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Type I interferons inhibit the generation of tumor-associated macrophages

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Abstract

Tumor-associated macrophages (TAM) are very abundant in tumors and are thought to play a major role in promoting tumor growth. The generation of TAM is positively regulated by several cytokines, including colony stimulating factor-1 (CSF-1) and monocyte chemoattractant protein-1 (CCL2). However, endogenous factors that suppress the generation of TAM within tumors have not been previously identified. An earlier study showed that endogenously produced type I interferons (IFN) suppressed tumor growth via their effects on hematopoietic cells rather than through direct effects on tumor cells. Therefore, we used mouse tumor models to investigate the effects of endogenously produced type I IFNs on the generation of TAM. We found using immunohistochemistry and flow cytometry that TAM density was significantly increased in tumors of mice lacking the type I IFN receptor (IFN- α/β R^{-/-} mice) compared to wild type mice. Moreover, the increase in TAM density was associated with a significant increase in tumor growth rate and angiogenesis. The phenotype of TAM was similar in IFN- α/β R^{-/-} mice and wild type mice and tumors in both mice produced similar amounts of CSF-1 and CCL2. However, in vitro assays indicated that low concentrations of type I IFNs significantly inhibited the generation of bone marrow macrophages in response to CSF-1. These findings indicate that endogenously produced type I IFNs suppress the generation of TAM, which may in turn account for inhibition of tumor growth and angiogenesis.

Keywords

Cytokine; CSF-1; Cancer; Angiogenesis

Introduction

Therapeutic administration of high doses of recombinant IFN- α has been used to inhibit tumor angiogenesis and growth [1–4]. The effects of IFN- α are mediated in part by suppression of endothelial cell motility and survival due to inhibition of production of pro-angiogenic factors such as VEGF, basic fibroblast growth factor (bFGF), and interleukin-8 [1,5–8]. It is also known that low level endogenous production of IFN- α and IFN- β (collectively, the type I IFNs) can suppress tumor growth and inhibit tumor angiogenesis, though the immunological mechanisms responsible for this effect are not well understood. For example, tumor growth and angiogenesis was found to be significantly increased in IFN α/β R $^{-/-}$ mice, which lack a functional type I IFN receptor [9]. In another study, it was also shown that the inhibitory effects of endogenously produced type I IFNs on tumor growth were mediated by their effects on hematopoietic cells, rather than through direct effects on tumor cells [10]. However, it was not established in the prior studies which hematopoietic cells were the targets for the tumor inhibitory effects of type I IFNs.

Tumor-associated macrophages (TAM) are a potential target for the tumor inhibitory effects of endogenous type I IFNs [11,12]. For example, increased numbers of TAM has been found to correlate with reduced survival times in human cancer patients, including patients with breast, prostate, and endometrial cancer [13–15]. In mice with reduced numbers of TAM due to lack of CSF-1 production, there was also decreased tumor growth and decreased tumor angiogenesis [16]. In addition, chemical depletion of TAM in mouse tumor models has also been shown to decrease tumor growth and angiogenesis [17,18]. TAM can promote tumor growth by stimulating tumor cell invasion into surrounding tissues, by increasing tumor cell growth and survival, by triggering tumor metastasis, and by stimulating angiogenesis [11,12]. For example, TAM may stimulate tumor angiogenesis by producing VEGF in hypoxic regions of the tumor [19,20]. TAM are thought to be derived primarily from circulating monocytes, which are recruited into tumor tissues in response to cytokines such as CCL2 and CSF-1 [21, 22].

The survival and differentiation of monocytes into TAM is critically dependent on local tumor production of CSF-1 [23]. Both experimentally and clinically, CSF-1 production by tumors correlates positively with TAM density [16,24]. A connection between type I IFNs and CSF-1 was noted previously in *in vitro* studies, where it was found that low concentrations of type I IFNs inhibited the generation and proliferation of bone marrow derived macrophages in response to exogenous CSF-1 [25,26].

Therefore, we conducted studies to investigate the effects of endogenous type I IFNs on the generation of TAM, using mouse tumor models. The effects of endogenous type I IFNs on tumor growth, tumor angiogenesis, and the density and phenotype of TAM were investigated, using wild type tumors implanted subcutaneously in IFN- α/β R $^{-/-}$ and wild type mice. Production of pro-angiogenic cytokines and monocyte/macrophage regulatory chemokines by tumor tissues or purified TAM was assessed using specific ELISA or by real-time RT-PCR analysis. The effects of type I IFNs and tumor conditioned medium on generation of bone marrow macrophages was evaluated *in vitro*. Based on the results of these studies, we concluded that suppression of TAM generation may account for the tumor growth inhibitory effects of endogenously produced type I IFNs.

Materials and methods

Animals

Breeding pairs of mice lacking the type I interferon receptor (IFN α/β R $^{-/-}$) on the 129 Sv/Ev background were kindly provided by Dr. Philippa Marrack (National Jewish, Denver, CO) and

Dr. Michel Auget (Swiss Institute for Experimental Cancer Research, Ecole Polytechnique Fédérale de Lausanne, 155, Chemin des Boveresses, 1066 Epalinges s/Lausanne, Switzerland). Age and sex-matched wild type 129 Sv/Ev mice, containing a fully functional type I interferon receptor, were obtained from Taconic Laboratories (Germantown, NY). Animal studies were approved by the Institutional Animal Care and Use Committee at Colorado State University.

