Immune suppressive activity of myeloid-derived suppressor cells in cancer requires inactivation of the type I interferon pathway

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Myeloid-derived suppressor cells (MDSC) are pathologically activated neutrophils and monocytes with potent immune suppressive activity. These cells play an important role in accelerating tumor progression and undermining the efficacy of anti-cancer therapies. The natural mechanisms limiting MDSC activity are not well understood. Here, we present evidence that type I interferons (IFN1) receptor signaling serves as a universal mechanism that restricts acquisition of suppressive activity by these cells. Downregulation of the IFNAR1 chain of this receptor is found in MDSC from cancer patients and mouse tumor models. The decrease in IFNAR1 depends on the activation of the p38 protein kinase and is required for activation of the immune suppressive phenotype. Whereas deletion of IFNAR1 is not sufficient to convert neutrophils and monocytes to MDSC, genetic stabilization of IFNAR1 in tumor bearing mice undermines suppressive activity of MDSC and has potent antitumor effect. Stabilizing IFNAR1 using inhibitor of p38 combined with the interferon induction therapy elicits a robust anti-tumor effect. Thus, negative regulatory mechanisms of MDSC function can be exploited therapeutically.
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yeloid-derived suppressor cells (MDSC) are pathologically activated neutrophils (PMN) and monocytes (Mon) with potent immune-suppressive activity. These cells have a distinct transcriptional profile, biochemical features and, most importantly, exhibit potent ability to suppress immune responses. The total population of MDSC consists of three groups of cells: the most abundant (>75%) relatively immature, PMN-MDSC; the less abundant population of pathologically activated monocytes—(M-MDSC); and a small population of early myeloid precursors present in humans. MDSC accumulation is described in many pathologic conditions, but they play an especially prominent role in cancer as major regulators of immune responses and one of the limiting factors for cancer immunotherapy. In recent years, the clinical role of MDSC has emerged. Results showed a positive correlation of MDSC in peripheral blood (PB) with cancer stage and tumor burden in many types of cancer. Elevated MDSC in the circulation was found to be an independent indicator of poor outcomes in patients with solid tumors. Recent studies demonstrated values of MDSC in predicting response to therapy in many types of cancer. The circulating MDSC negatively correlated with objective clinical response to check-point inhibitors.

Furthermore, the literature on the role of IFN1 in the control of MDSC is rather controversial. Reports suggesting that IFN1 stimulates the generation or suppressive activities of MDSC via sustaining expression of PD-L1 are contradicted by the studies where administration of IFN1 inducers such as poly(I:C) or CpG led to decreased numbers of MDSC, and undermined suppressive activities of MDSC. Whereas activation of STING and ensuing production of IFN1 by tumor irradiation was suggested to induce MDSC, the opposite results were reported by studies using the forced expression of STING; however, the latter effects were not dependent on IFN1. Thus, the critical questions regarding the possible role of IFN1 in the regulation of MDSC suppressive activity as well as the mechanisms by which the effects of IFN1 on MDSC are inactivated in the tumor microenvironment remain to be answered.

Results

MDSC in cancer patients and TB mice exhibit low expression of type I interferon receptor. Careful analysis of the previously obtained transcriptome of mouse spleen PMN and PMN-MDSC revealed a substantial downregulation of the IFNAR1 mediated pathway in PMN-MDSC (Supplementary Fig. 1A). We found 911 genes that were significantly downregulated (FDR < 20%) in PMN-MDSC compared to PMN. Among these genes, there was a significant enrichment of interferon-induced genes (2.4-fold over random chance, p = 7 x 10^-6 by Fisher exact test). Similar observations were made during the analysis of the transcriptome of blood PMN and PMN-MDSC (based on LOX-1 expression) in cancer patients (Supplementary Fig. 1B). These results suggested that the interferon pathways are suppressed in the PMN-MDSC in cancer patients and this phenotype is recapitulated in the PMN-MDSC from the TB mice.

