Research paper

Concomitant type I IFN and M-CSF signaling reprograms monocyte differentiation and drives pro-tumoral arginase production

Yuanyuan Tong, Luyang Zhou, Limin Yang, Panpan Guo, Yanlan Cao, F. Xiao-Feng Qin, Jianghuai Liu

A R T I C L E  I N F O
Article history:
Received 21 June 2018
Received in revised form 19 November 2018
Accepted 28 November 2018
Available online 7 December 2018

Keywords:
Type I IFN
M-CSF
Arginase
Monocyte maturation
Tumor-associated macrophages
Anti-tumor immunity

A B S T R A C T
Background: Type I IFN-based therapies against solid malignancies have yielded only limited success. How IFN affects tumor-associated macrophage (TAM) compartment to impact the therapeutic outcomes are not well understood.

Methods: The effect of an IFN-inducer poly(I:C) on tumor-infiltrating monocytes and TAMs were analyzed using a transplantable mouse tumor model (LLC). In vitro culture systems were utilized to study the direct actions by poly(I:C)-IFN on differentiating monocytes.

Results: We found that poly(I:C)-induced IFN targets Ly6C+ monocytes and impedes their transition into TAMs. Such an effect involves miR-155-mediated suppression of M-CSF receptor expression, contributing to restricting tumor growth. Remarkably, further analyses of gene expression profile of IFN-treated differentiating monocytes reveal a strong induction of Arg1 (encoding arginase-1) in addition to other classical IFN targets. Mechanistically, the unexpected Arg1 arm of IFN action is mediated by a prolonged STAT3 signaling in monocytes, in conjunction with elevated macrophage colony-stimulating factor (M-CSF) signaling. Functionally, induction of Arg1 limited the therapeutic effect of IFN, as inhibition of arginase activity could strongly synergize with poly(I:C) to enhance CD8+ T cell responses to thwart tumor growth in mice.

Conclusions: Taken together, we have uncovered two functionally opposing actions by IFN on the TAM compartment. Our work provides significant new insights on IFN-mediated immunoregulation that may have implications in cancer therapies.

© 2018 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Type I IFNs (IFN-Is) are a family of closely related cytokines produced by the innate immune system in response to specific pathogen- or danger-associated molecular patterns [1]. They function via a common, broadly expressed IFN-1 receptor to activate Jak1/Tyk2, which in turn drive signal transduction mainly via the STAT1/STAT2/IRF9 complex called ISGF3. ISGF3 acts as a transcription factor to induce a number of interferon-stimulated genes (ISGs) that mediate IFN-1s potent antiviral, anti-proliferative and immunomodulatory functions [2,3].

The antitumor activities by IFN-Is have long been recognized and are mediated by both tumor-intrinsic and -extrinsic mechanisms [4,5]. Importantly, in defending against solid tumors, IFN-I-mediated enhancements of the innate, as well as the adaptive arms of antitumor immunity are essential [6]. In humans, IFN-I therapies are approved for treatment of several types of cancers [6,7]. More recently, IFN-I-inducing pattern recognition receptor agonists used in either stand-alone or combinatorial regimens have also shown promising results in cancer clinical trials [8,9]. Additionally, in response to conventional and new generations of therapies, the endogenous IFN-Is produced in the tumor microenvironment are shown to represent key determinants of treatment outcomes [7,10]. Nevertheless, data have also emerged that IFN-I can have tumor-promoting roles under certain contexts [11,12]. In addition, effective cancer treatments by IFN-I or IFN-I-inducers are often limited by systemic toxicities [6]. Such problems may have arisen from the complexities of tumor-associated immune cell types, whose varied responses to IFN-I may not become integrated optimally.

Tumor-associated macrophages (TAMs) are abundant in solid tumors [13]. They play key roles in promoting angiogenesis, tumor growth and metastasis, and in suppressing adaptive antitumor immunity. Their abundance has been generally correlated to poor prognosis of cancer.
patients [14]. The phenotypic and functional features of TAMs are rather diverse in different tumors or even within the same tumors, consistent with the tremendous plasticity of macrophages corresponding to changing environments [15]. Furthermore, besides macrophages, the tumors also contain circulation-borne monocytes that are not only the major precursors to TAMs, but are themselves highly responsive to cues within the tumor microenvironment [16]. The remarkable heterogeneity within this lineage compartment also presents a significant challenge to cancer treatments where tumor-associated monocytes/macrophages have been shown to play complex yet influential roles in determining the therapeutic outcomes in different models [13,14].

Despite strong clinical relevance, the impacts of IFN-I-based therapies on the behaviors of tumor-associated monocytes/macrophages are not fully understood. Although a previous report showed that systemic administration of TLR3 agonist poly(I:C) could directly cause TAMs to undergo tumoricidal, M1-type polarization in a mouse model [17], potential impacts of IFN-I signaling on the TAM compartment was not investigated. In this report, we revealed that the M-CSF-dependent, tumor-associated differentiating monocytes respond to poly(I:C)-induced IFN with a strong up-regulation of arginase-1, which subsequently blunts the cytokine's anti-tumor actions. Our work therefore shed some light on the undesirable tumor-promoting mechanisms associated with IFN-I-based therapies.
BM monocytes. The precursor cells were induced for differentiation toward macrophages using M-CSF. The supernatant from minced LLC tumors was also used in some experiments to drive macrophage differentiation [18]. Briefly, the tumors (n = 6, day-10 after inoculation) were cut into small pieces and then homogenized in 5 ml RPMI1640/g tumor tissues. After centrifugation and filtration (0.45 μm), the supernatants of minced tumors were obtained. In some occasions, the levels of M-CSF or type I IFNs within the supernatants were determined using ELISA kits according to manufacturer’s instructions. Otherwise, the above supernatants were mixed with basic culture medium 1:1 and fed to mouse BM mononuclear cells.

2.3.4. Gene expression by quantitative real-time PCR. RNA preparation and qPCR were performed as previously described [19]. The real-time PCR was performed on ABI Step One Plus using gene-specific primers (Supplemental Table 1–1, 1–2). The results were normalized to housekeeping gene GAPDH and HPRT. The quantification of mature microRNAs was also carried out using established protocols [20]. Briefly, total RNA was first polyadenylated. After extraction, the samples were reverse-transcribed using a universal adaptor-adding primer (5′-GCCGTCAGATCTAGCTAAACGATACAGTTTTGTTTTTTTTTTC-3′). Consequently, a same reverse primer sequence was used for quantitation of all microRNAs (5′-GCCGTCAGATCTAGCTAAACGATACAGTTTTGTTTTTTTTTTC-3′). All forward primer sequences are shown in Supplemental Table 1–3. The microRNA expression levels were normalized to those of 5S rRNA and U6 snRNA.