Tumor cell lines and tumor models

A tumor cell line (MCA 1.2) derived from 129 Sv/Ev mice was generated by s.c. injection of methylcholanthrene (MCA) in peanut oil. The cutaneous fibrosarcoma tumor that developed was passaged in vivo twice in wild type 129 mice. The MCA 1.2 tumor cells were maintained at low passage number in modified Eagle's complete medium with 10% FBS, 0.2 mM L-glutamine, 1 mM HEPES buffer, and 0.1 mM nonessential amino acids, 50 IU/ml penicillin and 50 µg/ml streptomycin (all from Invitrogen, Carlsbad, CA), and incubated at 37°C in 5% CO₂. To assure mycoplasma-free conditions, cells were routinely tested by MycoSensor PCR Assay Kit (Stratagene, La Jolla, CA). Tumors were established in either wild type or IFN α / β R^{-/-} mice by the intradermal inoculation of 1×10^6 MCA2.1 cells over the hip. Tumor diameters were measured every 2–3 days using calipers and mice were euthanized once the tumor diameter reached 1.0 cm.

Tumor immunohistochemistry

Tumor tissues were embedded in Tissue-Tek OCT compound (TedPella Inc., Redding, CA) and snap-frozen in isopentane. Tissues were cryosectioned to a thickness of 4 µm and adhered to glass slides (Superfrost, VWR International, West Chester, PA). For assessment of macrophage infiltration, macrophage specific monoclonal antibodies F4/80 (clone BM8, eBiosciences) and CD68 (clone FA11, Serotec, Raleigh, NC) were utilized. After fixation in acetone and blocking nonspecific binding, tissues were incubated first with the primary antibody, followed by donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA), then with streptavidin–HRP and AEC substrate (Vector Laboratories, Burlingame, CA), followed by hematoxylin counterstain. For assessment of CCR2 expression by tumor macrophages, sections were incubated with a goat anti-CCR2 antibody (Abcam, Cambridge, MA), followed by the appropriate secondary antibody. Negative controls included incubation with irrelevant mAb and omission of the primary antibodies.

For quantitation of tumor microvessel density (MVD), a mAb directed against murine CD31 (clone 309, eBiosciences, San Diego, CA) was used. Staining of acetone fixed tissues was carried out as above. Four photomicrographs at 10 $\times$ magnification were obtained by digital camera (Leica Microscope equipped with digital camera with SPOT Advanced Imaging software). Utilizing Adobe Photoshop and Reindeer Graphics Quantitative Analysis Plug-ins (Asheville, NC), the photomicrographs were converted to binary images and the number of microvessels per section was determined digitally, as described previously [27]. MVD values for each tumor sample were calculated based on the average number of vessels for 4 fields.

Flow cytometry and cell sorting

Flow cytometry was used to assess and quantitate leukocytes infiltrating tumor tissues. Tumor tissue was collected, minced, digested for 20 min in a solution of 1.5 mg/ml collagenase, 65 µg/ml soybean trypsin inhibitor, 1.5 µg/ml DNase I (all from Sigma Chemical Co, St Louis, MO). Single cell suspensions from digested tumor tissues were prepared by Ficoll density gradient centrifugation (LSM Lymphocyte Separation medium; MP Biomedicals, Aurora, OH). Single cells suspensions were resuspended in FACS buffer (PBS with 2% FBS and 0.1% sodium azide) at a concentration of 2.5×10^5 to 1×10^6 cells per well. The cells were then immunostained with directly labeled antibodies, all purchased from eBiosciences (San Diego, CA unless otherwise noted): anti-F4/80 (APC; clone BM8), CD11b (APC-Cy7 clone M1/70),

Gr-1 (PE-Cy7; clone RB6-8C5), and NKG2D (biotin, clone CX5). Nonspecific binding of antibodies was blocked by pre-incubation of cells in normal mouse serum and anti-FcRIII antibody (clone 24.G2), plus 0.2 mg/ml human immunoglobulin IgG. Staining was carried out at 4°C for 20 min, followed by washing in FACS buffer. In most cases, cells were fixed in 1% paraformaldehyde for 30 min and stored in FACS buffer at 4°C before analysis. Flow cytometry was performed using a Cyan ADP flow cytometer (Dako-Cytomation, Ft Collins, CO). Data analysis was carried out using Summit software (Dako-Cytomation).

Tumor-associated macrophages (TAM) were purified by flow cytometric sorting, using a MoFlo cell sorter (Dako-Cytomation). Briefly, tumor tissues from wild type and IFN- α / β R^{-/-} mice were prepared by enzymatic digestion and Ficoll density gradient purified as described above. The cells were then resuspended in balanced salt solution with 2% FBS and immunostained with antibodies to CD11b and F4/80. The cells were then positively sorted for CD11b and F4/80 double-positive cells. The purity of the sorted population was routinely >85%. Total RNA was extracted from sorted macrophages and used for quantitative real time PCR analysis.

