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Activation of IRF3 contributes to IFN- γ and ISG54 expression during the immune responses to B16F10 tumor growth

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Abstract

Interferon Regulatory Factor (IRF-3) has been shown to contribute to immune control of B16 melanoma tumor growth. We have shown previously that IRF-3 has a role in IFN- γ -induced expression of pro-apoptotic interferon stimulated gene 54 (ISG54) in macrophages and IFN- γ in T cells. To investigate the IRF3-IFN- γ -ISG54 nexus, we injected C57Bl/6 (B6) and IRF3KO mice s.c. with luciferase-producing B16-F10 tumor cells. Tumor growth as measured by luciferase levels was similar between B6 and IRF3KO mice at day 2 and 6, but was significantly greater at day 9 in IRF3KO mice compared with B6 mice. Transcription factor assays on splenic protein extracts after tumor inoculation revealed peak activation of IRF3 and IRF7 at day 6 in B6 tumor-bearing mice but not in IRF3KO tumor-bearing mice. Likewise, significant induction of IFN- γ occurred in spleens and tumors in B6 mice from day 6–9 but failed to occur in tumor-bearing IRF3KO mice. Previous reports from other labs showed that the anti-tumor properties of IFN- γ are the result of cell cycle arrest. Using B16F1 cells or B16F1 cells deficient in IFN- γ receptor (B16-IRFGRKO), we found that IFN- γ alone and in synergy with the TLR3/IRF3 agonists, poly I:C, decreased B16F1 cell growth in significant correlation with increased ISG54 expression. Moreover, IFN- γ alone increased expression of the cell cycle inhibitor, p27Kip while IFN- γ plus poly I:C increased cleaved Caspase-3 in B16 cells. Thus, it is likely that an IFN- γ /IRF3/ISG54 nexus can significantly contribute to tumor cell control during anti-tumor immune responses.

Keywords

IFN- γ ; ISG54; IRF-3; B16 melanoma; Poly I:C

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1. Introduction

IRF3 is a key transcription factor for development of innate antiviral immunity [1]. However, some studies suggested that there are links between IRF3 and anti-cancer immune responses [2] [3]. We reported that mice deficient in IRF3 had impaired NK cell responses, decreased T cell production of Interferon-(IFN)- γ , and accelerated melanoma growth[4]. In contrast, treatment of tumor-bearing mice or responding T cells with polyinosinic:polycytidylic (poly I:C), which activates IRF3, decreased melanoma growth and increased IFN- γ production, respectively. In addition, we unexpectedly found that IRF3 contributes intrinsically to T cell production of IFN- γ [5]. Therefore, IRF3 and compounds that activate IRF3 contribute to anti-cancer immunity by bringing about IFN- γ production from T cells.

IFN- γ from T cells is a key cytokine in anti-tumor immunity through its ability to induce cell cycle arrest by increasing expression of the cell cycle inhibitor protein p27Kip [6]. However, IFN- γ could also induce apoptosis in theory through Interferon Stimulated Genes (ISG) like ISG54 (Aka: IFIT2) [7] [8], which is one of several tetratricopeptide repeat protein ISGs[9]. Moreover, expression of ISG54 has been shown to be dependent on activation of IRF3 [5] [10]. In addition, ISG54 is a potent anti-viral factor because it is an inducer of apoptosis [8] in virus infected cells, thus limiting viral spread. However, ISG54 has also been found to be an antitumor factor limiting growth of oral squamous cell carcinomas [11] and adenocarcinomas [12], most likely through the induction of apoptosis. Therefore, we hypothesize that IRF3 contributes to anti-cancer immunity by supporting IFN- γ production and expression of ISG54 [10].

The research herein looks at the relationship between IFN- γ , IRF3, and ISG54 in antitumor immunity using the B16 melanoma model. The data reveal that IRF3 is more involved in antitumor immunity in the time-frame corresponding with development of adaptive immunity rather than innate immunity. Specifically, IRF3 is activated in the spleen at day 6 post B16F10 cell implantation, which coincides with increased expression of IFN- γ at the spleen and at the tumor site. We also show that, in addition to induction of p27Kip, IFN- γ induces ISG54 in B16 cells, which correlates with caspase-3 activation and decreased growth of B16 cells. Thus an IFN- γ /IRF3/ISG54 nexus is involved in anti-tumor immune responses.

2. Materials and Methods

2.1. Cells

B16F10-Luc cells that constitutively produce Luciferase were obtained from Caliper Life Sciences (Hopkinton, MA). B16F1-ISG54-SEAP cells that are stably transfected with a plasmid that expresses secreted embryonic alkaline phosphatase (SEAP) under the control of the ISG54 promoter-5X ISRE were obtained from InVivogen (San Diego, CA). B16F1-ISG54-SEAP cells that do not express the IFN- γ Receptor (IFNGR-KO) were also obtained from InVivogen. All B16 cells were maintained in RPMI with 10% FBS and 50 μ g/ml gentamycin.

2.2. Mice

Female C57Bl/6 (B6) mice 6–8 weeks of age were obtained from Harlan-Sprague Dawley (Envigo, Indianapolis, IN). IRF3 deficient (IRF3KO) mice, originally derived by Tadatsugu Taniguchi of the University of Tokyo [13], were offspring of breeder pairs obtained from Dr. Karen Mossman, McMaster University. The absence of IRF3 in IRF3KO mice was verified by western blot. Experimental animal procedures using mice were approved by and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center.