2.3.5. Microarray analysis. The MACS-purified mouse BM monocytes from a cohort of 12 mice divided into two triplicated groups and treated with M-CSF (20 ng/ml) without or with IFNβ (100 U/ml) for 60 h. The samples were probed against Agilent SurePrint G3 Mouse Gene Expression Microarray 8x60K and data were analyzed via R/bioconductor. The raw and processed data were deposited at GEO (GSE115392) and can be openly accessed.

2.3.6. Culture of primary cells and cell lines. The primary mouse macrophages and fibroblasts were cultured as described [21]. The cell lines (LLC, Raw264.7, 293T and L929) were all from American Type Culture Collection and cultured according to recommended conditions. Lipofectamine 3000 (Invitrogen) was used for transfection of Raw264.7 cells, 293T or BM mononuclear cells. The miR-155 mimic, chemically modified miR-155 inhibitor and the negative control oligos (Genomeditech) (Supplemental Table 1–4) were transfected at a final concentration of 40 nM. For transfection of BM mononuclear cells, the freshly harvested cells were cultured in medium containing M-CSF for 2 h and then were transfected with 25 nM of oligos.

2.3.7. Luciferase reporter assay. The full-length 3’UTRs of mouse Csf1r (NCBI accession, NM_001037859) and Pu.1 (NCBI accession, NM_011355) was amplified from cDNA from macrophages and cloned into the xbaI site of pGL3 3’-UTR reporter vector using homologous recombination kit (Vazyme #C112–02). The reporter constructs were used to transfetct 293T cells together with either the miR-155 mimic or the control oligo. After 24 h, the cells were harvested and the lysates were analyzed using the Dual-Luciferase Reporter Assay System (Promega).

2.3.8. Immunostaining and histology. Tissue processing and immunofluorescence staining were performed as previously described [21]. Briefly, fixed tumor tissues were embedded in Tissue-Tek OCT compound. The sections (6-μm) were incubated with fluorophore-conjugated primary antibodies or unconjugated primary antibodies (ARG1 (BD); F4/80 (Biolegend)). When necessary, further staining with secondary antibodies (Invitrogen) was applied. The images were taken using an Olympus FX1000 confocal microscope. When the cultured monocytes/macrophages were study subjects, the cells were cytospun onto slides.

After fixation in methanol for 5 min, the slides were either subjected to immunofluorescence or H&E staining.

2.3.9. Sorting of monocytes and macrophages using flow cytometry. FACS sorting was performed on BD FACSaria III high speed sorter. For cell sorting from tumors, the single cell suspensions of tumors (n = 6, day-14 after inoculation) were prepared as described earlier. The following populations were collected: CD45+CD11b+Ly6G−Ly6C+ F4/80− (monocytes) and CD45+CD11b+Ly6G−Ly6C+ F4/80− (macrophages). For cell sorting from differentiated monocytes in vitro, after 3 days treatment with M-CSF ± IFNβ (100 U/ml), the following populations were collected: Ly6C+ F4/80− (monocytes) and F4/80+ (macrophages).

2.4. Statistical analysis

All data presented in this study are derived from at least two independent experiments. The effects of treatments on tumor growth were presented as the average measurements (±SD) from at least 6 tumors. Cell abundances determined by flow cytometry are quantified using samples from independent experiments. Cytokine levels were measured by ELISA using prepared supernatants from three individual tumors. Cell counting were presented as average values (±SEM) from at least three random microscopic fields. Levels of protein bands of interest in comparison to a loading control have been determined by densitometry and average values (± variances) from independent experiments are presented. As in standard practices, qPCRs or reporter assays were initially measured using four or three technical replicates. Nevertheless, the presentation of essential results from these quantitative assays reflect biological, but not technical replicates. When applicable throughout the study, Student t-tests were performed between given sample groups to determine the P values. If not specifically indicated, asterisks were used to mark the P values in graphs (N.S.: not significant; *: P < 0.05; **: P < 0.01; ***: P < 0.001; ****: P < 0.0001; *****: P < 0.00001).

3. Results

3.1. IFN-I targets Ly6C+ monocytes to inhibit monocyte-to-TAM differentiation

Intraperitoneal (i.p.) administration of TLR3 agonist poly(I:C) induces IFN-I/cytokine release, resulting in an apparent systemic IFN response [22]. Previously, such treatment was shown to elicit anti-tumor effects attributable to several IFN-dependent or -independent mechanisms of immune activation [23]. However, the relevant roles by IFN signaling in tumor-associated monocyte/macrophage compartment had not been clearly characterized. To this end, a transplantable mouse tumor model (LLC) with well-known presence of a significant macrophage compartment was chosen [24]. When introduced i.p., poly(I:C) caused notable inhibition of tumor growth throughout the course of the treatment (Figs. 1A and S1A). The tumor samples harvested at the last time point from the poly(I:C) group showed induction of an interferon-stimulated gene (ISG), i.e. Ifg5, validating the engagement of an IFN response in tumors (Fig. S1B). Consistent with previous observations [17], a number of M1-type, pro-inflammatory markers were also found to be up-regulated in tumors of the poly(I:C) group. Interestingly, the mRNA levels of a macrophage-selective marker F4/80 (encoded by Emr1) showed consistent decrease in the poly(I:C) group (Fig. S1B), suggestive of a decrease in the numbers of tumor-associated macrophages (TAMs). Immunofluorescent microscopy of tumor sections further confirmed such a finding (Fig. 1B, C). It is of note that such intriguing observations on decrease of TAM numbers by IFN signaling in tumor-associated monocyte/macrophage compartment were not previously reported.

As TAMs are largely derived from circulating Ly6C+ monocytes that extend the tumors [13], we extended our analyses to the overall myeloid populations within tumors, including the monocyte...
compartment. When the tumor-associated myeloid cells were selected under the sequential CD45+ and CD11b+ gates, we could then separately examine the numbers of Ly6G+ granulocytic lineage cells as well as the Ly6C+ population that contains both the F4/80+ macrophages and Ly6C+ monocytes [25]. The numbers of Ly6G+ cells were not significantly affected by poly(I:C) in tumors (Fig. S1C, right). Further analyses of the Ly6G+ population in tumors revealed an expected decrease of F4/80+ macrophages (Fig. 1D). In an apparent reverse pattern, the Ly6C+ monocyte subset showed marked increase in the poly(I:C) group (Figs. 1D, E and S1C). In the spleen from the same mice, there were apparent increases in both Ly6G+ cells and Ly6C+ monocytes (Fig. S1C, left), consistent with expansion of such myeloid-derived suppressor cells (MDSC)-like cells by CpG (another IFN inducer) observed previously [26]. The apparent expansion of splenic Ly6C+ monocytes and the evident decrease in tumor macrophage/monocyte ratios collectively suggested that poly(I:C) treatment substantially reduced monocyte-to-TAM conversion.