ELISA assays for cytokines

In some experiments, tumor conditioned medium was generated by collecting freshly harvested tumor tissues, and under sterile conditions cut and weighed the tumor tissues into approximately 1 mm cubes, and then placing 30 mg of tumor tissue in 1 ml of serum-free media (X-Vivo 15, Biowhittaker, Walkersville, MD) in a tissue-culture incubator for 24 h. The supernatants were then harvested and frozen at -70°C until ELISA analysis was performed. The following cytokine assay kits were used to quantitate cytokine concentrations in tumor conditioned medium. Colony stimulating factor-1 (R&D Biosystems, Minneapolis, MN), CCL2 (MCP-1) (BD Biosciences, San Jose, CA), VEGF (R&D Biosystems), KC (R&D Biosystems), IL-1 β (R&D Biosystems) IFN- γ (R&D Biosystems) and CSF-1 (R&D Biosystems). ELISAs were performed as per manufacture's instructions. For detection of mouse IFN- α an ELISA was developed as previously described using a rat anti-mouse IFN- α capture antibody (PBL, Piscataway, NJ) and a rabbit anti-IFN- α detection antibody (US Biological, Swampscott, MA) [28,29]. ELISAs were developed with tetramethylbenzidine substrate (Sigma Chemical Co, St Louis, MO) and absorbance measured at 450 nm with an optical plate reader (Thermo Labsystems, Waltham, MA).

Macrophage migration assay

A transwell migration was performed as previously described [30]. Non-elicited peritoneal macrophages (PM) were lavaged from the abdomen of wild type and IFN α / β R^{-/-} mice with 5 ml of ice cold hanks. PM were washed twice and resuspended in serum-free media at a final concentration of 2×10^6 cells/ml. 100 μ L of cells were added to the upper chamber of a 24-well transwell with 8 μ m pore size (BD biosciences, Bedford, MA). Cells were allowed to equilibrate for 30 min at 37°C in 5% CO₂. As a positive control stimulus for macrophage migration, 100 ng/ml recombinant murine CSF-1 (PeproTech Inc, Rock Hill, NJ) was used. The effects of tumor conditioned medium on macrophage migration were evaluated. Samples for testing were added to the lower chamber of the transwell plate. After 3 h incubation at 37°C in 5% CO₂, the cells remaining on the upper chamber were wiped off with a cotton swab. The cells that migrated to the lower filter of the membrane were fixed in 75% ethanol, washed with water, and stained with hemotoxylin. Migrated cells were quantified by averaging the counts of 5 random 200 $\times$ fields.

Bone marrow macrophage culture

Bone marrow macrophage cultures were generated from wild type or IFN α / β R^{-/-} mice. Briefly, bone marrow was flushed from the femurs and tibias with balanced salt solution plus 2% FBS,

as described previously [31,32]. Cells were washed and RBC were lysed using NH_4Cl solution. Cells were plated at 2×10^5 /well in a 96 well plate (Corning Lifesciences, Acton, MA) in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS, 0.2 mM L-glutamine, 1 mM HEPES buffer, and 0.1 mM nonessential amino acids, 50 IU/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin (all from Invitrogen, Carlsbad, CA) and incubated at 37°C in 5% CO_2 for 2 h. After 2 h, non-adherent cells were removed and the cells were re-fed complete medium. Adherent macrophages were cultured for 3–5 days prior to assay.

Quantitation of macrophage survival and proliferation

Numbers of viable macrophages in culture were quantitated using an MTT (3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which was performed as previously described [33]. Briefly, cells were pulsed for 2 h with MTT solution (Sigma-Aldrich), then the cells were lysed with acidified propanol and the optical density in lysed wells was determined by optical density reader (Multiscan Ascent, Thermo Labsystems, Cambridge, MA). Macrophage proliferation was quantitated by ^3H thymidine uptake. Briefly, cells were pulsed overnight with 1 μCi ^3H (Amersham Biosciences, Waltham, MA), then harvested by automated beta counter (Wallac Microbeta, Waltham, MA). Proliferation was expressed as counts per minute incorporated ^3H .

Effects of CSF-1 and type I IFNs on macrophage generation

The effects of endogenous CSF-1 produced by tumor cell lines on the generation of bone marrow macrophages were assessed. Adherent bone marrow macrophages from wild type and $\text{IFN-}\alpha/\beta\text{R}^{-/-}$ mice were incubated in serial dilutions of tumor-conditioned medium. Tumor conditioned medium was generated by incubating confluent cultures of MCA2.1 tumor cells for 72 h, then removing the medium, sterile filtering, and freezing aliquots at -80°C prior to use. As a positive control, cells were also incubated with 50 ng/ml recombinant murine CSF-1 (PeproTech Inc). CSF-1 concentrations in tumor conditioned medium were also assessed by ELISA.

Endogenous CSF-1 in tumor conditioned medium was neutralized by incubating tumor-conditioned medium with 50 ng/ml CSF-1 neutralizing polyclonal Ab (goat anti-mouse, R&D Biosystems, Minneapolis, MN). Equivalent amounts of purified irrelevant goat IgG (R&D Systems) was used as an irrelevant antibody control. Cultures were incubated at 37°C in 5% CO_2 for 96 h. Viable bone marrow macrophages were quantitated using MTT assay.

The ability of recombinant type IFN to inhibit the responsiveness of bone marrow macrophages to exogenous CSF-1 was also assessed. Freshly adherent bone marrow macrophages from wild type and $\text{IFN-}\alpha/\beta\text{R}^{-/-}$ mice were treated with 40 ng/ml recombinant mouse CSF-1 (R&D Biosystems) alone or with CSF-1 + 25 ng/ml recombinant mouse $\text{IFN-}\alpha$ or 25 ng/ml recombinant mouse $\text{IFN-}\beta$ (both from PBL Biomedical Laboratories, Piscataway, NJ). Cultures were incubated at 37°C in 5% CO_2 for 96 h and macrophages numbers were quantitated by MTT assay as noted above.