2.3. Tumor model

For B16 tumors, 10^6 B16-F10 cells in 100 μ l PBS were injected subcutaneously (s.c.) into the dorsal hindquarter of B6 or IRF3KO mice. At day 2, 4, 6, 7, 9, and 12 after tumor injection, areas of the dermis were excised, inverted, black tumors identified, extracted, placed into cold PBS, filtered through a 70 micron nylon filter, and then cells were treated with Luciferase Cell Culture Lysis Reagent of Promega (Madison, WI). Luciferase in lysates was measured using a luciferase kit of Promega and a Turner Design TD-20/20 Luminometer. Protein concentrations in lysates were determined using the BioRad (Hercules, CA) DC protein assay kit. Relative luciferase in tumor lysates was calculated by dividing luciferase units with protein concentration in each sample. IFN- γ concentrations in each tumor lysate was determined using the IFN- γ ELISA kit from eBiosciences (ThermoFisher) and normalized to total protein concentration in each lysate. SEAP in supernatants of B16F1-ISG54-SEAP cells was determined using the QUANTI-Blue™ kit from InvivoGen (San Diego, CA).

2.4. Isolation of splenic mononuclear cells

Spleens from tumor bearing mice were extracted and placed into DMEM with 10% FBS and 50 μ g/ml gentamycin. Cells were dispersed using 70- μ m mesh screens; washed in PBS; treated with ACK lysing buffer (ThermoFisher), washed again; and cells were lysed with either complete lysis buffer from for Transcription Factor analysis (Active Motif) or RNA lysis buffer of the Purelink total RNA extraction kit from Ambion/Invitrogen (ThermoFisher), according to the manufacturer's specifications.

2.5. Transcription Factor Assays

TransAM® IRF-3 and IRF-7 DNA-binding ELISA kits for activated IRF-3 and IRF-7 were obtained from Active Motif (Carlsbad, CA). Activated IRF-3 and IRF-7 were evaluated according to the manufacturer's specifications. Briefly, spleen cell protein lysates were added to 96-well plates coated with the IRF3 consensus sequence 5'-GAAACTGAACT-3' or the IRF7 consensus sequence 5'-GAAAGCAAAAAGAGAAGTAGAAAGTAACT-3'. After incubation at room temperature for 1 h, wells were washed 3 $\times$ before addition of rabbit anti-IRF3 or anti-IRF7, followed by HRP-conjugated anti-rabbit IgG and TMB substrate. Color development was measure with an ELISA plate reader at 450 nm with 655 nm reference. Relative IRF3 or IRF7 activity was normalized to total protein concentration in the lysates.

2.6. Quantitative RT-PCR

IFN- γ mRNA in spleen mononuclear cell lysates was quantified as we have done before. Briefly, 100 ng to 1 μ g of RNA was reverse transcribed. IFN- γ sense (5' TGCATCTTGGCTTTGCAGCTC TTCCTCATGGC 3') and antisense (5' TGGACCTGTGGG TTGTGACCTCA AACTTGGC 3'), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense (5' TTGTCAGCAATGCATCCTGCAC 3') and antisense (5' ACAGCTTTCAGAGGGGCCATC 3') were used to run quantitative PCRs with an ABI Prism 7000 thermal cycler at 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. Cycle thresholds (CT) of sample were normalized to the CT of GAPDH for that sample (CT) and then normalized to the average CT of the control samples (CT), after which data were expressed as relative levels of mRNA using 2^{-CT} .

2.7. In vitro tumor cell proliferation

To measure B16F10-Luc cell viability in response to IFN- γ and poly I:C in vitro, we used the CellTiter-Blue® Cell Viability Assay from Promega or trypan blue staining. Briefly, B16F10-Luc cells were seeded at 10,000 or 75,000 cells per well of a 96-well plate in complete DMEM after which 0, 0.05, 0.5 or 5.0 μ g/ml of poly I:C (InVivogen) with or without 2, 20, or 50 ng/ml recombinant IFN- γ from BioLegend, (San Diego, CA). After 48, 72, or 96 h CellTiter-Blue Resazurin reagent was added to cell cultures and 590 nm fluorescence was measured after 4 h according to manufacturer's specifications. Alternatively, B16F10-Luc cells were seeded at 50,000 cells per well of a 24-well tissue culture plate, treated with poly I:C with or without IFN- γ and incubated for 48 h. At that time, cells were detached using TrypLe reagent (InVitrogen), suspended in 50% cell culture media, and counted using trypan blue and a Countess™ II FL Automated Cell Counter (InVitrogen).

2.8. Caspase 3/7 activation

Caspase 3/7 activation in B16F10-Luc cells treated with IFN- γ and poly I:C was evaluated using the CellEvent™ Caspase-3/7 Green Detection Reagent of Invitrogen. Briefly, B16F10-Luc cells were seeded at 50,000 cells per well of a 24-well tissue culture plate, treated with poly I:C with or without IFN- γ and incubated for 48 h. At that time, cells were detached, suspended in 50% cell culture media, and 50 μ l of the DEVD-peptide reagent was added to each well. Cells were then incubated at 37°C for 60 min and then green fluorescent cells were quantified using the Countess™ II FL Automated Cell Counter.

