C with anti-CD16 monoclonal antibody (2.4G2) to block potential nonspecific binding and

with the fluorochrome-coupled antibodies against the following molecules for 30 min at 4°C: CD3ε, CD4, CD8, CD11b, CD11c, CD40, CD45R (B220), CD80, CD86, H-2K^b, and I-A^b/I-E^b (BD) and PDCA (Miltenyi Biotec). For tetramer staining, cells were labeled with PE-MHC class I tetramers (Beckman Coulter or Proimmune) consisting of murine H-2K^b complexed to either SIYRYYGL (SIY) peptide or SIINFEKL (OVA) peptide, anti–CD8-APC, anti–B220-PerCP-Cy5.5, and anti–CD4-PerCP-Cy5.5. For CFSE dilution analysis, splenocytes were labeled with DAPI, anti–CD8-APC, and 1B2-PE (anti–2C-TCR; Kranz et al., 1984). For TCR tetramer staining, cells were stained with anti–CD11c-APC, anti–CD11b-PerCp.Cy5.5, and biotin-2C-m67 TCR tetramer (Holler et al., 2003), followed by streptavidin-PE. FACS analysis was performed using FACSCanto or LSR II cytometers with FACSDiva software (BD). Data analysis was conducted with FlowJo software (Tree Star). For DC sorting experiments, pooled tumor-draining lymph node cells were stained with anti–CD11c-APC and sorted in a FACSAria cell sorter (BD).

Quantitative real-time RT-PCR. Total RNA was purified using the RNeasy mini kit (QIAGEN) and analyzed in a 7300 Real Time PCR System (Applied Biosystems) using primer and probe sets from TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan-based quantification. The results are expressed as $2^{-\Delta Ct}$ using GAPDH or 18s as endogenous control.

BM chimeras. Mice were maintained on trimethoprim and sulfamethoxazole for at least 3 d before the start of the experiment. Groups of 3–5 mice were lethally irradiated (900 rad) and maintained on antibiotics. The following day, pooled tibial and femoral BM cells from donor mice were ACK-lysed and 10×10^6 cells were injected retroorbitally into recipient mice. Mice were allowed to reconstitute for at least 8 wk before tumor challenge.

Allogeneic MLR. MLR stimulation to generate effector cells for cytokine and CTL analysis was adopted from a previously published protocol (Gajewski et al., 1995). Total T cells were purified from spleens by negative selection with antibodies and magnetic beads from StemCell Technologies according to the manufacturer's protocol. These responder cells were plated at 10^6/well containing stimulator cells consisting of allogeneic T cell–depleted irradiated (5,000 rad) splenocytes at 5×10^6/well. After 5 d, cells were used in chromium release assays and ELISA.

Chromium release assay. Chromium release assays were performed as previously described (Kacha et al., 2000). Briefly, ^{51}Cr-labeled targets (2×10^3) were plated with effectors cells at the indicated E/T ratios from 100:1 to 3.7:1. After 4 h of incubation at 37°C, 50 μl of supernatant was transferred to a LumaPlate-96 (PerkinElmer) and allowed to dry overnight. Plates were then counted using a TopCount-NXT plate reader (PerkinElmer). Percent specific lysis was calculated using standard methods.

Cytokine ELISA. For cytokine analysis, tissue culture–treated 96-well flat bottom plates were coated with either DPBS alone, 2C11 (1 μg/ml; anti-CD3ε), or 2C11 and PV-1 (2 μg/ml; anti-CD28) in DPBS overnight at room temperature and washed with culture medium. Effector cells were incubated on the antibody-coated plates overnight, and supernatants were collected for measurement of IFN-γ concentration by ELISA. Mouse IL-2 and IFN-γ antibody sets were obtained from BD. Cytokine concentrations were determined with the Softmax PRO data analysis program (Molecular Devices).

2C CD8$^+$ T cells purification, CFSE staining, and adoptive transfer. CD8$^+$ T cells were purified from spleens of 2C/RAG2$^{-/-}$ mice by negative selection with antibodies and magnetic beads from StemCell Technologies according to the manufacturer's protocol. T cells were stained with 2.5 mM CFSE at room temperature for 6 min and thoroughly washed with an excess volume of cold FCS. 10^7 CFSE-labeled T cells in 100 μl of DPBS were transferred by retroorbital injection into venous plexus of anesthetized mice. Mice were challenged with 10^6 B16.SIY cells, and 7 d later splenocytes from recipient mice were analyzed by flow cytometry.

BMDC immunization. BMDCs were generated according to a modified version of a published protocol (Inaba et al., 1992). BM cells from the tibiae and femora were ACK-lysed and incubated for 10 d in complete DME medium with 20 ng/ml rmGM-CSF (R&D Systems) with the addition of 200 ng/ml LPS (Sigma-Aldrich) for the last 24 h. On day 10, cells were exposed to 10 μM SIY peptide for 1 h at 37°C and washed 3 times with DPBS. Mice were injected s.c. in the flank with 10^6 BMDCs in 100 μl DPBS.

Adherent splenocyte stimulation of CD8$^+$ T cells. Splenocytes were ACK-lysed, irradiated (5,000 rad), and plated at 10^6 cells/well. After 2 h of incubation, nonadherent cells were removed by 2 washes with DPBS. To stimulate CD8$^+$ T cells, SIY peptide (10 μM) or culture medium was added to the adherent splenocytes, followed by the addition of 5×10^4 naive 2C/RAG2$^{-/-}$ CD8$^+$ T cells/well. Supernatants were collected after 18 h and IL-2 production was determined by ELISA.

Statistical methods. Differences between datasets were analyzed with the two-sided Student's t test, and correlation was analyzed with Pearson test and Prism software (GraphPad).

Online supplemental material. Fig. S1 shows that in CD11c-DTR transgenic mice treated with diphtheria toxin, the majority of CD8α$^+$ DCs and pDCs, and a fraction of mDCs, were depleted and that these cell populations are critical for spontaneous CD8$^+$ T cell priming to tumor-associated antigens. Fig. S2 shows that CD8α$^+$ DCs fail to accumulate in the tumors grown in Stat1$^{-/-}$ mice. Fig. S3 shows that parental and SIY-expressing B16 melanomas grow faster in Batf3$^{-/-}$ and IFN-α/βR$^{-/-}$ mice compared with wild-type. Table S1 shows selected immune-related genes up-regulated with B16 tumors, including a panel of IFN-inducible genes. Table S2 shows that there is no difference in surface expression levels of CD40, CD80, and CD86 in CD11c$^+$ tumor-draining lymph node cells from WT and IFN-α/βR$^{-/-}$ mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101159/DC1.

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