IRF-1 Expression Is Essential for Natural Killer Cells to Suppress Metastasis

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Abstract

IFN-γ promotes tumoral immune surveillance, but its involvement in controlling metastases is less clear. Using a mouse model of pulmonary metastases, we show that local IFN-γ treatment inhibits formation of metastases through its regulation of IRF-1 in tumor cells. IRF-1 is an IFN-γ-induced transcription factor pivotal in the regulation of infection and inflammation. IRF-1 blockade abolished the inhibitory effect of IFN-γ on tumor metastases, whereas ectopic expression of IRF-1 phenocopied the inhibitory effects of IFN-γ. IRF-1 did not affect the survival of tumor cells in the circulation or their infiltration into lungs, but it was essential to support the pulmonary attraction and activation of natural killer (NK) cells. Depleting NK cells from mice abolished the protective effect of IFN-γ or IRF-1 on metastases. In addition, cytotoxicity assays revealed that tumor cells expressing IRF-1 were targeted more effectively by NK cells than IRF-1 nonexpressing tumor cells. Moreover, NK cells isolated from lungs inoculated with IRF-1–expressing tumor cells exhibit a greater cytotoxic activity. Mechanistic investigations revealed that IRF-1–induced NK cell cytotoxicity was independent of perforin and granzyme B but dependent on the NK cell activating receptor DNAM-1. Taken together, our findings establish IRF-1 as an essential mediator of the cross-talk between tumor cells and NK cells that mediate immune surveillance in the metastatic niche. Cancer Res; 71(20); 6410-8. ©2011 AACR.

Introduction

The immune surveillance hypothesis suggests that the immune system constantly monitors the organism, recognizes, and eradicates abnormal or cancer cells (1). In this process, cells of the innate and adaptive immunity are involved. Both, NK and T cells are known to induce lysis of target cells by releasing soluble membrane disrupting proteins such as perforin and granzyme B or by initiating apoptotic pathways triggered by death receptors (2). IFN-γ plays a pivotal role in promoting antitumor responses. Mice deficient in IFN-γ or the IFN-γ receptor show increased tumor development or the IFN-γ receptor show increased tumor development – induced NK cell cytotoxicity was independent of perforin and granzyme B and metastases (3). Therefore, IFN-γ promotes direct tumor suppressive effects by inhibiting cellular proliferation (4), promoting apoptosis by upregulation of caspases, CD95 or TRAIL (5), inhibiting angiogenesis (6), and promoting host antitumor immunity by enforced expression of MHC molecules or components involved in antigen processing.

One of the intracellular targets of IFN-γ is IRF-1, a transcriptional activator. IRF-1 is induced by IFN-γ and other cytokines like type I IFNs, TNF-α, (IL)-1, IL-6, platelet-derived growth factor, granulocyte macrophage colony-stimulating factor, dsRNA viruses, and genotoxic stress (7). Studies on IRF-1 knockout mice revealed that IRF-1 favors T-cell differentiation into helper T cells and its involvement in the differentiation and function of NK cells (8, 9). IRF-1 exerts a variety of biologic processes including inflammation, antiviral response, immune regulation, and tumor suppression. The efficiency of IRF-1 in tumor suppression in mice was first observed due to its ability to revert IRF-2–induced transformation of NIH3T3 cells and the inhibition of tumor formation in nude mice (10). Furthermore, the role of IRF-1 in immune responses was shown by the induction of antitumoral immune responses mediated by CD4+ and CD8+ T cells (11). Tumor-suppressive functions of IRF-1 are exhibited by the induction of target genes involved in cell-cycle control or apoptotic processes (12). Interestingly, deletion of IRF-1 alone does not promote tumorigenesis. However, it enhances the extent of tumors arising from p53 deletions (13).

On the basis of the hypothesis that stimulation of the intrinsic activity of IRF-1 upon IFN-γ addition should induce antitumor activities, we administered IFN-γ to mice with experimental pulmonary lung metastases. Intranasal application was able to inhibit the development of metastases, whereas this effect was partially reverted by suppression of...
IRF-1 by short hairpin RNA (shRNA). We show that the IRF-1–mediated repression of lung metastases is dependent on NK cells. Systemic analysis revealed that IRF-1 expression influences the tumor microenvironment by the expression of CXCL11, which results in the attraction of CXCR3-positive NK cells. Evidence is provided that the IRF-1–mediated protective effect on lungs is mediated by an upregulation of TRAIL and DNAX accessory molecule 1 (DNAM-1) on NK cells and of CD155 and DR5 on tumor cells.