2.9. PAGE and western blot analysis

Ten μ g of protein from cell lysates were run on PAGE gels as we have done before[14] and transferred to a nitrocellulose membrane. The membrane was treated with blocking buffer for 1 h at room temperature, followed by incubation in 1:1000 dilution rabbit anti-cleaved Caspase-3 (Cell Signaling #9664), 1:2000 dilution rabbit anti-ISG54 (Thermo-Fisher #PA3-845), 1:1000 dilution of rabbit anti- p27Kip (Cell Signaling 3688). or 1:500 mouse anti-tubulin E7 (Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences, Iowa City, IA) and then goat anti-mouse-IgG-Alexa Fluor680-

(1/5000) or donkey anti-rabbit-IgG-IRDye® 800CW. The membrane was washed three times and then scanned with a LICOR Odyssey® Infrared Imaging System.

2.10. Statistical analyses

Student's t test was used to determine the significance of differences between means; $p < 0.05$ was considered significant. A two-way ANOVA was used to determine the significance of synergism between IFN- γ and poly I:C. A correlation analysis was used to determine the correlation between ISG54 promoter activity and cell viability due to IFN- γ and poly I:C treatments of B16F1 cells.

3. Results

3.1. IRF3 contributes to control of tumor growth

Previously we reported that B16F10 tumor growth was greater in mice deficient in IRF3 compared with wild-type mice [4]. However, tumor size for that report was measured starting at day 9 when tumors were palpable and measureable by caliper. Anti-tumor immunity can be divided into early innate anti-tumor immunity dominated by NK cells [15] [16] and later adaptive anti-tumor immunity when effector T cells emerge [17, 18]. B16F10-Luc cells, which constitutively express luciferase, permit measurement of tumors at earlier time-points by quantifying relative luciferase. To determine the impact of IRF3 on early vs. later tumor growth, we subcutaneously injected C57Bl/6 and IRF3KO mice with B16F10-Luc cells. On day 2, 6, and 9, dermis at injection sites was overturned to basal sides to isolate and extract the visible but not palpable melanomas. Luciferase activity in these extracted early melanomas revealed that at day 2 and 6, both C57Bl/6 and IRF3KO mice exhibited similar tumor growth (Fig. 1). However at day 9, tumor growth was significantly greater in IRF3KO compared with C57Bl/6 mice. These data suggest that IRF3 contributes to reducing tumor growth during the time when adaptive immunity emerges but not at the time when innate anti-tumor immunity is prominent.

3.2. IFN- γ expression is impaired in IRF3KO mice during tumor growth

Previously, we reported that IRF3 contributes to IFN- γ expression from effector T cells during antiviral immune responses [19]. To determine the dynamics of IFN- γ expression we injected B16F10 cells into C57Bl/6 mice and quantified IFN- γ in spleens and tumors by qRT-PCR and ELISA, respectively, during tumor growth. Expression of IFN- γ at the spleen that occurred at day 2 and 4 increased significantly by day 6, 7, and 9 during tumor growth (Fig. 2A). Likewise, the concentration of IFN- γ at tumor sites increase significantly from day 2, 4, and 6 compared with days 7, 9, and 12 of tumor growth (Fig. 2B). Therefore, peak IFN- γ expression in the spleen during responses to tumor implantation took place from day 6–9 after tumor injection. To determine the impact of IRF3 on IFN- γ expression, splenic IFN- γ expression was evaluated at day 6 in a separate cohort of tumor-bearing C57Bl/6 and IRF3KO mice. Deficiency of IRF3 significantly impaired expression of IFN- γ at the spleen at day 6 after tumor injection (Fig. 2C). Overall, these data suggest that IRF3 contributes significantly to the IFN- γ component of the immune response to tumor growth.

3.3. IRF3 and IRF7 are activated in splenic mononuclear cells during tumor growth

IRF3 is a constitutively expressed transcription factor that is present in cytoplasm in an inactive conformation. It is activated by hyperphosphorylation during viral infections in response to viral nucleic acids whereby it induces transcription of ISGs, including a second transcription factor, IRF7 [1, 20]. IRF3, as well as IRF7 activation is measured by its hyperphosphorylation, dimerization, nuclear localization, or ability to bind to promoter elements of ISGs [21]. To quantify IRF3 and IRF7 activation during tumor growth, spleen protein extracts from tumor bearing C57Bl/6 and IRF3KO mice were used in transcription factor assays measuring IRF3 and IRF7 binding to consensus oligonucleotide sequences, indicating their activation [22]. In C57Bl/6 tumor-bearing mice, significant IRF3 (Fig. 3A) and IRF7 (Fig. 3C) activation was detected in the spleen at day 6 compared with day 4, 7, and 12 post tumor inoculation. A separate cohort of tumor-bearing C57Bl/6 and IRF3KO mice revealed that significant activation of IRF3 (Fig. 3B) and IRF7 (Fig. 3D) is not activated in spleens of IRF3KO tumor-bearing mice compared with C57Bl/6 mice. Therefore, activation of IRF3 and IRF7 in tumor bearing mice coincides with increased production of IFN- γ .