Quantitative RT-PCR

Tumor and macrophage mRNA expression was assessed using quantitative real time RT-PCR. Tumor tissues were homogenized in lysis buffer using a rotostator tissue homogenizer. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA) and an on-column DNase treatment was performed (Qiagen). 1 μg of RNA was used to generate first strand cDNA using a MMLV reverse transcription kit as per manufactures protocol (Invitrogen). PCR was carried out in 25 μl reaction volumes using iQ SYBR Supermix (Bio-Rad, Hercules, CA) and amplification was carried out using the iQ iCycler system (Bio-Rad). Negative controls were preformed with water and primer specificity was assessed by melt curve analysis. All PCR

reactions were preformed in duplicate and the average Ct value was used to calculate relative expression when normalized to HPRT housekeeping gene for each sample. The primer sequences used were previously published and included primers for mouse *c-fms*, CCR2, CXCL9, CXCL10, bFGF, PDGFA-D, UPA, MMP9, EGF and HPRT [34–40].

Statistical analyses

For comparisons between two treatment groups, Student's *t* test was used. In experiments with multiple groups of mice, statistical differences between treatment groups were compared using ANOVA and Tukey's multiple means comparisons test. Statistical analyses were done using Prism5 software (GraphPad, San Diego, CA). A *p* value <0.05 was considered statistically significant for these analyses.

Results

Endogenous production of type I interferons suppresses tumor growth

Treatment with high doses of recombinant IFN- α has been shown previously to inhibit tumor growth and angiogenesis [1,2,41–43]. However, in the current study we wished to investigate the role of endogenous production of type I IFNs in regulating the generation of TAM. In a previous study, it was determined that growth of primary tumors was significantly increased in IFN- α/β R^{-/-} mice [9]. In those studies growth of human melanoma xenografts was evaluated in a *nu/nu* mouse tumor model. In the current study, we used a syngeneic tumor model in immune competent mice to investigate in greater detail the mechanisms that might account for the increased tumor growth and angiogenesis observed previously in the IFN- α/β R^{-/-} mice.

First, tumor growth kinetics were compared between wild type mice and IFN- α/β R^{-/-} mice (*n* = 5 per group) challenged with wild type syngeneic MCA 2.1 tumor cells. As in the previous studies in *nu/nu* mice, we also found that the rate of tumor growth was significantly increased in IFN- α/β R^{-/-} mice compared to wild type animals (Fig. 1). Since the MCA2.1 tumor was derived from an IFN- α/β R^{+/+} mouse, these results indicated that the inability of host cells rather than tumor cells to respond to endogenously type I IFNs most likely accounted for the observed differences in tumor growth rates. We also noted that serum concentrations of IFN- α and IFN- β were undetectable in both wild type and IFN- α/β R^{-/-} mice with tumors (data not shown).

Endogenous production of type I IFNs suppresses tumor angiogenesis

Since tumor growth was significantly increased in IFN- α/β R^{-/-} mice, we next investigated mechanisms that might account for accelerated tumor growth. First, tumor angiogenic responses were assessed in IFN- α/β R^{-/-} mice versus wild type mice. We found that tumors in IFN- α/β R^{-/-} mice had a significantly higher MVD than did tumors in wild type mice (Fig. 2). This result was in agreement with a previous report, though as noted before that study used human tumor xenografts in immunodeficient mice [9].

Production of angiogenic cytokines by tumors in wild type and IFN- α/β R^{-/-} mice

It is possible that endogenously produced type I IFNs may inhibit tumor angiogenesis by suppressing the production of pro-angiogenic cytokines. Therefore, we investigated whether concentrations of IL-1 β , VEGF, and IL-8 were increased in tumor tissues from IFN- α/β R^{-/-} mice compared to tumors from wild type mice. To address this question, tumor explants from wild type and IFN- α/β R^{-/-} mice (*n* = 4 mice per group) were cultured in vitro in complete medium for 24 h and concentrations of IL-1 β , VEGF, and KC (mouse homolog of IL-8) in supernatants were determined by specific ELISA. We found that although all 3 cytokines were

spontaneously secreted by tumor tissues in vitro, there were no significant differences in cytokine concentrations between wild type versus IFN- α/β R^{-/-} mice (data not shown).

We also assessed the expression of additional genes known to be associated with tumor angiogenesis, using real time PCR. Total RNA was extracted from tumor tissues of wild type and IFN- α/β R^{-/-} mice ($n = 4$ per group) and was subjected to quantitative PCR amplification. Gene expression levels of the following pro-angiogenic and anti-angiogenic factors were assessed: bFGF, platelet derived growth factor (PDGFA-D), (matrix metalloproteinase) MMP-9, (urokinase) uPA, CXCL9, CXCL10, and epidermal growth factor (EGF). Primers were designed based on published primer sequences, as noted in "Materials and methods". Using this approach, we did not detect significant differences in the level of gene expression for any of these 5 genes evaluated in tumor tissues of wild type versus IFN- α/β R^{-/-} mice (data not shown). We concluded therefore that differences in tumor expression of bFGF, PDGF, CXCL9, CXCL10, MMP-9, uPA, and EGF did not account for either the increased tumor angiogenesis or the increased rate of tumor growth noted in IFN- α/β R^{-/-} mice.