We next tried to establish an in vitro culture system to model the dynamics of monocyte-to-macrophage transition in the control and poly(I:C) group of tumors. M-CSF/M-CSF receptor (CSF1R) is the main ligand/receptor system that drives macrophage lineage commitment and terminal differentiation [27,28]. Indeed, when the mononuclear cells harvested from the BM of naïve mice were cultured in the presence of recombinant M-CSF for two days, a major cell population labeled positively with the macrophage marker F4/80 emerges, accompanying a decrease in their Ly6C+F4/80+ monocyte precursors (Fig. S1D) [29]. To examine the monocyte differentiation cues within the tumor microenvironment, we prepared the supernatant fractions from minced tumors tissues. Such fractions were found to contain high levels of M-CSF (Fig. 1F), consistent with others’ observations with the LLC cell line [30]. The control tumor supernatant sufficed to drive the appearance of a F4/80+ population from BM mononuclear cells in 48 h. The F4/80+ and Ly6C+F4/80+ populations can also be grossly distinguished by their relative levels of forward scatter (FSC) and side scatter (SSC) (Fig. S1E, right), confirming their respective macrophage and monocyte identities [31]. Moreover, a specific inhibitor against CSF1R tyrosine kinase (GW2580) [32] substantially reduced tumor supernatant-induced conversion of Ly6C+F4/80+ monocytes into F4/80+ macrophages (Fig. S1E, left). These results established M-CSF as a major monocyte maturation signal within the LLC tumors.

Compared to the control group, the poly(I:C) group of tumor supernatants drove the conversion of fewer Ly6C+F4/80+ monocyte precursors into F4/80+ cells (Fig. S1F). Although the latter F4/80+ cells appeared to express higher levels of Ly6C, the size and granularity of the cells validated their macrophage identities. Therefore, our observations in vivo (Fig. 1D) are recapitulated by in vitro examinations of tumor supernatant-dependent monocyte differentiation, suggesting that solubile cues within the tumor microenvironment are responsible for poly(I:C)-dependent inhibition of monocyte-to-TAM differentiation. Since the levels of M-CSF in tumors from poly(I:C)-treated mice were higher (Fig. 1F), poly(I:C) might have engaged inhibitory signals against monocyte maturation. We tested the contribution by IFN-Is, as the latter were previously shown to inhibit monocyte-to-macrophage differentiation [33]. Expectedly, the levels of both IFNα and IFNβ were significantly elevated in the poly(I:C) group of tumor supernatant (Fig. 1G). Moreover, when the neutralizing antibody against IFN-1 receptor (IFNAR1) was added together with poly(I:C) group of tumor supernatant to BM mononuclear cells, the percentages of macrophages and monocytes in the culture (48 h) were normalized to levels achieved by the control supernatant (Figs. 1H and S1G). The antibody also normalized Ly6C levels in macrophages (Fig. S1H), consistent with a role of IFN in up-regulating Ly6C [34].

We next examined the direct effect of IFN-I on differentiation of MACS-purified mouse BM Ly6C+F4/80+ monocytes (Fig. S1I). Judged by the criteria of plate-aderences (Fig. 1I), morphology (Figs. 1J, S1J) and immunophenotypes (Figs. 1K, S1K), we firmly established that IFN-I can directly act on Ly6C+ monocytes to impede monocyte-to-macrophage transition. It is important to note that, when IFN was added to BM cells that had been previously differentiated under M-CSF for 3 days, no subsequent decrease in adherent cells were observed (data not shown), arguing against a potential role of IFN on mature macrophage survival. Similar to the situation in mouse cells, IFN also inhibited the differentiation of human PBMCs toward macrophages, as judged by cells’ plate-aderences (Fig. S1L) and down-regulation of macrophage markers (Fig. S1M, N). Taken together, our in vivo and in vitro observations strongly suggest that systemic poly(I:C)-induced IFN-I can target tumor-associated monocytes to inhibit their M-CSF-dependent transition into TAMs.

3.2. IFN inhibits the expression of CSF1R protein in differentiating monocytes and macrophages

Although similar inhibitory effects by IFN-I on monocyte maturation had been noted previously [33,35], the underlying mechanisms were not understood. ERK1/2 signaling plays an essential role on CSF1R-dependent macrophage differentiation [27]. Indeed, time-dependent increases in ERK1/2 phosphorylation were observed in M-CSF-driven, mouse differentiating monocytes (Fig. 2A). Notably, we found that these cells had reduced levels of pERK1/2 at 24 h following IFN treatment, which later recovered at 48 h (Fig. 2A). To simplify data interpretations, we treated the terminally differentiated, M-CSF-starved macrophages (BMDMs and peritoneal macrophages (PMs)) with or without IFN for overnight and then stimulated with M-CSF for 10 min. As expected, IFN treatment led to an apparent induction of STAT1, the product of a classical ISG. Importantly, the immediate M-CSF signaling was blunted by IFN (Figs. 2B and S2A). It is worth noting that overnight M-CSF withdraw together with IFN treatment on macrophages did not result in apparent global effects such as notable changes in their numbers or morphologies (data not shown).

To further understand IFN’s inhibitory effect on proximal M-CSF signaling, we examined the levels of cell surface CSF1R. During M-CSF-driven monocyte differentiation, cell surface CSF1R levels gradually increased in a population of transitional monocytes (Fig. 2C) [29]. Such increases in CSF1R were apparently suppressed by IFN in time points from 24 h to 72 h. Interestingly, the mRNA levels of Csfr1 were not reduced significantly within the first 48 h of treatment (Fig. 2D). Since CSF1R is indispensable for M-CSF signaling [27,28], our results therefore strongly suggest that IFN-mediated down-regulation of cell surface CSF1R levels accounts for its inhibitory effect on monocyte-to-macrophage transition.