3.4. IFN- γ and poly I:C decrease growth of B16 cells and increase ISG54 expression in vitro

IFN- γ contributes to control of B16 tumor cell growth through cell cycle arrest by inducing the cell cycle inhibitor protein, p27Kip [6]. Moreover, we reported that poly I:C, which is a TLR3 agonist that activates IRF3, also decreases B16F10 tumor cell growth [4]. To determine if IFN- γ can synergize with poly I:C in reducing tumor cell growth, we utilized B16F1 cells that express SEAP under the control of an ISG54 promoter (B16F1-SEAP) compared with B16F10 cells, and B16F1-SEAP cells deficient in the IFN- γ receptor (B16F1-SEAP-IFNGRKO). We incubated these cells with poly I:C in the presence or absence of 20 or 50 ng/ml recombinant IFN- γ . Using a Cell-Titer Blue kit to measure viable cells at 48 h, poly I:C and IFN- γ each alone, significantly decreased growth of B16F1-SEAP and B16F10 cells (Fig. 4A, C). IFN- γ alone failed to decrease growth of B16F1-SEAP-IFNGRKO cells (Fig. 4B), but interestingly it synergized slightly but significantly to enhance poly I:C-decreased growth of B16F1-SEAP-IFNGRKO cells.

In addition to cell cycle arrest, IFN- γ also induces ISG54 in cells [7]. ISG54 is a critical anti-viral ISG that activates Caspase-3 dependent apoptosis [8]. To determine the extent to which IFN- γ and/or poly I:C induce ISG54 we used B16F1-SEAP and B16F1-SEAP-IFNGRKO cells. At 24 h, poly I:C alone significantly induced ISG54 promoter activity, as measured by SEAP production, in B16F1-SEAP and B16F1-SEAP-IFNGRKO cells (Fig. 5A & B). Moreover, IFN- γ , alone induced ISG54 promoter activity in B16F1-SEAP cells but not B16F1-SEAP-IFNGRKO cells. Furthermore, IFN- γ and poly I:C exhibited significant synergism in the enhancement of ISG54 promoter activity. In addition, reduction in B16F1-SEAP cell growth, but not B16F1-SEAP-IFNGRKO cells, was significantly correlated with increases in ISG54 promoter growth in response to poly I:C with IFN- γ (Fig. 5C). Therefore increased expression of ISG54 in tumor cells could contribute to IFN- γ control of tumor cell growth.

To determine the effects of suboptimal concentrations of IFN- γ or poly I:C on B16 cell viability and ISG54 promoter activity, we incubated B16F1-SEAP cells with 2 ng/ml IFN- γ without or with 0.05 to 5 μ g/ml of poly I:C (Fig. 6A), or with 5–20 ng/ml IFN- γ without or with 0.1 to 10 μ g/ml poly I:C (Fig 6B). The loss of B16 cell viability correlated with the concentration of poly I:C in the presence of IFN- γ (Fig. 6A). Likewise, ISG54 promoter activity correlated with varying concentrations of poly I:C in the presence of varying concentrations of IFN- γ (Fig. 6B). Therefore, the synergism between IFN- γ and poly I:C in limiting B16 cell growth and ISG54 expression can be seen at a range of doses.

3.5. IFN- γ activates Caspase-3 in B16 cells

The data so far show that IFN- γ can induce ISG54 promoter activity. ISG54 is a potent anti-viral factor of the innate immune response due to its ability to associate with STING (MITA), activate Caspase-3, and induce apoptosis through the intrinsic pathway at mitochondria [9]. To determine if IFN- γ with or without poly I:C can limit growth of and activate Caspase-3 in B16 cells, we incubated B16F10 cells with IFN- γ for 48 h. We then counted the cells microscopically and treated the cells with a DEVD peptide probe that is fluorogenic only when it is cleaved by active Caspase-3. As seen previously, IFN- γ significantly decreased the number of B16F10 cells and addition of poly I:C significantly reduced B16F10 cell number further (Fig. 7A). Moreover, IFN- γ significantly increased the percentage of B16F10 cells with active Caspase-3 at 48 h. Unexpectedly, addition of poly I:C did not activate Caspase-3 and attenuated the increase in active Caspase-3 induced by IFN- γ at 48 h (Fig. 7B).

IFN- γ has been shown to control tumor cell growth by cell cycle arrest through induction of cyclin dependent kinase inhibitors, such as p27Kip [23]. To clarify and confirm the effect of IFN- γ and poly I:C on cell cycle arrest and apoptosis, we collected cell lysates at 24 and 48h from a separate cohort of B16F10 cells stimulated with IFN- γ and poly I:C, and performed western blots for cleaved caspase-3, p27Kip, and ISG54. IFN- γ or poly I:C, alone, induced slight Caspase-3 activation at 24 h (Fig. 7C). However, IFN- γ and poly I:C together synergized to activate more Caspase-3 at 24 h compared with either factor alone. Interestingly, at 48 h poly I:C attenuated the increased Caspase-3 activation in B16F10 cells induced by IFN- γ as seen with the 48-h fluorogenic Caspase-3 activation assay. As expected IFN- γ alone induced the cyclin dependent kinase inhibitor, p27Kip, at 24 and 48 h. Interestingly, poly I:C attenuated the IFN- γ induction of p27Kip at both time points. Thus one means by which IFN- γ limits B16 growth is through activation of Caspase-3, presumably through induction of proapoptotic ISG54, while a second means is through induction of proteins involved in cell cycle arrest, such as p27Kip.