Macrophage density is significantly increased in tumors of IFN- α/β R^{-/-} mice

Since macrophage density is known to correlate with angiogenic responses, we next compared the number of TAM in tumors from wild type and IFN- α/β R^{-/-} mice. Using flow cytometry, we found that the number of CD11b⁺/F4/80⁺ macrophages was significantly increased ($p < 0.05$) in tumor tissues of IFN- α/β R^{-/-} mice compared to tumors in wild type animals (Fig. 3).

To further assess macrophage infiltration in tumors, tumor tissues were examined by immunohistochemistry. We observed that tumor tissues from IFN- α/β R^{-/-} mice had marked increases in the density of F4/80⁺ macrophages compared to tumors from wild type mice (Fig. 4a, b). In addition, immunohistochemical analysis also revealed a marked increase in the density of CD68⁺ macrophages in tumors from IFN- α/β R^{-/-} mice (Fig. 4c, d). The results of these analyses indicated therefore that the density of tumor infiltrating macrophages was another major difference between tumors of wild type and IFN- α/β R^{-/-} mice.

However, the increase in leukocytes in tumors of IFN- α/β R^{-/-} mice was restricted to macrophages. For example, significant differences in the numbers of tumor-infiltrating CD11b⁺/Gr-1⁺ neutrophils were not found (Fig. 5a). Nor were the numbers of CD11c⁺ DC or NKG2D⁺ NK cells in tumor tissues significantly different between wild type and IFN- α/β R^{-/-} mice (Fig. 5b, c). However, based on the phenotype of the neutrophils assessed in this study, we cannot exclude that some of these cells may have also represented myeloid suppressor cells [44,45]. We also did not observe significant differences in the numbers of CD4⁺ or CD8⁺ T cells in tumors of wild type versus IFN- α/β R^{-/-} mice (data not shown). Thus, we concluded that CD11b⁺/F480⁺ TAM were the primary hematopoietic cell type that exhibited significant differences in tumors of wild type versus IFN- α/β R^{-/-} mice.

Production of CCL2 and CSF-1 are not significantly different in tumors of wild type and IFN- α/β R^{-/-} mice

We next investigated mechanisms that might account for the increased density of TAM in tumors of IFN- α/β R^{-/-} mice. The chemokine CCL2 is a key regulator of monocyte recruitment into tissues, where they then differentiate into macrophages [21,22,46,47]. Therefore, we measured the concentration of CCL2 released spontaneously by tumor tissues from wild type and IFN- α/β R^{-/-} mice following overnight in vitro culture. We found that CCL2 production by tumors from IFN- α/β R^{-/-} mice was not significantly different from CCL2 production by tumors from wild type mice (Fig. 6). In addition, CCL2 concentrations in lysates prepared from freshly collected tumor tissues were also not significantly different between IFN- α/β R^{-/-} and wild type mice (data not shown).

CSF-1 production by tumor tissues was also assessed, since CSF-1 is a major regulator of both monocyte recruitment and differentiation as well as macrophage survival in tissues [16,21, 23,48,49]. After overnight culture of tumor biopsies, the concentrations of CSF-1 released into supernatants were not significantly different between tumors of wild type and IFN- α/β R^{-/-} mice (Fig. 6). In addition, CSF-1 concentrations in tumor lysates prepared without culture were also not significantly different (data not shown). Therefore, we concluded that differences in tumor production of CCL2 or CSF-1 were not responsible for the striking differences in TAM density that were observed in tumors of IFN- α/β R^{-/-} mice compared to wild type mice.

To rule out the possibility that TAM expression of receptors for CCL2 or CSF-1 might account for the differences in macrophage infiltration of tumors in wild type and IFN- α/β R^{-/-} mice, the surface expression of CCR2 (the CCL2 receptor) and *c-fms* (the CSF-1 receptor) on purified TAM isolated from tumor tissues was assessed. The TAM were purified by FACS sorting, using F4/80 and CD11b expression, from enzymatically digested tumor tissues. Real-time PCR analysis revealed that transcription of *c-fms* and CCR2 genes was not significantly different between wild type and IFN- α/β R^{-/-} mice (data not shown). We also assessed CCR2 expression on tumor tissues by IHC and found only very low levels of expression in tumor tissues of wild type mice and undetectable expression in tumors of IFN- α/β R^{-/-} mice (data not shown). These results suggested therefore that differences in expression of the receptors for CCL2 or CSF-1 by TAM were unlikely to account for the observed differences in the density of TAM in tumors of wild type versus IFN- α/β R^{-/-} mice.

Monocyte migration in wild type and IFN- α/β R^{-/-} mice

The marked increase in TAM in tumors of IFN- α/β R^{-/-} mice could also have resulted from increased recruitment of monocytes into the tumors from the bloodstream in response to stimuli other than CCL2 or CSF-1. For example, it could be possible that monocytes and macrophages from mice lacking the type I IFN receptor were more sensitive to chemokines produced by tumor tissues than monocytes or macrophages from wild type mice. To address this question, we evaluated whether tumor-conditioned medium elicited differential migratory responses in macrophages obtained from wild type compared to IFN- α/β R^{-/-} mice. Using a transwell migration assay similar to what has been reported previously for use with PM, we found that migratory responses to tumor-conditioned medium were similar between peritoneal lavage cells from wild type and IFN- α/β R^{-/-} mice (Fig. 7) [50]. In addition, peritoneal lavage cells from both types of mice responded equivalently to a CCL2 gradient (Fig. 7). Thus, differential responsiveness to macrophage recruitment signals also did not appear to explain the increase in TAM density noted in tumors of IFN- α/β R^{-/-} mice.