Fig. 1. IFN-I targets Ly6C+ monocytes to inhibit monocyte-to-TAM differentiation. (A) The mice were injected (s.c.) with 1 × 106 LLC cells in the flanks. Starting on day 5 after tumor implantation, IFN-I-inducer poly(I:C) was administered (i.p.) every two days. The changes in tumor sizes were determined (± SD, n = 6, P value is marked for the last time point). Arrows indicate treatments. (B–E) Tumors were harvested after 4 treatments. (B) Sections were analyzed by F4/80 immunofluorescence microscopy (scale: 50 μm). (C) Quantitation from 6 random fields (± SD) is presented (P value marked). The myeloid compartments in disintegrated tumor tissues (6 combined) were analyzed by FACS (D and E). Representative plots are shown (D). In (E), the mean percentages (± SD) of macrophages (Mφ) and monocytes (Mono) are presented (three independent experiments, P values for both subsets marked) (F–H) Tumor-burden mice (day 9) were mock- or poly(I:C)-treated for 12 h and the tumors were harvested. In (F and G), supernatants were prepared from individual minced tumors (n = 3). The levels of M-CSF (F) or IFNα/β (G) in the supernatants were determined by ELISA (±SD, P values marked). In (H), the BM mononuclear cells were fed with the supernatants (sup) from minced tumor tissues (six tumors combined). Control IgG or IFNAR-blocking antibody (10 μg/ml) were included in the medium. In 48 h, cells were FACS-analyzed for Ly6C and F4/80. Average abundance of these subsets (relative) from two independent experiments are presented (± data range). (I–K) MACS-purified monocytes were cultured in 20 ng/ml M-CSF ± IFNα (100 U/ml). The extents of cells’ plate-aderences are shown in (I) (scale: 100 μm). (J) Cells were subjected to cytospin/H&E staining and their constitutions were quantitated (SEM, three random fields). (J) Cells were also analyzed by FACS after 24 h of treatments.
Similar down-regulation of cell surface CSF1R was also observed in IFN-treated mature macrophages and a macrophage cell line, Raw264.7 (Fig. S2B). As expected, the mRNA levels of Csf1r in parallelly treated BMDMs and Raw264.7 cells were not affected (Fig. S2C). We further found that IFN-mediated down-regulation of cell surface CSF1R in BMDMs was blunted by pre-treatment of the cells with a selective Jak inhibitor, at a dose resulting in partial inhibition of ISG induction (Fig. S2D, E), establishing the participation of Jak pathway in such an effect.

Using flow cytometry analyses of fixed/permeablized cells (Fig. S2F), as well as Western blotting (Fig. 2E), we further demonstrated down-regulation of total CSF1R protein within 24 h of IFN treatment in BMDMs. To examine the turnover of CSF1R, BMDMs were pre-treated with or without IFN and then added with protein synthesis inhibitor cycloheximide (Fig. 2F and S2G). Consistent with previous findings [36], CSF1R protein showed fast turnover. Nevertheless, its half-life was not further accelerated following IFN treatment. Taken together, our results indicated miR-155 in regulating CSF1R (Fig. 3E). Additionally, transfection with an miR-155 inhibitor oligo led to up-regulation of miR-155 and down-regulation of CSF1R in whole tumors (Fig. 3F, I). Together, this provided evidence for the role of IFN-CSF1R inhibitory circuit in the non-myeloid MEFs or L929 cells (Fig. S3C). We noted that miR-155 was previously shown to regulate some targets mainly at the level of mRNA translation [40]. Furthermore, miR-155 was known to regulate some targets at the level of mRNA translation [41,42].

Next, we probed the relevance of IFN-miR-155-CSF1R inhibitory circuit in tumor-associated monocyes/macrophages in vivo. Indeed, poly(I:C) treatment of LLC tumor-burden mice led to notable up-regulation of miR-155 and down-regulation of CSF1R in whole tumors (Fig. 3H, I). To functionally define the role of IFN-CSF1R inhibitory circuit in tumors, CSF1R inhibitor GW2580 was used together with poly(I:C) in tumor-bearing mice to further impede CSF1R function. As expected, the combinatorial treatment led to additive inhibition of TAM numbers, as well as macrophage marker Emr1 mRNA (Fig. S3D, E). Furthermore, in general agreements with many other studies of CSF1R targeting (reviewed in [13]), GW2580 enhanced poly(I:C)-mediated control of tumor progression (Fig. 3J), pointing to an anti-tumoral role by the engaged miR-155-CSF1R inhibitory circuit in the macrophage compartment.
3.4. IFN-I signaling in differentiating monocytes unexpectedly leads to strong induction of arginase-1

Our results so far revealed that the differentiating Ly6C⁺ monocytes represent a major IFN-responding cell type within the tumor microenvironment, where the rate of their conversion into TAMs is impeded by IFN. As IFN-I was known to engage diverse, cell type-specific responses [43], we next utilized microarray analyses to examine the functional role of IFN (60 h) on differentiating monocytes (Fig. 4A). Expectedly, many classical ISGs appeared in the up-regulated genes. Gene ontology (GO) analysis revealed that genes whose functions related to interferon response, host defense and immune activation were enriched in the up-regulated gene list (Fig. S4A). Consistently, IFN-mediated induction of some common proinflammatory markers was confirmed by qPCR (Fig. S4B). To our surprise, the gene exhibiting the greatest fold increase by IFN in such a system was Arg1 (Fig. 4B), encoding arginase-1, whose remarkable induction pattern was confirmed by qPCR (Fig. S4B). Arginase-1 catalyzes a key step in arginine catabolic pathway and is mostly known to play a pro-tumor role in tumor-associated myeloid cells [44]. The extent of Arg1 induction by IFN in differentiating monocytes greatly exceeded those of several other immunosuppressive genes that previously reported to be IFN-inducible (Fig. S2B) [10]. Furthermore, genes within the GO term of “arginine transport” are enriched within the list of IFN-up-regulated genes from the microarray (Fig. S4A). These striking results implicated that IFN-treated differentiating monocytes indeed also engaged an arginase-dependent protumoral program.