4. Discussion

In this report we utilized the B16 melanoma mouse model and several genetically modified B16 cell lines *in vitro* to understand the relationship between IRF3, ISG54, and IFN- γ in anti-tumor immunity. Using wild-type and IRF3KO mice with luciferase-expressing B16F10 tumor cells, we were able to measure tumor growth as early as day 2 post tumor cell inoculation, which is a time prior to development of anti-tumor effector cells of adaptive

immunity. Alternatively, this early time frame of innate anti-cancer immunity is expected to be dominated by NK cells [24], which could have a significant impact on the trajectory of tumor growth [16]. Instead, we found that IRF3 contributes to limiting B16 melanoma growth at day 9 and beyond following B16 inoculation. This is the period when effector T cells producing IFN- γ can be found in secondary lymphoid tissue and tumors [25, 26]. Therefore, it is clear that, as far as anti-tumor immunity is concerned, IRF3 has a greater impact on adaptive than innate anti-tumor immunity. Previously, we reported that at day 16 after B16F10 tumor inoculation, NK cells infiltration into the tumor and stimulation of IFN- γ from NK cells with poly I:C was impaired in IRF3KO compared with wild-type mice [4]. Thus, it is possible that NK cell involvement in anti-tumor immunity, which is expected to be sustained all throughout adaptive T cell immune responses, is indeed impaired along with anti-tumor T cells in IRF3KO mice.

Interestingly, we showed here that IFN- γ production at the spleen, a major secondary lymphoid tissue involved in adaptive anti-tumor immunity, was significantly increased in wild-type mice starting at day 6 post B16F10 tumor inoculation. The dynamics of the IFN- γ response in the spleen and tumor are consistent with previous reports [25]. However, IRF3 deficiency impaired expression of splenic IFN- γ at this time. Previously, we found that T cells from IRF3KO mice had significant impairments in T cell IFN- γ during the response to Influenza A virus infection compared with wild-type mice [19]. These data strongly suggested that IRF3 plays a significant role in expression of IFN- γ from T cells. Based on this suggestion and a large aggregate of scientific literature on IRF3 and APC cytokines, it was reasonable to speculate that the impairments in IFN- γ expression during T cell responses from IRF3KO mice were solely related to deficient production of APC cytokines. Previous reports from our lab and other labs showed that IRF3 plays a significant role in IL-12[4, 27, 28], IL-15[4, 29], and IL-6 [30] expression from APCs, all of which contribute to effector T cell responses to antigen. However when we added exogenous IL-12, IL-15, or IL-6 to responding T cells from IRF3KO mice, IFN- γ expression was not restored to levels found with wild-type responding T cells [19]. More recently, we showed that incubating enriched T cells from IRF3KO mice with DCs from wild-type mice during in vitro T cell responses also failed to restore IFN- γ production [5]. These unexpected findings prompted us to focus on IRF3 and T cell responses and led us to postulate that IRF3 functions intrinsically in T cell IFN- γ production during immune responses. A preliminary experiment where we found that enriched T cells from IRF3KO mice responding to anti-CD3/anti-CD28 exhibited significantly less IFN- γ than those from wild-type mice (data not shown) supports this notion. However, the clear contribution of IRF3 in optimum T cell production of IFN- γ notwithstanding, the precise mechanism for IRF3 in transcriptional regulation of IFN- γ remains enigmatic.

While the role for IRF3 in IFN- γ production from T cells remains unclear, in the present report some light was shed on the role for IFN- γ in control of B16 cell growth. The data herein indicated that IFN- γ induces expression of ISG54 in B16 cells, which correlated significantly with reduction in B16 cells growth. While IFN- γ has been shown to induce cell cycle arrest [6], IFN- γ has also been reported to stimulate expression of several key ISGs, with ISG54 being proapoptotic [7]. IFN- γ through its receptor system stimulates activation of STAT1, which dimerizes and functions as a transcriptional activator at Interferon gamma-