Materials and Methods

Mice

All experiments were done with 8- to 12-week-old mice. Balb/c mice were purchased from Harlan. IFNAR1–/– mice (14) on Balb/c background were kept under specific pathogen-free conditions at German Cancer Research Centre. Rag2–/– and NMRInu/nu mice were bred under specific pathogen-free conditions at the Helmholtz Centre for Infection Research. All animal experiments were done according to the guidelines of the ethics committee of Landesamt für Verbraucherschutz und Lebensmittelsicherheit of Lower Saxony. Mice were treated with 2 mg/mL doxycycline in the drinking water. Water was changed every second day. Recombinant mouse IFN-γ (peripheral blood lymphocytes) was administered intranasally.

Cell lines

CT26 colon carcinoma cell line was purchased from American Type Culture Collection. Authentication of cells was confirmed by nanoplex PCR by the German Biological Resource Centre. Cells were stably transfected with pVBC3HA to express hemagglutinin from Influenza virus PR8 to generate CT26HA cells. CT26HA cells were infected with recombinant lentiviruses containing the IRF-1 gene under the control of the tetracycline promoter to generate CT26HA/IRF-1 cells. Cells were maintained in Dulbecco’s Modified Eagle Medium (Invitrogen) supplemented with 10% (v/v) FCS (Lonza) and 1% tetracycline. Cells were monitored by CD3+ immunodotting. CD4+ and CD8+ cells were isolated by treatment with 20 μg/mL of anti-CD4, CD8+ and 7-AAD was added and cells were stained. Efficiency of NK cell depletion was more than 95% in blood, spleen, and lungs monitored by CD3+“NKp46+” staining.

Transwell assays

Tumor cells were seeded in a 24-transwell assay plate (Costar) with a pore size of 3 or 5 μm and treated with doxycycline or left untreated. NK cells, sorted from spleen or lungs, were seeded in the upper chamber. Five, 24, and 48 hours after seeding migration rate and activation status of NK cells were determined.

RT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen) and reverse transcribed with Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences). Quantitative real-time PCR (RT-PCR) analysis was done with a LightCycler (Roche) and the Quantitect SYBR Green PCR Kit (Qiagen). The data are represented as ratios relative to the values of actin. Standard curve analysis was done for relative quantification. Actin-for_TGGAATCTCTGTGGCATTGAAAC, actin-rev_TAAAACGGTGTTCAAGCTGGCAGC; muCXCL11-for_AGTAACGGCTTGGCACAAGATG, muCXCL11-rev_GCATTCTCAAGAGCTGGCAGC.

Cytotoxicity assay

NK cells were isolated from spleen or lungs. Tumor cells were stained with CFSE according to the manufacturer’s instructions (Invitrogen) and incubated for 4 hours with NK cells in the indicated effector:target ratio 7-AAD was added and cells were analyzed. Percentage of killing was calculated from the ratio of living CFSE−/−7-AAD+ tumor cells. Involvement of specific NK cell was by treatment with 20 μg/mL of DNAM-1 blocking antibodies.

Results

Local IFN-γ administration inhibits pulmonary metastases formation

Tumor cells injected i.v. into syngenic Balb/c mice attach on pulmonary vessel walls and form tumors, a procedure that simulates the late steps of metastases. To examine the influence of local IFN-γ administration on metastasis formation, we used CT26HA cells expressing hemagglutinin (HA) as a model tumor antigen. Expression of HA had no influence on metastases formation. To examine metastatic tumor growth, mice were inoculated with 5 × 105 CT26HA or CT26HA/IRF-1 colon carcinoma cells. Lungs were isolated and analyzed as described.
One of the key mediators of IFN-γ-induced effects is IRF-1. To investigate whether IRF-1 expression in the tumor cells is responsible for the IFN-γ-mediated tumor-suppressive effect, IRF-1 expression was downregulated by shRNAs in CT26HA cells (Supplementary Fig. S2). Mice inoculated with these cells showed no significant decrease in lung metastasis formation upon IFN-γ treatment (Fig. 1B). These data suggest that IFN-γ–induced inhibition of metastases is mediated by the expression of IRF-1.