We further confirmed the induction of arginase-1 protein in IFN-stimulated (60 h) differentiating monocytes (Fig. 4C). A marked up-regulation in total STAT1 protein (an ISG) in comparison to its previously noted low baseline level in differentiating monocytes [45] was used as a positive control. Note that at such a late time point, pSTAT1 became difficult to detect and was therefore not used in our studies as controls (Fig. S4C). In the ensuing experiment, it was found that Arg1...
Fig. 4. IFN-1 signaling in differentiating monocytes unexpectedly leads to strong induction of arginase-1. (A–C) The MACS-purified BM monocytes were cultured in M-CSF ± IFN for 60 h. Microarray analyses were performed. Heatmap of differentially expressed genes is presented in (A). (B) Genes with the greatest fold-changes are presented (Arg1 highlighted). (C) Protein samples were analyzed by Western blot (WB). Average quantifications of normalized ARG1 levels (± range) from two independent experiments are marked. (D–F) Mouse BM mononuclear cells (D), mature BMDMs (E) or human PBMCs were treated w/M-CSF ± IFNβ for indicated times (PBMC with IFNα for 5 days). RNA samples were analyzed by qPCR. Average data (± range) from two independent experiments are presented. (G and H) Relative levels of Arg1 mRNA as normalized to its liver levels (G) or ARG1 protein (H) in control and treated tumors (day 14) are presented. In (G), data were from 3 independent experiments (±SEM, P value marked). In (H), some normal tissue samples were included. Quantifications of normalized tumor ARG1 levels were averaged (± range, n = 2). (I) BM mononuclear cells were cultured within the control or poly(I:C) group of supernatants from minced tumor tissues for 60 h in two independent experiments. The poly(I:C) group was also added with neutralizing antibody against IFN receptor (αR1). The mRNA levels of Arg1 were determined (± range). (J and K) BM mononuclear cells were cultured in M-CSF ± IFNα for 60 h. After cytospin, the slides were then co-stained with indicated antibodies for immunofluorescence analyses ((J), scale: 50 μm). Arrowheads point at positively stained cells. In (K), the cells positive for Arg1 (A+), F4/80 (F+) or both markers (F + A+) were quantitated (±SEM, 3 fields, P values between given groups marked). (L and M) In (L), sections of tumors (day 14) were immunostained as in (J). In (M), tumor associated monocytes or macrophages were sorted by flow cytometry in two independent experiments. Samples from sorted cells were subjected to WB analysis. Average quantifications of normalized ARG1 levels are marked (± range). (N and O) Tumor-burden mice were treated ± poly(I:C) ± GW2580 and tumors were harvested (day 14). The samples were subjected to qPCR (N) or WB analyses (O), respectively. Average quantifications from two independent experiments are presented (± range). (P) Tumor-burden mice were treated with ± poly(I:C) ± CCR2 antagonist RS504393 (CCR2-A) on day 6 after tumor implantation. Tumors were harvested on day 14 and subjected to qPCR analyses (± range, n = 2).
induction by IFN was apparently not the consequence of a differentiation block, as the mRNA levels of Arg1 remained relatively unchanged during monocyte maturation (Fig. S4D). Interestingly, unlike Isg15, a classical ISG whose induction peaked around 24 h, Arg1 mRNA induction by IFN became much more substantial at 48 h (Fig. 4D). Moreover, in contrast to IFN-Î·, type II IFN used at the same concentration caused minimal up-regulation of Arg1 (Fig. S4E). Intriguingly, in differentiated bone marrow-derived macrophages (BMDMs) cultured under M-CSF, IFNβ failed to stimulate Arg1 mRNA (Fig. 4E). Therefore, Arg1 induction by IFN-Î· in monocytes is both stimulus- and differentiation stage-restricted. Moreover, we confirmed that in human peripheral blood mononuclear cells (PBMCs) cultured under M-CSF, IFN-Î· treatment also led to a notable up-regulation of ARG1 mRNA (Fig. 4F).

Consistent with results from cultured monocytes, increased expression of arginase-1 at both mRNA (~10 fold induction) and protein levels were seen in the whole LLC tumors from poly(I:C)-treated mice, while no such trend was observed in the lungs or livers from the same animals (Fig. 4G, H). The restricted ARG1 induction by poly(I:C)-IFN in tumors is likely to be attributed to their ability to continuously recruit infiltrating monocytes where the IFN-ARG1 axis can be subsequently engaged.

We next used supernatants from mixed tumors to examine whether poly(I:C)-stimulated IFN-Î· in the tumor microenvironment may contribute to inducing Arg1 expression in differentiating monocytes. Notably, compared to the control group, the poly(I:C) group of supernatant caused a much higher expression of Arg1 in the BM culture, which could be largely prevented by a neutralizing antibody against IFNAR1 (Fig. 4I and S4J). It is worth noting that direct Poly(I:C) treatment of BM mononuclear cells did not lead to induction of Ifnb or Arg1 mRNAs (Fig. S4G), consistent with their low expression of TLR3 [46]. Moreover, when BMDMs were treated with poly(I:C), Arg1 mRNA levels were only moderately induced (Fig. S4H, ~5-fold). Since tumor-associated myeloid cells represent a relatively small percentage of the whole tumor mass, such a moderate, direct induction of Arg1 mRNA is not possible to account for its substantial changes in whole tumors (see Fig. 4G). Therefore, the results from the above in vitro characterization experiments strongly support a model that poly(I:C)-induced IFN-Î· subsequently acts on tumor-infiltrating monocytes, driving marked induction of arginase-1.

During monocyte-to-macrophage differentiation, cells belonging to either stages co-exist. Therefore, we determined the maturation status of the cells expressing high levels of Arg1 following IFN stimulation. Remarkably, in IFN-treated BM mononuclear cells, high levels of Arg1 were mostly localized in the newly formed F4/80+ macrophages (arrowheads, <50% of the total cells) (Fig. 4, K). Similarly, poly(I:C)-induced ARG1 expression in tumors was largely localized in F4/80+ TAMs (Fig. 4L). Such a macrophage-restricted ARG1 induction in tumors were also corroborated by analysis of Arg1 mRNA and protein levels in sorted monocytes and macrophages from tumors (Figs. 4M and S4I). Consistent with the restriction of poly(I:C)-dependent ARG1 expression in the TAM compartment, pharmacological depletion of TAMs (see Figs. S3D, E) by CSF1R inhibitor GW2580 led to reduction of Arg1 mRNA and protein levels in tumors from poly(I:C)-treated mice (Fig. 4N, O). Such an Arg1-reducing effect by GW2580 is likely to contribute to its enhancement of poly(I:C)-mediated antitumor control seen earlier (see Fig. 3J).