associated sequence (GAS) promoter elements [31] (Fig. 8). Interestingly, the ISG54 promoter does not have GAS elements but rather ISRE sequences that are bound by IRF3 dimers, IRF7 dimers, or ISGF3 transcription factors [32]. However, our data clearly show that IFN- γ synergizes with transcription factors activated by poly I:C to induce the maximal expression of ISG54 in B16 melanoma cells. In support of the data here, we recently reported that IFN- γ stimulates ISG54 expression in RAW264.7 cells and poly I:C activated IRF3 contributes to IFN- γ -stimulated expression of ISG54 [5]. ISGs are a class of molecules induced by interferons that play a significant role in limiting viral infections and whose antiviral effect includes induction of apoptosis in virus infected cells [8]. We therefore postulated that IFN- γ could limit growth of tumor cells by inducing apoptosis through ISG54. Previous reports showed that ISG54 associates with MITA (aka STING) and this complex is an apoptotic trigger for apoptosis through the mitochondrial intrinsic pathway [11] (Fig. 8). Indeed we showed here that treatment of B16F10 cells with IFN- γ limited B16F10 cell growth and induced significant levels of active caspase-3 and ISG54. IFN- γ -induced apoptosis is brought about by ISG54, which is one member of a class of antiviral ISGs termed tetratricopeptide repeat proteins. The tetratricopeptide repeat proteins include ISG54, ISG56, and ISG60 (Aka: IFIT2, IFIT1, and IFIT3). However, ISG54 induced apoptosis in target cells is counteracted by ISG60, which blocks this apoptosis. It is unclear here to what extent IFN- γ induces ISG60 in B16F10 cells. Expression of ISG54 was reported to be a key factor limiting growth of oral squamous cell carcinomas [11] and adenocarcinomas [12]. Moreover, expression of ISG54 depends on IRF3 [5, 10], which itself is a potent anti-viral factor [11]. Earlier reports showed that IRF3 has non-transcriptional effects. IRF3 itself dimerizes with Bax contributing to induction of Cytochrome-C release from mitochondria and apoptosis [33]. Overall, the data here suggest that poly I:C through TLR3 on tumor cells activates innate antiviral immune components such as IRF3 that plays a role in expression of ISG54 during anti-tumor immune responses (Fig. 8). IFN- γ signaling through its IFN γ R signaling pathway stimulates expression of a key cell cycle arrest protein, p27Kip in tumor cells. However, the IRF3 activation pathway appears to synergize with the IFN- γ /IFN γ R signaling pathway to induce high levels of ISG54 expression and apoptosis of tumor cells.

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References

1. Wathlet MG, Lin CH, Parekh BS, Ronco LV, Howley PM, Maniatis T. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. *Mol Cell*. 1998; 1(4):507–18. [PubMed: 9660935]
2. Tokunaga T, Naruke Y, Shigematsu S, Kohno T, Yasui K, Ma Y, Chua KJ, Katayama I, Nakamura T, Hishikawa Y, Koji T, Yatabe Y, Nagayasu T, Fujita T, Matsuyama T, Hayashi H. Aberrant expression of interferon regulatory factor 3 in human lung cancer. *Biochem biophys res com*. 2010; 397(2):202–207.

3. Wang C, Zhuang Y, Zhang Y, Luo Z, Gao N, Li P, Pan H, Cai L, Ma Y. Toll-like receptor 3 agonist complexed with cationic liposome augments vaccine-elicited antitumor immunity by enhancing TLR3-IRF3 signaling and type I interferons in dendritic cells. *Vaccine*. 2012; 30(32):4790–9. [PubMed: 22634298]
4. Moore TC, Kumm PM, Brown DM, Petro TM. Interferon response factor 3 is crucial to poly-I:C induced NK cell activity and control of B16 melanoma growth. *Cancer letters*. 2014; 346:122–128. [PubMed: 24368188]
5. Guinn Z, Lampe AT, Brown DM, Petro TM. Significant role for IRF3 in both T cell and APC effector functions during T cell responses. *Cell Immunol*. 2016; 310:141–149. [PubMed: 27641636]
6. Matsushita H, Hosoi A, Ueha S, Abe J, Fujieda N, Tomura M, Maekawa R, Matsushima K, Ohara O, Kakimi K, T Cytotoxic. Lymphocytes Block Tumor Growth Both by Lytic Activity and IFN γ -Dependent Cell-Cycle Arrest. *Cancer Immunol Res*. 2015; 3(1):26–36. [PubMed: 25127875]
7. Ito S, Ansari P, Sakatsume M, Dickensheets H, Vazquez N, Donnelly RP, Larner AC, Finbloom DS. Interleukin-10 inhibits expression of both interferon α - and interferon γ - induced genes by suppressing tyrosine phosphorylation of STAT1. *Blood*. 1999; 93(5):1456–63. [PubMed: 10029571]
8. Stawowczyk M, Van Scoy S, Kumar KP, Reich NC. The Interferon Stimulated Gene 54 Promotes Apoptosis. *J Biol Chem*. 2011; 286(9):7257–7266. [PubMed: 21190939]
9. Zhou X, Michal JJ, Zhang L, Ding B, Lunney JK, Liu B, Jiang Z. Interferon induced IFIT family genes in host antiviral defense. *Int J Biol Sci*. 2013; 9(2):200–8. [PubMed: 23459883]
10. Nakaya T, Sato M, Hata N, Asagiri M, Suemori H, Noguchi S, Tanaka N, Taniguchi T. Gene induction pathways mediated by distinct IRFs during viral infection. *Biochem Biophys Res Com*. 2001; 283(5):1150–6. [PubMed: 11355893]
11. Lai KC, Chang KW, Liu CJ, Kao SY, Lee TC. IFN-induced protein with tetratricopeptide repeats 2 inhibits migration activity and increases survival of oral squamous cell carcinoma. *Mol Cancer Res*. 2008; 6(9):1431–9. [PubMed: 18819931]
12. Feng X, Wang Y, Ma Z, Yang R, Liang S, Zhang M, Song S, Li S, Liu G, Fan D, Gao S. MicroRNA-645, up-regulated in human adenocarcinoma of gastric esophageal junction, inhibits apoptosis by targeting tumor suppressor IFIT2. *BMC cancer*. 2014; 14(1):1–12. [PubMed: 24383403]
13. Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, Nakaya T, Katsuki M, Noguchi S, Tanaka N, Taniguchi T. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN- α /beta gene induction. *Immunity*. 2000; 13(4):539–48. [PubMed: 11070172]
14. Moore TC, Al-Salleh FM, Brown DM, Petro TM. IRF3 polymorphisms induce different innate anti-Theiler's virus immune responses in RAW264.7 macrophages. *Virology*. 2011; 418(1):40–8. [PubMed: 21810534]
15. Pahl J, Cerwenka A. Tricking the balance: NK cells in anti-cancer immunity. *Immunobiology*. 2017; 222(1):11–20. [PubMed: 26264743]
16. Talmadge JE, Meyers KM, Prieur DJ, Starkey JR. Role of natural killer cells in tumor growth and metastasis: C57BL/6 normal and beige mice. *J Natl Cancer Inst*. 1980; 65(5):929–35. [PubMed: 6933263]
17. Yarchoan M, Johnson BA 3rd, Lutz ER, Laheru DA, Jaffee EM. Targeting neoantigens to augment antitumor immunity. *Nat Rev Cancer*. 2017; 17(4):209–222. [PubMed: 28233802]
18. Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, Vyth-Dreese FA, Dellemijn TA, Antony PA, Spiess PJ, Palmer DC, Heimann DM, Klebanoff CA, Yu Z, Hwang LN, Feigenbaum L, Kruisbeek AM, Rosenberg SA, Restifo NP. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8 $^{+}$ T cells. *J Exp Med*. 2003; 198(4):569–80. [PubMed: 12925674]
19. Moore TC, Vogel AJ, Petro TM, Brown DM. IRF3 deficiency impacts granzyme B expression and maintenance of memory T cell function in response to viral infection. *Microbes Infect*. 2015; 17(6):426–39. [PubMed: 25777301]