Type I IFNs inhibit macrophage responsiveness to CSF-1

Type I IFNs have been shown previously to inhibit the responsiveness of bone marrow macrophages to CSF-1 in vitro [25]. Moreover, it is also known that CSF-1 stimulates release of type I IFNs, thus establishing a potential autoregulatory loop [26]. However, the effects of type I IFNs on CSF-1 responsiveness have not been previously evaluated in the context of tumors or TAM, nor have the specific effects of recombinant IFN- α or IFN- β on macrophage responsiveness to CSF-1 been assessed.

Therefore, we first determined whether tumor cells spontaneously produced type I IFNs. By ELISA, we found that in vitro cultured MCA2.1 tumor tissues spontaneously released low concentrations of IFN- α (in the range of 5 pg/ml; data not shown). Concentrations of IFN- β in cultured tumor supernatants were below the level of detection of the ELISA (<100 pg/ml; data not shown). By quantitative real-time PCR, low levels of transcription of both IFN- α and IFN- β genes in tumor tissues were detected (data not shown). Thus, the tumor cells used in our studies spontaneously produced low levels of type I IFNs.

Next, *in vitro* assays were used to determine whether the low levels of type I IFNs produced by tumor cells had an effect on the generation of macrophages. Bone marrow derived macrophages isolated from wild type or IFN- α/β R^{-/-} mice by brief adherence to tissue culture plastic were incubated with serial dilutions of supernatants from cultured tumor tissues. We observed that tumor conditioned medium strongly stimulated the development of macrophages in this assay (Fig. 8). By 96 h in culture, greater than 80% of the adherent cells were found to be CD11b⁺/F480⁺ macrophages (data not shown). To determine the extent to which the generation of macrophages by tumor conditioned medium was dependent on CSF-1, a neutralizing antibody was used to deplete CSF-1 from tumor supernatants (Fig. 8). Treatment of supernatants with CSF-1 neutralizing antibody significantly reduced ($p < 0.05$) the number of macrophages generated in culture, indicating that the major macrophage stimulatory factor present in tumor conditioned medium was CSF-1. Importantly, we also noted that the generation of macrophages was significantly greater ($p < 0.001$) in bone marrow macrophage cultures derived from IFN- α/β R^{-/-} mice, compared to cultures derived from wild type mice.

The bone marrow macrophage assay was also used to directly compare the inhibitory effects of recombinant murine IFN- α and IFN- β on macrophage generation in response to recombinant CSF-1. First, we noted that the bone marrow macrophage response to recombinant CSF-1 was similar in wild type and IFN- α/β R^{-/-} bone marrow cells (Fig. 9). However, when wild type macrophages were cultured with recombinant CSF-1 (25 ng/ml) plus recombinant IFN- α (25 ng/ml), there was significant inhibition (67% inhibition; $p < 0.001$) of macrophage generation. Similarly, culture of macrophages with CSF-1 plus recombinant IFN- β (25 ng/ml) also resulted in significant inhibition (87% inhibition; $p < 0.001$) of macrophage generation. These experiments were repeated, using cell proliferation as the read-out (Fig. 10). Very similar results were also seen with respect to cell proliferation, with significant inhibition ($p < 0.01$) of proliferation observed following addition of recombinant IFN- α or IFN- β to the macrophage cultures. These results indicated that IFN- α and IFN- β appeared to be equally effective in suppressing the macrophage response to CSF-1.

Finally, when bone marrow macrophages derived from IFN- α/β R^{-/-} mice were incubated with CSF-1 plus either IFN- α or IFN- β , the inhibitory effects of IFN- α and IFN- β were almost completely eliminated. Thus, we concluded that type I IFNs potently suppressed the stimulatory effects of CSF-1 on macrophages. Based on the preceding results, it was apparent that the significant decrease in generation of macrophages in response to tumor-conditioned medium observed in wild type bone marrow cultures (Fig. 8) compared to bone marrow from IFN- α/β R^{-/-} mice was most likely mediated by the low concentrations of type I IFNs present in the tumor conditioned medium. Taken together, these results indicated that in wild type animals, type I IFNs generated in tumors blocked the macrophage stimulatory effects of CSF-1 and suppressed the generation of TAM.

Discussion

Studies were conducted here to investigate the role of type I IFNs in regulating the numbers of TAM in tumor tissues. A key finding was that the density of TAM was markedly increased in tumors developing in IFN- α/β R^{-/-} mice. The tumors also grew more rapidly and had significantly increased angiogenesis in the IFN- α/β R^{-/-} mice. The low concentrations of type I IFNs that were spontaneously released by tumor cells were sufficient to inhibit the macrophage stimulatory properties of CSF-1. Thus, endogenously produced type I IFNs appeared to be important negative regulators of the generation of TAM in tumors.