Monocyte recruitment to tumors largely involves CCR2 signaling [15]. Therefore, to further support that the infiltrating monocytes in poly(I:C)-treated tumors subsequently differentiate into the ARG1high TAMs, we administered poly(I:C) together with a CCR2 antagonist [47]. Such inhibitor substantially reduced the levels of tumor-associated monocytes in the poly(I:C) group (Fig. S4J). The numbers of TAMs were also reduced, but to a lesser extent, likely attributed to the longer life-spans of macrophages than those of monocytes [48]. Importantly, CCR2 blockade greatly reduced poly(I:C)-mediated Arg1 mRNA induction in tumors (Fig. 4P), functionally linking monocyte recruitment to subsequent ARG1 induction in TAMs under the context of poly(I:C) stimulation. Consistent with an expected, pro-tumoral role of Arg1 in TAMs, CCR2 antagonist further enhanced poly(I:C)-mediated control of tumor progression (Fig. S4K).

Besides arginase-1, the rate of arginine catabolism are controlled by the levels of transporter CAT2b (Slc7a2) and arginase-2 (Arg2) [44,49]. Indeed, similar to that of Arg1, the mRNA levels of Arg2 and Slc7a2 were induced by poly(I:C) in tumors, but not in the lungs or livers (Fig. S4L). In contrast, no changes in these markers were induced by IFN in cultured LLC cells (Fig. S4M). Collectively, our results have unveiled a non-canonical function of IFN-Î· in tumor-infiltrating monocytes, i.e. induction of a group of genes associated with arginine catabolism.

### 3.5. IFN-ARG1 axis in differentiating monocytes is mediated by sustained STAT3 activation, in conjunction with M-CSF signaling

Besides activating the canonical ISGF3 complex, IFN-Î· was also known to activate STAT3 in some cell types [2]. Interestingly, STAT3 was previously shown to up-regulate Arg1 transcription in MDCs [50]. Consequently, we tested the contribution of STAT3 signaling to the IFN-ARG1 axis in differentiating monocytes. Mature BMDMs were used as a negative control (see Fig. 4E). With 30 min of treatment by IFN, STAT3 was similarly activated in both BM mononuclear cells and BMDMs (Fig. S5A). However, in longer period of treatment (24 or 48 h), only in IFN-treated, differentiating monocytes, pSTAT3 levels were still notably elevated above the control levels (Fig. 5A, B). Based on the established role by a sustained STAT3 activation in driving an anti-inflammatory program [51], it is plausible that such a difference in STAT3 activation dynamics may have underlie the monocyte-to-macrophage transitional phase-specific Arg1 induction by IFN. In the ensuing validation experiments, we used a specific inhibitor against STAT3, i.e. Stattic [52]. Stattic greatly reduced IFN-mediated induction of Arg1 mRNA and protein, correlating to its abolishment of IFN-dependent increase of pSTAT3 (Fig. 5C, D). In contrast, the induction of Isg15 was not affected. These results functionally link STAT3 activation to the IFN-ARG1 axis in differentiating monocytes. Moreover, we also examined the dependence of the IFN-miR-155-CSF1R inhibitory axis on STAT3 activity. Interestingly, IFN-mediated induction of miR-155, as well as down-regulation of CSF1R and macrophage marker Emr1 in the differentiation monocytes were unaffected by Stattic treatment (Figs. 5E, S5B). These results demonstrate that the IFN-ARG1 axis and IFN’s inhibition on monocyte maturation can be separated by their different dependence on STAT3.

Since IFN-mediated Arg1 mRNA induction follows a slow kinetics (Fig. 4D) and the subsequent expression of Arg1 protein appears to be concomitant with M-CSF-driven monocyte-to-macrophase conversion (Fig. 4), we considered the possibility that M-CSF might serve as a “signal two” to cooperate with IFN in monocytes, driving Arg1 induction. To test this, IFN was added to monocytes cultured with different concentrations of M-CSF. While such differences in M-CSF dosage did not affect the general IFN responses (Fig. 5F, see Mx1 levels), the induction of Arg1 mRNA by IFN was indeed substantiated by increasing concentrations of M-CSF. To analyze the cellular compartment that mainly contribute to the M-CSF-dependent enhancement of Arg1 expression, we sorted the monocytes and macrophages from the control or IFN-treated mononuclear cells cultured under different doses (5 or 20 ng/ml) of M-CSF (Fig. S5C). Consistent with the bulk cell analyses (see Fig. 5F), both sorted populations exhibited marked up-regulation of Mx1, in a pattern unaffected by the M-CSF concentration (Fig. 5G).

Importantly, M-CSF-dependent enhancement of Arg1 induction by IFN was mostly attributed to the macrophage compartment, where IFN-dependent Arg1 induction was much more substantial.

As M-CSF also represents a critical survival signal for monocytes in vitro [27], it was not feasible to treat monocytes with IFN alone for long term, in the absence of M-CSF. Alternatively, we considered to add IFN to naïve BM mononuclear cells cultured with another myeloid
growth factor, GM-CSF. We observed high levels of Arg1 mRNA in GM-CSF-treated cells (Fig. S5D), consistent with a previous work [53]. However, co-addition of IFN to the GM-CSF-present cultures did not further increase Arg1 mRNA levels, despite that Isg15 was robustly induced. Taken together, our results show that M-CSF, but not GM-CSF “licenses” IFN-driven Arg1 induction in monocytes under conversion toward macrophages. Mechanistically, although increasing M-CSF concentrations led to increments in ARG1 expression, the pY705 STAT3 levels were similar among the samples (Fig. 5H), suggesting that M-CSF and IFN signaling interact at nodes beyond STAT3 Y705 phosphorylation.

3.6. Poly(I:C) and arginase inhibitor exhibit synergistic anti-tumor effects in mice

Our work thus far demonstrates that IFN can parallelly drive downregulation of CSF1R as well as induction of ARG1 in TAMs. Due to the generally considered pro-tumoral role of arginase-1 [44], it stands as another promising TAM-related therapeutic target. We considered to combine poly(I:C) treatment with administration of a commercially available arginase inhibitor, i.e. Nor-NOHA [49]. Interestingly, in cultured differentiating monocytes, Nor-NOHA treatment led to near abrogation of IFN-induced Arg1 mRNA (Fig. 6A), similar to others’ observations with the inhibitor [49,54]. As a control, the induction of a classical ISG, Isg15 was not affected. Since arginase-mediated usage of L-arginine can have a secondary effect on the NOS2-NO axis, an important redox-dependent signaling hub [55,56], our results with Nor-NOHA points to an intriguing possibility that redox signaling might regulate IFN-ARG1 induction.