20. Sarkar SN, Peters KL, Elco CP, Sakamoto S, Pal S, Sen GC. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. *Nat Struct Mol Biol.* 2004; 11(11):1060–7. [PubMed: 15502848]
21. Gautier G, Humbert M, Deauevieu F, Scuiller M, Hiscott J, Bates EE, Trinchieri G, Caux C, Garrone P. A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. *J Exp Med.* 2005; 201(9):1435–46. [PubMed: 15851485]
22. Petro TM. Modulation of IL-12 p35 and p40 promoter activity by smokeless tobacco extract is associated with an effect upon activation of NF-kappaB but not IRF transcription factors. *Int immunopharm.* 2003; 3(5):735–45.
23. Bahram F, Hydbring P, Tronnorsjo S, Zakaria SM, Frings O, Fahlen S, Nilsson H, Goodwin J, von der Lehr N, Su Y, Luscher B, Castell A, Larsson LG. Interferon-gamma-induced p27KIP1 binds to and targets MYC for proteasome-mediated degradation. *Oncotarget.* 2016; 7(3):2837–54. [PubMed: 26701207]
24. Kurosawa S, Harada M, Matsuzaki G, Shinomiya Y, Terao H, Kobayashi N, Nomoto K. Early-appearing tumour-infiltrating natural killer cells play a crucial role in the generation of anti-tumour T lymphocytes. *Immunology.* 1995; 85(2):338–46. [PubMed: 7642226]
25. Kirk CJ, Hartigan-O'Connor D, Mule JJ. The dynamics of the T-cell antitumor response: chemokine-secreting dendritic cells can prime tumor-reactive T cells extranodally. *Cancer Res.* 2001; 61(24):8794–802. [PubMed: 11751401]
26. Gao Y, Yang W, Pan M, Scully E, Girardi M, Augenlicht LH, Craft J, Yin Z. Gamma delta T cells provide an early source of interferon gamma in tumor immunity. *J Exp Med.* 2003; 198(3):433–42. [PubMed: 12900519]
27. Dahlberg A, Auble MR, Petro TM. Reduced expression of IL-12 p35 by SJL/J macrophages responding to Theiler's virus infection is associated with constitutive activation of IRF-3. *Virology.* 2006; 353(2):422–432. [PubMed: 16824573]
28. Goriely S, Molle C, Nguyen M, Albarani V, Haddou NO, Lin R, De Wit D, Flamand V, Willems F, Goldman M. Interferon regulatory factor 3 is involved in Toll-like receptor 4 (TLR4)- and TLR3-induced IL-12p35 gene activation. *Blood.* 2006; 107(3):1078–84. [PubMed: 16219795]
29. Soudja SM, Ruiz AL, Marie JC, Lauvau G. Inflammatory Monocytes Activate Memory CD8(+) T and Innate NK Lymphocytes Independent of Cognate Antigen during Microbial Pathogen Invasion. *Immunity.* 2012; 37(3):549–62. [PubMed: 22940097]
30. Moore TC, Cody L, Kumm PM, Brown DM, Petro TM. IRF3 helps control acute TMEV infection through IL-6 expression but contributes to acute hippocampus damage following TMEV infection. *Virus Res.* 2013; 178(2):226–33. [PubMed: 24140628]
31. Mirkovitch J, Decker T, Darnell JE Jr. Interferon induction of gene transcription analyzed by in vivo footprinting. *Mol Cell Biol.* 1992; 12(1):1–9. [PubMed: 1729591]
32. Sweeney SE, Kimbler TB, Firestein GS. Synovocyte innate immune responses: II. Pivotal role of IFN regulatory factor 3. *J Immunol.* 2010; 184(12):7162–8. [PubMed: 20483755]
33. Chattopadhyay S, Yamashita M, Zhang Y, Sen GC. The IRF-3/Bax-Mediated Apoptotic Pathway, Activated by Viral Cytoplasmic RNA and DNA, Inhibits Virus Replication. *J. Virol.* 2011; 85(8):3708–3716. [PubMed: 21307205]

Highlights

- IRF3 is activated during the immune response to B16 melanomas
- IRF3 contributes to expression of IFN- γ during the response to B16 melanomas
- IFN- γ reduces B16 melanoma growth through cell cycle arrest and apoptosis
- Synergistic induction of ISG54 with IFN- γ and the TLR3/IRF3 agonist, poly I:C

Fig. 1.