**IRF-1 decreases metastatic tumor growth and prolongs survival of mice**

Overexpression of IRF-1 inhibits proliferation, reverses transformation (16), and induces tumor-specific immune responses (11, 17). To determine the mechanisms of IRF-1 on metastasis formation, we generated a CT26HA tumor cell line expressing IRF-1 under the control of a tetracycline-dependent promoter (CT26HA/IRF-1). IRF-1 was only slightly induced upon doxycycline treatment (Supplementary Fig. S3A). Induction of IRF-1 in this cell line did not influence proliferation or transformation (Supplementary Fig. S3B and C), but mice inoculated with IRF-1–expressing tumor cells showed significantly increased survival rates (Fig. 2A). Development of lung metastases was analyzed 3 weeks after tumor cell inoculation. A significant decrease of lung colonies was found in mice inoculated with IRF-1–expressing tumor cells (Fig. 2B and C). This indicates that IRF-1 expression inhibits experimental lung metastases and prolongs survival.

**IRF-1 negatively influences initiation and maintenance of metastases**

Inhibition of metastases could be due to reduced survival in circulation or by retarding initiation of micrometastases in the lungs. To define the level of inhibition, IRF-1 induction was initiated at different time points to tumor cell inoculation. The number of metastases was reduced in all animals independent of the time point of IRF-1 induction (Fig. 2D). This observation indicates that IRF-1 expression reduces development of metastatic nodules in the lungs rather than affecting the survival of the cells in circulation or their settling in the lungs.

**IRF-1 initiates NK cell accumulation in metastatic lungs**

As IRF-1 expression had no direct growth inhibitory effect on the tumor cells, we assumed that immune cells might be involved and analyzed immune cell populations of the metastatic lungs (Fig. 3A and B). No significant differences between IRF-1 induced and noninduced lungs were observed in the number of tumor-infiltrating granulocytes. The percentage of macrophages and dendritic cells was decreased upon IRF-1 expression. Interestingly, IRF-1 induction led to a significantly higher percentage of NK cells in metastatic lungs.

Previous work with s.c. tumor models showed that tumor suppression by IRF-1 was mainly dependent on CD8+ and CD4+ T cells (11, 17). In the pulmonary lung metastasis model, the number of CD8+ T cells is not significantly altered by IRF-1 expression, but the number of CD4+ T cells is increased (Fig. 3B). Further analysis revealed no change in the Treg cell subset (CD4+CD25+FoxP3) of CD4+ T cells (Fig. 3C).
NK cells are essential for IRF-1-mediated metastases inhibition

Elimination of tumor cells could be T-cell dependent. To investigate whether the increased number of lung metastases by IRF-1 was T-cell dependent, we inoculated T-cell-deficient nude mice with CT26HA/IRF-1 tumor cells and determined the effect of IRF-1 induction on tumor burden. The effect of IRF-1 was comparable with that of wild-type mice (Fig. 4A). Thus, T lymphocytes are not responsible for the IRF-1-dependent restriction of metastases.

NK cells are known to be responsive to tumor cells. We therefore used Rag2<sup>−/−</sup> mice to determine the impact of NK cells on IRF-1-mediated inhibition of metastases (Fig. 4B). Induction of IRF-1 by doxycycline treatment did not lead to a reduction in metastasis formation, suggesting that NK cells are involved in IRF-1-mediated inhibition of tumor development.

To further clarify the role of NK cells, we depleted NK cells using anti-asialo-GM1 antibody (Fig. 4C). Mice depleted of NK cells showed enhanced metastasis development, regardless of IRF-1 expression (Fig. 4D). We conclude that NK cells are involved in IRF-1-mediated inhibition of metastases development.