These findings are important because TAM are acknowledged as playing a major role in promoting tumor growth, invasion, and metastasis. For example, TAM stimulate angiogenesis by producing pro-angiogenic molecules such as VEGF, IL-8, bFGF, and angiopoietin [12,

51]. Evidence linking macrophage infiltration in tumors and angiogenesis has recently been provided by studies in *op/op* mice, which fail to produce CSF-1 [16,52,53]. In addition, chemical depletion of TAM using liposomal clodronate has also been shown to elicit a significant decrease in tumor angiogenesis [18]. Thus, we propose that the increased tumor growth rate and angiogenic responses noted in tumors in $\text{IFN-}\alpha/\beta\text{R}^{-/-}$ mice in the present study were a consequence of the greatly increased numbers of TAM present in these tumors.

While it is known that CSF-1 plays an important role in recruiting and promoting the survival of TAM in tumors, much less is known about factors that may suppress macrophage generation within tumors. Therefore, identification of type I IFNs as significant inhibitors of TAM generation has important implications. For one, it is possible that inhibition of tumor angiogenesis by recombinant $\text{IFN-}\alpha$ may be due in part to inhibition of TAM generation, in addition to the direct inhibitory effects of $\text{IFN-}\alpha$ on endothelial cell migration [1,6,54]. Although we found that type I IFNs did not affect macrophage migration across a transwell membrane, it remains possible that type I IFNs could alter vascular adhesiveness or the vasculature architecture in a way that would inhibit intravasation of monocytes. It is also possible that type I IFNs may suppress the generation of myeloid suppressor cells, which would in turn impact the generation of TAM and tumor growth [44,45,55].

Our in vitro studies demonstrated that the endogenous release of low levels of type I IFNs by tumor cells inhibited macrophage generation (see Fig. 8). We observed that the MCA tumor cell line used in these studies spontaneously produced low concentrations of $\text{IFN-}\alpha$. However, it is also possible that in vivo, stromal cells in the tumor may also contribute to local type I IFN production, as has been shown previously [9]. Further, the effects of type I IFNs in our studies appeared to be due primarily to inhibition of macrophage responsiveness to CSF-1. Similar findings were reported by Hamilton et al. who demonstrated that the inhibitory effects of LPS and $\text{TNF-}\alpha$ on bone marrow macrophage generation were due largely to stimulation of type I IFN release and subsequent inhibition of CSF-1 responsiveness [25]. A previous report also noted that endogenously produced type I IFNs accelerated tumor growth, metastasis, and angiogenesis in $\text{IFN-}\alpha/\beta\text{R}^{-/-}$ mice with xenografted human tumors [9]. We have now extended those earlier findings using a syngeneic tumor model in fully immune competent mice. Based on our results, we therefore suggest that the accelerated tumor growth and exuberant angiogenic responses observed in tumors in $\text{IFN-}\alpha/\beta\text{R}^{-/-}$ mice may be due to increased numbers of TAM found within these tumors. These findings are important inasmuch as they describe a new mechanism by which type I IFNs may inhibit tumor progression and angiogenesis.

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Fig. 1.

Comparison of tumor growth rates in IFN- α/β R^{-/-} and wild type mice. Tumors were established in wild type or IFN- α/β R^{-/-} mice ($n = 5$ per group) by intradermal injection of 1×10^6 MCA 2.1 fibrosarcoma cells per mouse. Tumor growth was assessed by serial measurement of two tumor dimensions using calipers and the mean tumor diameter ($\pm$ SEM) for each group was calculated. Tumor growth was significantly ($p < 0.05$) increased in IFN- α/β R^{-/-} mice, compared to wild type mice, as assessed by repeated measures ANOVA. Similar results were obtained in two additional experiments

Fig. 2.

Comparison of tumor angiogenesis in wild type and IFN- α/β R $^{-/-}$ mice. Cutaneous MCA 2.1 tumors were established in wild type and IFN- α/β R $^{-/-}$ mice. At 21 days after tumor implantation, the mice were euthanized and tumor tissues were collected and processed for immunohistochemistry, using an antibody to CD31 to identify microvessels, as described in "Materials and methods". The mean ($\pm$ SEM) tumor microvessel density (*MVD*) was calculated for each group of mice. The *MVD* in tumors of IFN- α/β R $^{-/-}$ mice was significantly higher (** $p < 0.01$) than in wild type mice, as assessed by Student's *t* test. Similar results were obtained in two additional experiments. (hpf = mean number of microvessels per $4 \times 10\times$ power fields)

Fig. 3. Assessment of tumor associated macrophages in tumors of IFN- α/β R^{-/-} and wild type mice by flow cytometry. Tumor tissues from wild type and IFN- α/β R^{-/-} mice ($n = 5$ per group) were enzymatically digested and immunostained for detection of CD11b⁺/F4/80⁺ tumor-associated macrophages and then analyzed by flow cytometry, as described in “Materials and methods”. In **a**, a typical dot plot of CD11b⁺/F4/80⁺ tumor-associated macrophages is shown. In **b**, the mean percentage of tumor-associated macrophages from tumors of wild type and IFN- α/β R^{-/-} mice ($n = 5$ per group) is shown. There was a significant increase ($*p < 0.05$) in the percentage of tumor-associated macrophages in tumors of IFN α/β R^{-/-} mice, compared to tumors of wild type mice, as assessed by Student's t test. These data are representative of three independent experiments

Fig. 4. Immunohistochemical analysis of F4/80⁺ and CD68⁺ macrophages in tumors of IFN- α / β R^{-/-} and wild type mice. Tumor tissues were processed for immunohistochemical localization of macrophages, using two macrophage-specific antibodies (F4/80, *top row, a, b*) and CD68 (*bottom row, c, d*), as described in “Materials and methods”. Macrophage density was consistently higher in tumors of IFN- α / β R^{-/-} animals compared to wild type animals. In addition, the macrophages appeared to be uniformly distributed throughout the tumor tissues and not clustered in any particular location. These images are representative of the histologic appearance of tumor tissues from five different mice in each group

Fig. 5.