IRF3 deficiency impairs control of B16 tumor growth and IFN- γ expression. C57Bl/6 and IRF3KO mice were subcutaneously injected with 10^6 B16-F10-Luc melanoma cells. Tumor growth of individual mice was monitored day 2, 6, and 9 days post injection by analyzing relative luciferase activity at the tumor sites. Data are means of relative luminescence $\pm$ standard error

Fig. 2.

IRF3 deficiency impairs IFN- γ expression during tumor growth. (A,B) Data are relative IFN-g activity in C57Bl/6 (A) spleens and (B) tumor sites as measured by qRT-PCR or ELISA, respectively, or (C) in spleens of tumor-bearing C57Bl/6 and IRF3KO mice as measured by qRT-PCR. n = 4–5; * indicates $p < .05$ for differences between mouse strain or differences between tissue from non-inoculated or inoculated mice.

Fig. 3.

IRF3 and IRF7 are activated during B16 tumor growth. C57Bl/6 and IRF3KO mice were subcutaneously injected with 10^6 B16-F10-Luc melanoma cells. (A) IRF3 or IRF7 (C) activation in spleens at day 0, 4, 6, 7, and 12 after B16F10-Luc inoculation into C57Bl/6 mice or (B) IRF3 or IRF7 (D) activation at day 6 after B16F10-Luc inoculation into C57Bl/6 or IRF3KO mice as measured by IRF3 or IRF7 TransAM assay. $n = 4-5$; * indicates $p < .05$ for differences between mouse strains.

Fig. 4. IFN- γ and poly I:C synergism controls growth of B16 melanoma cells. (A) B16F1-SEAP, (B) B16F1-SEAP-IFNGRKO, and (C) B16F10 cells were treated with poly I:C (10 μ g/ml) without or with 20 or 50 ng/ml recombinant IFN- γ . After 48 h cell relative cell viability was assessed by measuring 590 nm fluorescence from reazurin reagent of a Cell Titer Blue kit. Representative data from three experiments; n = 4; * indicates p < 0.05 for differences between cell cultures with or without IFN- γ ; ** indicates significant interaction between presence of IFN- γ with poly I:C as measured by ANOVA, p < 0.05..

Fig. 5.

IFN- γ and poly I:C induce ISG54 promoter activity in inverse proportion of B16 melanoma cell growth. (A) B16F1-SEAP or (B) B16F1-SEAP-IFNGRKO cells were treated with poly I:C (10 μ g/ml) without or with 20 or 50 ng/ml recombinant IFN- γ . After 48 h cell relative SEAP activity was assessed. Representative data from three experiments; n = 4; C; ** indicates significant interaction between presence of IFN- γ with poly I:C as measured by ANOVA, $p < 0.05$. (C) Correlation analysis of individual Cell-titer blue data plotted with ISG54 promoter activity data in B16F1-SEAP cells.

Fig. 6.

Poly I:C and IFN- γ reduce cell viability (A) and induce ISG54 promoter activity (B) of B16 melanoma cells in dose dependent fashion. B16F1-SEAP cells (10^4) were treated with varying amounts of poly I:C (A) without or with 2 ng/ml recombinant IFN- γ (A) or varying amounts of IFN- γ without or with varying amounts poly I:C (B). After 48 h cell relative cell viability (A) or SEAP activity (B) was assessed. Representative data from three experiments; $n = 5$. * indicates $p < 0.05$ for differences between cell cultures with or without IFN- γ or with or without poly I:C.

Fig. 7.

Control of B16 melanoma cell growth with IFN- γ involves Caspase 3 activation and cell cycle arrest protein, p27Kip. B16F10-Luc cells (5×10^4) were treated with varying amounts of recombinant IFN- γ with or without 10 μ g/ml poly I:C. After 48 h (A) cell viability was assessed using Trypan Blue staining or (B) percentage of cells with active Caspase 3 was assessed using a fluorogenic DEVD probe. Representative data from two experiments; n = 4; * indicates p < 0.05 for differences between cell cultures with or without IFN- γ ; ** indicates p < 0.05 for differences between cell cultures with poly I:C and the indicated level of IFN- γ . (C) B16F10-Luc cells (1×10^6) were treated with IFN- γ with or without poly

I:C; after 24 and 48 h, 10 µg of cell lysate protein was Western blotted for cleaved caspase-3, p27Kip, ISG54, and beta-tubulin in B16F10 cells treated with IFN- γ with or without poly I:C for 24 or 48 h.

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Fig. 8.
Graphic representation of results and conclusions of the synergism between poly I:C and IFN- γ on B16 melanoma cells.