In tumor-bearing mice, Nor-NOHA alone showed a modest inhibitory effect on tumor growth, consistent with most pre-clinical studies targeting arginases [24,49,57,58]. Nevertheless, Nor-NOHA clearly synergized with poly(I:C) in slowing tumor growth through the course of the treatment (Fig. 6B). As a control, the body weights of mice were not significantly different among experimental groups (Fig. 6C). In resemblance to the pattern of Arg1 mRNA in cultured monocytes (Fig. 6A), poly(I:C)-induced ARG1 protein in tumors were
greatly reduced by Nor-NOHA co-treatment (Fig. 6D). Since arginase activities from TAMs were known to suppress an effective anti-tumor immune response [13], we harvested tumors at an early time point of treatment (day 3 after treatment). Importantly, a group of activation markers for T cells were most strongly up-regulated in the co-administration group (Fig. 6E), whereas induction of an IFN response by poly(I:C) was not further promoted. Furthermore, the mRNA levels of general CD8+ (but not of the CD4+, not shown) T cell markers showed similar trends as the above activation markers, suggesting synergistic increase in tumoricidal T cells (Fig. 6F). Interestingly, as a marker that is shared by natural killer (NK) cells and activated CD8+ T cells [59], the mRNA levels of Klrk1 (encoding NKG2D) was also highest in the co-treatment group. Nevertheless, a potential involvement of NK cells was not further tested. Additional flow cytometry analyses showed that the numbers of CD8+ T cells in tumors were significantly higher in the co-treatment group (Fig. 6G), whereas the abundance of CD4+ T cells were rather low in all groups. These results suggest that arginase inhibition may potentiate the immune-stimulatory action by poly(I:C), leading to more effective anti-tumor T cell responses.

4. Discussions

IFN-Is have been exploited as anti-cancer drugs in the clinics. Nevertheless, how IFN signaling impacts the monocyte/macrophage lineage compartment to shape an anti-tumor response was not clearly
appeared to also require parallel M-CSF signaling (Figs. 5F, G, H and S5D). Further in vitro and in vivo experiments revealed a surprising regulatory axis where IFN-I exposure during this particular monocyte maturation stage leads to a robust induction of arginase-1 in subsequently matured TAMs (Fig. 4). Originally considered as an M2 macrophage marker induced by IL-4/IL-13 in the mouse system, ARG1 is a potent immuno-suppressive enzyme whose expression is now found to be upregulated in different myeloid cells by a variety of stimuli including growth factors, hypoxia and even some pro-inflammatory stimuli [44,46,61]. Interestingly, while IL-4 and IL-13 fail to reproduce the Arg1-inducing effect in human PBMCs [61,62], the IFN-Arg1 axis can be similarly engaged in mice and human systems (see Fig. 4D and F), implicating the functional significance of such regulation. Although IFN-I is mostly known to be immunostimulatory against tumors, our results suggest an additional theme of regulation that a robust ARG1 up-regulation by IFN in monocyte-derived TAMs may be counter-effective.

Mechanistically, we find that the IFN-ARG1 pathway is most probably mediated by a long-duration STAT3 signaling engaged by IFN selectively in the differentiating monocytes, but not in the mature macrophages (Fig. 5A, B, C, D). Similar distinctions in basal pSTAT3 levels were previously described between MDSCs and TAMs and were attributed to their differences in specific phosphatase activities [63]. Whether such possibility applies to our system warrants future investigations. Nevertheless, our results are consistent with the notion that the functional heterogeneities in macrophages may be attributed to their differences in maturation status and the resulting diversities in cellular responses [61]. Furthermore, the correlation between the sustained STAT3 activation in differentiating monocytes and the relative slow kinetics of Arg1 induction associated with the maturation progress (Fig. 5A, B and Fig. 4D, J) implies that STAT3 may participate in a multi-dimensional regulatory program, which leads to the eventual, remarkable activation of Arg1 transcription. In support of such a notion, we found that IFN-mediated Arg1 induction in BM mononuclear cells appeared to also require parallel M-CSF signaling (Figs. 5F, G, H and SS). Such a “licensing” effect by M-CSF on IFN-ARG1 axis is consistent with this growth factor’s known involvement in differentiation/maintenance of macrophages exhibiting M2-like, tolerant phenotypes [28]. As the nature, strength and duration of M-CSF signaling, as well as the associated molecular effects are conceivably different between monocytes that were either undifferentiated or undergoing conversion into macrophages [27], we speculate that such distinctions may be another crucial determinant to further restrict high Arg1 induction by IFN selectively in newly differentiated macrophages (see Figs. 4J and S5G). To place our data into a broader perspective, we favor a hypothetical, monocytic-centric, “two-signal” model that the integration of signals from the inflammatory mediators (signal 1) and myeloid growth factors (signal 2) in the monocyctic precursors critically determines the function of subsequently derived macrophages. As many growing tumors are continuously infiltrated by monocytes [16], further testing the above model and potentially probing the associated mechanistic principles would shed more light on the molecular basis for the ever-elusive natures of TAMs. Regarding the immediate clinical relevance, we suggest that the scenario of IFN-I driving TAM ARG1 expression may particularly operate in many solid tumors that produce high levels of M-CSF [13], subsequently influencing their responses to IFN-I.

Consistent with a pro-tumoral role by the IFN-ARG1 axis in monocytes/TAMs, blockade of CCR2-dependent monocyte recruitment to tumors (Fig. S4K), suppression of CSF1R-mediated monocyte-to-macrophage maturation (Fig. 3), or inhibition of arginases (Fig. 6B) all led to improvement of poly(1:C)-mediated anti-tumor control. On the practical side, results from our preclinical, co-treatment experiments have pointed to several potential targeting strategies to combine with IFN-I-based therapies or IFN-inducing conventional therapies, especially for tumors highly expressing M-CSF. Focusing on the poly(1:C) and arginase inhibitor co-treatment regimen that was most effective in our study, we observed significantly enhanced CD8+ T cell responses (Fig. 6). Therefore, our work has revealed an IFN-driven, monocyte/macrophage-centered “checkpoint” pathway whose targeting may subsequently lead to unleashing the adaptive anti-tumor immunity. It is worth noting that for all the above mentioned IFN-sensitizing targets (CCR2, CSF1R and ARG1), there are multiple designed drugs in clinical development [28,58,64], making more advanced translational research approachable.