All responses to IFN1 are determined by the cell surface levels of the IFN1 receptor, which, in turn, is tightly controlled by ubiquitination and degradation of its IFNAR1 chain. We compared IFNAR1 expression on the surface of PMN and Mon from healthy donors and patients with non-small cell lung cancer, pancreatic cancer, breast cancer, head and neck cancer, and colon cancer (Supplementary Fig. 2A). Although several patients demonstrated decreased expression of IFNAR1 in Mon as compared to healthy donors, in the entire group, the differences were not statistically significant (Fig. 1a). In contrast, cancer patients’ PMN-MDSC demonstrated markedly lower expression of IFNAR1 than PMN from healthy donors (Fig. 1a). Those differences in IFNAR1 expression were largely associated with the presence of MDSC in cancer patients. When Mon and M-MDSC, as well as PMN and PMN-MDSC, were compared in the same patients separated using established phenotypic criteria (Supplementary Fig. 2B), substantially lower amounts of the IFNAR1 on M-MDSC and PMN-MDSC than Mon and PMN were observed (Fig. 1b). Notably, the decrease in IFNAR1 expression was more pronounced in PMN-MDSC than in M-MDSC.

A similar analysis was performed on mouse MDSC, which were gated using established criteria: PMN-MDSC—CD11b–Ly6C–Ly6G–, M-MDSC—CD11b+Ly6C–Ly6G–. Cells with the same phenotype in naïve, tumor-free mice were defined as PMN and Mon, respectively (Supplementary Fig. 2C). We assessed the expression of IFNAR1 in MDSC from transplantable...
models of B16 melanoma, LLC lung carcinoma, CT26 colon carcinoma (Fig. 1c), and genetically engineered models of melanoma (RET) and pancreatic cancer (KPC) (Fig. 1d). In transplantable models, spleen PMN-MDSC demonstrated markedly lower expression of IFNAR1 than corresponding PMN from tumor-free mice. Although in some models there was a clear trend in the decrease of IFNAR1 expression in M-MDSC as compared to Mon, it did not reach statistical significance (Fig. 1c). In RET and KPC models, IFNAR1 levels were significantly lower in PMN-MDSC compared with PMN, whereas M-MDSC and Mon exhibited comparable same levels of the receptor (Fig. 1d). To safeguard against potential IFNAR1 cleavage by proteases, we avoided enzymatic tumor digestion by using ascites of EL4 inducible genes such as Irf7 and Isg15 (Fig. 2a and Supplementary Fig. 3A). Thus, both populations of MDSC in the blood of cancer patients, as well as in tumors of TB mice and PMN-MDSC in splens of TB mice exhibited marked inhibition of the IFN1-IFNAR1 pathway.

The biological role of IFNAR1 in MDSC. Given the difference in IFNAR1 levels in tumor and spleen MDSCs, we next compared side-by-side functional activities of MDSC in tumors and spleens. Tumor PMN-MDSC and M-MDSC were markedly more suppressive than spleen MDSC (Fig. 2b). This suggested a possible association between the functional activity of MDSC and the expression of IFNAR1. We asked whether the suppressive activity of MDSC could be canceled by providing high amounts of IFNAR1 ligand—IFNβ. PMN-MDSC and M-MDSC isolated from the spleen of TB mice were cultured for 2 h with IFNβ (2000U), extensively washed, and then used in a suppressive assay. This treatment completely abrogated PMN-MDSC and M-MDSC suppressive activity (Fig. 2c) indicating that IFN1 acts as a negative regulator of MDSC immune suppressive function.