Expression of IRF-1 in the tumor cells attracts and activates NK cells

To analyze whether induction of IRF-1 leads to the activation of NK cells, CD69 expression was assessed on lung NK cells. The number of NK cells was not altered, but frequency of NK cells expressing high levels of CD69 was significantly enriched in lungs of mice inoculated with tumors expressing IRF-1 (Fig. 5A). Using transwells which prevent contact between NK and tumor cells, we could show that soluble factors produced by IRF-1-expressing tumor cells induce activation of NK cells (Fig. 5B). This effect is even stronger when NK cells migrate through the membrane and are in direct contact with the IRF-1-expressing tumor cells (Fig. 5C).

The CXCR3 binding chemokine CXCL11 is known to attract NK cells. RT-PCR analysis of lungs revealed that CXCL11 mRNA was much more abundant if IRF-1 expression in the tumor cells had been induced (Fig. 5D). In addition, the frequency of tumor-infiltrating NK cells expressing the corresponding receptor CXCR3 was increased in mice upon IRF-1 induction (Fig. 5E), suggesting that expression of IRF-1 leads to the secretion of CXCL11 and attraction of NK cells via its activity on the CXCR3 receptor.
Retardation of tumor growth by IRF-1 is independent of type I IFN

Type I IFNs are critical for controlling NK cell–mediated antitumor responses (18). To investigate the role of type I IFN in IRF-1–mediated tumor suppression and NK cell activation, metastasis formation was investigated in IFNAR-/- mice. Mice treated with doxycycline to induce IRF-1 expression showed a significant decrease in the number of tumor nodules compared with the untreated controls (Fig. 5F). No differences were detectable between the IFNAR-/- animals and the heterozygous controls, indicating that type I IFN plays no role in NK cell-mediated inhibition of metastases upon IRF-1 expression.

NK cell cytotoxicity and sensitivity of tumor cells to NK cell–mediated killing is increased by IRF-1

The contact of IRF-1–expressing tumor cells with NK cells leads to a strong activation of NK cells. We investigated if expression of IRF-1 renders CT26HA/IRF-1 tumor cells susceptible to NK-cell cytotoxicity. In vitro activated spleenic NK cells show higher lytic activity against CT26HA/IRF-1 tumor cells expressing IRF-1 compared with nonexpressing tumor cells (Fig. 6A). In addition, NK cells isolated from lungs of mice harboring IRF-1–expressing tumors showed increased cytotoxicity to Raji cells compared with NK cells from noninduced animals (Fig. 6B). The data reveal that IRF-1 induction increases the sensitivity of tumor cells to NK cell-mediated killing and leads to a higher cytotoxic capacity of NK cells. NK cell responses against tumor cells are mediated by cytokine release, activating or inhibitory receptors. Production of IFN-γ, perforin, or granzyme B by NK cells was not affected by IRF-1 expression (data not shown) and neutralization of IFN-γ did not influence the effects of IRF-1 on metastasis formation (Fig. 6C). Surface protein analysis revealed that the effect of IRF-1 does not influence the expression of death receptor Fas, or the NKG2D ligands Rae-1 or H60. However, surface expression of MHC class I molecules, death receptor DR5 and the adhesion molecule CD155, was increased (Fig. 6D). Analysis of tumor-infiltrating NK cells revealed that IRF-1 induction had no influence on NKG2D and FasL expression (Fig. 6E). However, increased expression levels of the death receptor ligand TRAIL and the adhesion molecule DNAM-1 were detectable on tumor-infiltrating NK cells after IRF-1 induction (Fig. 6E).

NK cell-mediated killing of IRF-1 expressing tumor cells is dependent on DNAM-1

DNAM-1 and TRAIL play an important role in elimination of tumor cells. To investigate the role of DNAM-1 in NK cell–mediated cytotoxicity, we blocked DNAM-1 by antibodies.
Cytotoxicity of lung NK cells from doxycycline-treated mice was partially inhibited (Fig. 7A). Analysis of tumor cells isolated from lungs of IRF-1−/− mice showed decreased frequencies of DR5+ and CD155+ tumor cells compared with nontreated animals (Fig. 7B). To test whether DNAM-1 and TRAIL-dependent mechanisms could be responsible for IRF-1−/−mediated inhibition of metastases, we inoculated tumor cells in DNAM-1−/− mice and wild-type mice treated with TRAIL-blocking antibodies. IRF-1−/−mediated inhibition of metastases was completely abolished in DNAM1−/− mice (Fig. 7C), whereas blockade of TRAIL resulted only in partial but not complete reduction of metastases (Fig. 7D). These data indicate that NK cell-mediated elimination of tumors by IRF-1 is dependent on DNAM-1 and partially on TRAIL.