Our study also adds to the evidence that IFN-I can have complex immune-regulatory roles [7]. In contrast to the arginase-inducing aspect discussed above, our initially characterized IFN-miR155-CSF1R inhibitory axis in differentiating monocytes is likely to dampen the signaling via the IFN/M-CSF-ARG1 pathway and contribute to IFN’s anti-tumoral effect (Figs. 3) and 4N, O). These findings may be helpful for future treatment designs centered on targeting CSF1R. Despite mainly being considered as a TAM-selective drug target, CSF1R in the non-myeloid stromal cells can inadvertently complicate the therapeutic effect by pharmacological CSF1R inhibition [30]. As IFN-I induction of miR-155 shows monocytes/macrophages-selective pattern (see Figs. 3A, B and S2C), to combine lower dosage CSF1R inhibitors/binders [28] with IFN-I-based therapies (to induce the miR-155-CSF1R axis) may limit potential on-target, off-monocyte/macrophage activities by CSF1R targeting to favor therapeutic gain.

Collectively, the present study reveals some functionally opposing actions by IFN-I in tumor-associated monocytes/macrophages that significantly shape poly(1:C)-dependent treatment effects. Further characterization of such opposing actions by IFN-I via more detailed analyses of individual myeloid subsets shall unveil additional strategies to harness this cytokine’s potent immunoregulatory functions for cancer treatments.

Acknowledgements

We thank the NBRI-NJU for excellent mouse services. We acknowledge the Model Animal Research Center core facility and the Collaborative Liver Disease Research Program of NJU Medical School for institutional support.

Funding sources

This work is supported by a by National Natural Science Foundation of China grants (31771574, 31471313). The funders had no role in study design, data collection/analysis and interpretation of data.

Declaration of interests

The authors declare no competing interests.

Author contributions

Y.T., L.Z., L.Y., P.G. and Y.C. performed experiments. F.Q. and J.L. designed the experiments. Y.T., F.Q. and J.L. wrote and edited the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.11.062.

References

[1] Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010;140 (6):805–20.
Liu D, Chang C, Lu N, et al. Comprehensive proteomics analysis reveals metabolic
activities. Cancer Immunol Immunother 2005;54(5):375–86.

Gresser I, Maury C, Brouty-Boye D. Mechanism of the antimitotic effect of interferon in mice. Nature 1972;239(5368):167–8.

Dumno GP, Bruder K, Schmitt M, et al. A critical function for type I interferons in cancer immunoeediting. Nat Immunol 2005;6(7):722–9.

Parkeri BS, Rautela J, Hertzog PJ. Antimutat actions of interferons: implications for cancer therapy. Nat Rev Cancer 2006;16(3):131–44.

Zitvogel L, Galluzzi L, Kepp O, Smyth MJ, Kroemer G. Type I interferon in hematopoietic stem cells induces a myeloproliferative disorder. J Exp Med 2005;202(1):585–9.

Rojasman J, van Ouderen A, den Broeder BJ, vM, Wofch, Pothof J, Leenens J. MicroRNA-mediated down-regulation of M-CSF receptor contributes to maturation of mouse monocyte-derived dendritic cells. Front Immunol 2013;4:353.

Forster SC, Tate MD, Hertzog PJ. MicroRNA as type I interferon-regulated transcripts and modulators of the innate immune response. Front Immunol 2015;6:334.

Rabinovich I, Schuler-Rozenberg R, Caponni L, et al. Silencing of c-Fl/Fos expres-

Ruien L, Guth A, Kamstock D, Dow S. Type I interferon inhibits the generation of tumor-associated microRNAs. Cancer Immunol Immunother 2010;59(4):587–98.

Dumno GP, Bruder K, Schmitt M, et al. A critical function for type I interferons in cancer immunoeediting. Nat Immunol 2005;6(7):722–9.

Irito, S; Liu D; Chang C; Lu N; et al. Comprehensive proteomics analysis reveals metabolic
activities. Cancer Immunol Immunother 2005;54(5):375–86.

Gresser I, Maury C, Brouty-Boye D. Mechanism of the antimitotic effect of interferon in mice. Nature 1972;239(5368):167–8.

Dumno GP, Bruder K, Schmitt M, et al. A critical function for type I interferons in cancer immunoeediting. Nat Immunol 2005;6(7):722–9.

Liu D, Chang C, Lu N, et al. Comprehensive proteomics analysis reveals metabolic
activities. Cancer Immunol Immunother 2005;54(5):375–86.

Gresser I, Maury C, Brouty-Boye D. Mechanism of the antimitotic effect of interferon in mice. Nature 1972;239(5368):167–8.

Dumno GP, Bruder K, Schmitt M, et al. A critical function for type I interferons in cancer immunoeediting. Nat Immunol 2005;6(7):722–9.

Liu D, Chang C, Lu N, et al. Comprehensive proteomics analysis reveals metabolic
activities. Cancer Immunol Immunother 2005;54(5):375–86.

Gresser I, Maury C, Brouty-Boye D. Mechanism of the antimitotic effect of interferon in mice. Nature 1972;239(5368):167–8.

Dumno GP, Bruder K, Schmitt M, et al. A critical function for type I interferons in cancer immunoeediting. Nat Immunol 2005;6(7):722–9.

Liu D, Chang C, Lu N, et al. Comprehensive proteomics analysis reveals metabolic
activities. Cancer Immunol Immunother 2005;54(5):375–86.

Gresser I, Maury C, Brouty-Boye D. Mechanism of the antimitotic effect of interferon in mice. Nature 1972;239(5368):167–8.

Dumno GP, Bruder K, Schmitt M, et al. A critical function for type I interferons in cancer immunoeediting. Nat Immunol 2005;6(7):722–9.

Liu D, Chang C, Lu N, et al. Comprehensive proteomics analysis reveals metabolic
activities. Cancer Immunol Immunother 2005;54(5):375–86.

Gresser I, Maury C, Brouty-Boye D. Mechanism of the antimitotic effect of interferon in mice. Nature 1972;239(5368):167–8.

Dumno GP, Bruder K, Schmitt M, et al. A critical function for type I interferons in cancer immunoeediting. Nat Immunol 2005;6(7):722–9.

Liu D, Chang C, Lu N, et al. Comprehensive proteomics analysis reveals metabolic
activities. Cancer Immunol Immunother 2005;54(5):375–86.

Gresser I, Maury C, Brouty-Boye D. Mechanism of the antimitotic effect of interferon in mice. Nature 1972;239(5368):167–8.

Dumno GP, Bruder K, Schmitt M, et al. A critical function for type I interferons in cancer immunoeediting. Nat Immunol 2005;6(7):722–9.

Liu D, Chang C, Lu N, et al. Comprehensive proteomics analysis reveals metabolic
activities. Cancer Immunol Immunother 2005;54(5):375–86.

Gresser I, Maury C, Brouty-Boye D. Mechanism of the antimitotic effect of interferon in mice. Nature 1972;239(5368):167–8.