Tumor infiltrating leukocytes in tumor tissues of wild type and IFN- α/β R^{-/-} mice. Tumor tissues from wild type and IFN- α/β R^{-/-} mice ($n = 5$ per group) were enzymatically digested and immunostained for detection of neutrophils (a), NK cells (b), and dendritic cells (c), then analyzed by flow cytometry, as described in “Materials and methods”. The mean percentages of each cell type in tumors of wild type versus IFN- α/β R^{-/-} mice were compared using Student's t test and significant differences ($p < 0.05$) in the populations of each cell type were not found. Similar results were obtained in one additional experiment

Fig. 6.

Production of CCL2 and CSF-1 by tumors from wild type and IFN- α/β R $^{-/-}$ mice. Fresh tumor tissues were obtained from wild type and IFN- α/β R $^{-/-}$ mice ($n = 5$ per group) on day 21 after tumor inoculation. Equivalent 30 mg portions of viable tumor tissues were cultured for 24 h in 1 ml complete medium, as described in "Materials and methods". The concentration of CCL2 (a) and CSF-1 (b) in tumor supernatants was determined by specific ELISA. Mean cytokine concentrations released by tumors of wild type and IFN- α/β R $^{-/-}$ mice were compared statistically using Student's t test and significant differences ($p < 0.05$) were not found. Similar results were obtained in one additional experiment

Fig. 7.

Migration of macrophages from wild type and IFN- α/β R $^{-/-}$ mice in response to tumor-conditioned medium. PM were placed in the upper chamber of a Boyden chamber and their ability to migrate through a membrane toward a gradient of recombinant CCL2 or a gradient of tumor-conditioned medium (CM) was assessed, as described in “Materials and methods”. Statistical differences between wild type and IFN α/β R $^{-/-}$ macrophages were assessed using Student's *t* test and significant differences ($p < 0.05$) were not found. Similar results were obtained in one additional experiment

Fig. 8.

Effects of tumor conditioned medium and neutralization of CSF-1 on generation of bone marrow macrophages from wild type and IFN- α/β R^{-/-} mice. Adherent bone marrow macrophages were obtained from wild type and IFN- α/β R^{-/-} mice and cultured in triplicate wells in complete medium alone (control) or in tumor-conditioned medium from MCA2.1 cells (CM). In other wells, cells were cultured in CM plus 50 ng/ml anti-CSF-1 neutralizing goat antibody (Neutr Ab), or with CM plus the equivalent concentration of an irrelevant goat antibody (Irrel Ab). After 96 h in culture, the mean number of viable macrophages ($\pm$ SEM) was determined by MTT assay, as described in "Materials and methods". Statistical differences between mean values for the number of viable wild type versus IFN- α/β R^{-/-} macrophages were determined for each of the four treatment groups, using Student's *t* test (**p* < 0.05). Similar results were obtained in one additional experiment

Fig. 9.

Effects of type I IFNs on generation of bone marrow macrophages in response to CSF-1. Bone marrow macrophages were obtained from wild type and IFN- α / β R^{-/-} mice and cultured in triplicate wells for 96 h in complete medium alone (control) or with medium plus recombinant murine CSF-1 (CSF-1), as described in “Materials and methods”. In other wells, bone marrow cells were cultured in complete medium plus CSF-1 plus recombinant IFN- α (CSF-1 + IFN- α) or recombinant IFN- β (CSF-1 + IFN- β). After 96 h in culture, the mean number of viable macrophages ($\pm$ SEM) was determined by MTT assay, as described in “Materials and methods”. Statistical differences between mean values for the number of viable wild type versus IFN- α / β R^{-/-} macrophages were determined for each of the four treatment groups, using Student's *t* test (**p* < 0.05). Similar results were obtained in one additional experiment

Fig. 10.

Effect of type I IFNs on proliferation of bone marrow macrophages in response to CSF-1. Bone marrow macrophages were obtained from wild type and IFN- α/β R $^{-/-}$ mice and cultured in triplicate wells for 96 h in complete medium alone (control) or with medium plus recombinant murine CSF-1 (CSF-1), as described in “Materials and methods”. In other wells, bone marrow cells were cultured in complete medium plus CSF-1 plus recombinant IFN- α (CSF-1 + IFN- α) or recombinant IFN- β (CSF-1 + IFN- β). After 96 h in culture, macrophage proliferation was assessed by 3 H thymidine uptake assay, as described in “Materials and methods”. Statistical differences between mean values for wild type versus IFN- α/β R $^{-/-}$ macrophage proliferation were determined for each of the four treatment groups, using Student's *t* test (* $p < 0.05$). Similar results were obtained in one additional experiment