To test whether expression of CD155 and DR5 leads to recognition and elimination of only CD155DR5low tumor cells by NK cells we treated CT26/IRF-1 cells with doxycycline for 2 days and sorted CD155lowDR5low cells. Inoculation into mice resulted in a lesser reduction of metastases by IRF-1 in mice injected with CD155lowDR5low cells compared with unsorted controls (Fig. 7E). Taken together, these findings indicate that IRF-1−/−mediated elimination of tumor cells results from DR5 and CD155 expression on tumor cells.

Discussion

IRF-1 exhibits powerful tumor-suppressive effects in different animal models (11, 17, 19). Both intrinsic and systemic responses including adaptive immunity have been linked to IRF-1−/−induced tumor suppression (11, 12, 16). Here, we show that neither intrinsic nor adaptive effects but innate defense mechanisms play a key role in IRF-1−/− induced inhibition of lung metastases.

Earlier reports have shown that systemic treatment of mice with high doses of IFN-γ led to a partial reduction of lung metastases by a macrophage-dependent mechanism (20). We show that local administration of IFN-γ leads to a potent inhibition of lung metastases (Fig. 1A) and that IRF-1 is a major player of IFN-γ−mediated inhibition of metastases, shown by downregulation of IRF-1 (Fig. 1B) and ectopic expression of low IRF-1 concentrations (Fig. 2).

Ectopic expression of IRF-1 in tumor cells shows inhibition of proliferation and tumorigenicity (16, 21). These effects are mediated by the inhibition of cell-cycle progression or induction of apoptosis and require comparably high levels of IRF-1 protein (11). In this report, IRF-1 is expressed at very low levels and is not able to inhibit proliferation and anchorage-independent growth of CT26/HA cells in vitro.
Doxycycline. DR5lowCD155low tumor cells were sorted and i.v. injected into
lung nodules cells, dendritic cells and NK cells (Fig. 3). The role of NK cells
in the use of mutant mouse strains and depletion experiments (Figs. 3 and 4) showing that a host response mediated by NK cells in particular is necessary for the tumoricidal effect, whereas T and B cells are dispensable.

NK cells are important mediators of antitumor immunity. Mice with decreased levels of NK cells have increased susceptibility to experimental lung metastases and tumor growth (22). Chemokines like the CXCR3 ligands play important roles in NK cell migration (23). IFN-γ and IRF-1 induce CXCR3 ligands CXCL9, CXCL10, and CXCL11 (24). Our data show that ectopic expression of IRF-1 induces the expression of CXCL11 (Fig. 5E) and lung tumor-infiltrating NK cells express CXCR3 (Fig. 6B).

Type I IFN has an important role in immune surveillance and in restricting growth of transplantable tumors (25), including lung metastases (18). NK cell activation and cytotoxicity is induced by type I IFN (26). IRF-1 was identified as an inducer of IFN-β (27). Our data reveal that IRF-1–mediated NK cell activation by IFN-β is unlikely, as IFNAR−/− mice showed unaltered reduction of metastatic lung nodules (Fig. 5F).

In addition, cytoplasmic granule toxins and IFN-γ released by NK cells are identified for their essential role in tumor killing (28–30) are not involved in the IRF-1–mediated effect of metastases reduction. We found no influence of granzyme B or IFN-γ production in tumor-residing NK cells by IRF-1. NK cells can control liver metastases by TRAIL-mediated induction of apoptosis through binding to DR5 on the tumor cells (31). Doxycycline-treated mice show significantly higher amounts of tumor-residing TRAIL+ NK cells and lower numbers of DR5+ tumor cells in the lungs (Fig. 7B). However, metastases are still decreased in mice treated with TRAIL-blocking antibodies, which is indicative of a minor role of TRAIL in the IRF-1–mediated inhibition of metastases.

NK cells recognize and eliminate cells through a complex set of activating and inhibiting receptors (32–34). MHC class I molecules on CT26HA cells were induced rather than downregulated by IRF-1, suggesting that NK cell activation by the lack of “self” signals is unlikely. Thus, IRF-1 may tip the balance between activating and inhibitory signals toward activation by other mechanisms. Earlier studies indicated that expression of NKG2D ligands results in NK cell–mediated tumor cell killing even when the tumor cells express normal levels of MHC molecules (35, 36). However, expression of NKG2D ligands Rae-1 or H60 was not found to be altered by IRF-1 (Fig. 6D).

Another activating receptor, DNAM-1, was recently reported to play an important role in tumor immune surveillance by NK cells (37, 38). DNAM-1 binds to CD155 and CD112, both of which have been found to be upregulated in tumors (37). DNAM-1 participates in the NK cell–mediated lysis of certain tumor cells in collaboration with NK cell receptors (39, 40). Furthermore, DNAM-1 expression on NK cells is sufficient to eliminate tumor cells that lack expression of ligands for NKGD2 or other NK cell–activating receptors (41, 42). Antibody-mediated blocking of DNAM-1 decreases cytotoxicity of NK cells, whereas DNAM-1−/− mice are not able to control metastases upon IRF-1 expression. We found significantly lower numbers of CD155+ tumor cells in metastatic lungs upon IRF-1 induction (Fig. 7B) which could be the result of NK cell–mediated killing of the tumor cells. Thus, DNAM-1 plays.

( Supplementary Fig. S3). Thus, it is unlikely that IRF-1 prevents metastases by a direct antiproliferative effect on tumor cells in vivo. Comparison of cell populations in the metastatic lungs in the absence or presence of ectopic IRF-1 revealed differences in the magnitude of CD4+ T cells, B cells, dendritic cells and NK cells (Fig. 3). The role of NK cells as a major effector cell population gained further support by

Figure 7. DNAM-1 is critical for NK cell–mediated killing of IRF-1–expressing tumor cells. Mice received CT26HA/IRF-1 cells i.v. and were treated with (+ Dox) or without doxycycline (− Dox). After 3 weeks, lungs were isolated and analyzed. A, NK cells were purified from lungs and lysis of Raji cells was measured in the absence or presence of DNAM-1 blocking antibodies for the indicated E:T ratios (n = 5). B, HA+ tumor cells were analyzed for surface expression of CD155 and DR5 expression by flow cytometry. Data are representative of 2 independent experiments (n = 10). Mice were injected i.v. with CT26HA/IRF-1 cells and treated with doxycycline (− Dox) or left untreated (− Dox). C, 3 weeks after tumor cell injection, the number of lung nodules was determined in DNAM-1−/− mice. D, wild-type mice were i.p. injected with anti-TRAIL antibody on days −5, 0, 6, and 10. Data show the mean number of lung nodules ± SD and are representative of 2 independent experiments (n > 10). E, CT26HA/IRF-1 tumor cells were treated for 2 days with doxycycline. DR5+/−/CD155+ tumor cells were sorted and i.v. injected into Balb/c mice. Mice were treated with doxycycline (+ Dox) or left untreated (− Dox). Three weeks after tumor cell injection, the number of lung nodules was determined. Data show the mean number of lung nodules ± SD (n = 5). *, P < 0.05.
an important role in NK cell–mediated killing of tumor cells that express IRF-1. IRF-1 expression in tumor cells recruits and activates NK cells which promote destruction of transformed cells by a DNAM-1–dependent mechanism. However, IRF-1 expression does not inhibit metastasis formation completely. As depicted in Fig. 2A, all mice died within 10 weeks despite the protective effect of IRF-1. Injection of sorted CD155lowDR5low tumor cells revealed that tumor cells which can escape NK cell–mediated killing are not controlled by IRF-1 (Fig. 7E).

Overall, our study shows that IRF-1 is a key mediator of IFN-γ. Numerous studies indicate that different types of inflammatory reactions and individual proinflammatory cytokines lead to strong induction of IRF-1 (7). Because we have shown that even low expression of IRF-1 is sufficient to inhibit metastasis development and prolonged survival, IRF-1 induction by proinflammatory cytokines or small molecules is an attractive target for cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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