Next, we investigated whether loss of IFNAR1 signaling was sufficient to convert PMN or Mon to MDSC by using Ifnar1 KO mice. PMN or Mon from tumor-free Ifnar1 KO mice indeed lacked IFNAR1 (Supplementary Fig. 3B) and yet did not display suppressive activity (Fig. 2d). While antiviral effects of IFN1 can lacked IFNAR1 (Supplementary Fig. 3B) and yet did not display suppressive activity (Fig. 2d). While antiviral effects of IFN1 can
IFNAR1 protein in TB mice could phenocopy genetic ablation of the Ifnar1 gene thereby masking the latter phenotype. To test this hypothesis, we established EL4 tumors in Ifnar1 KO mice. These mice displayed markedly accelerated tumor growth as compared to WT mice (Supplementary Fig. 3C) and had the same proportion and an absolute number of spleen PMN-MDSC and M-MDSC as WT TB mice (Fig. 2e). PMN-MDSC isolated from spleen and tumors of WT and Ifnar1 KO EL4 TB mice had the same potent suppressive activity (Fig. 2f) suggesting that deletion of IFNAR1 is not sufficient to make MDSCs more suppressive in TB mice, since they may have already reached the maximum of immune suppression.

Then we asked whether the prevention of IFNAR1 down-regulation could affect the suppressive activity of MDSC. To address this question, we used the knock-in mice that express the Isg15 allele, which is resistant to ubiquitination and degradation because it lacks critical Ser526, whose phosphorylation enables the recruitment of β-TrCP E3 ligase. All tissues of these animals harbor homozygous Isg15 allele (Isg15SA or SA). Naïve Isg15SA mice did not exhibit an overt phenotype (this study and ref. 33). MDSC from SA TB mice had substantially higher expression of Irf7 than cells from wild-type (WT) TB mice (Supplementary Fig. 3D). As expected, PMN-MDSC from SA TB mice had markedly higher expression of Irf7 than WT mice (Supplementary Fig. 3C) and had the same potent suppressive activity (Fig. 2f) suggesting that deletion of IFNAR1 was reduced, however, the total number of PMN-MDSC and M-MDSCs in the spleen of tumor-bearing mice. IFNAR1 was reduced, however, the total number of PMN-MDSC and M-MDSCs in the spleen of tumor-bearing mice. IFNAR1 was reduced, however, the total number of PMN-MDSC and M-MDSCs in the spleen of tumor-bearing mice. IFNAR1 was reduced, however, the total number of PMN-MDSC and M-MDSCs in the spleen of tumor-bearing mice. IFNAR1 was reduced, however, the total number of PMN-MDSC and M-MDSCs in the spleen of tumor-bearing mice. The expression of Isg15SA allele (Isg15SA) and degradation of IFNAR1 from spleen or tumor were cocultured with antigen-specific CD8+ T cells (OT-1 splenocytes) at different ratios. T cell proliferation was evaluated in triplicate using [3H]-thymidine uptake and presented as a percentage based on positive proliferation in the absence of MDSC. In all panels, data are expressed as mean ± SD and the p values were calculated using unpaired two-sided Student’s t test, except when otherwise indicated. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns not significant.
Mechanism of regulation of MDSC function by IFNAR1. We next sought to delineate putative mechanisms by which down-regulation of the IFNAR1 provides a license for immune-suppressive activities of MDSC. To this end, WT and SA EL4 TB mice were treated with CD8 antibodies to eliminate differences in tumor growth, PMN-MDSC were collected 18 days after tumor inoculation and expression of genes implicated in MDSC suppressive activity was evaluated. PMN-MDSC from SA TB mice had markedly lower expression of Ptgs2 and downregulation of Ptg2, and Nox2 (Fig. 4a). ROS production is one of the hallmarks of PMN-MDSC activity. NOX2 is directly involved in the production of ROS by PMN-MDSC. Because of a substantial decrease in Nox2 expression, we assessed the level of ROS in PMN-MDSC. Both spleen and tumor PMN-MDSC from SA TB mice demonstrated a markedly lower level of ROS than WT mice (Fig. 4b). These results suggest that downregulation of IFNAR1 on MDSC is required for their ability to express several critical mediators of the immune-suppressive activity.

To gain further insight into the mechanisms delineating the effects of IFN1 on suppressive activities, we performed RNA sequencing of PMN-MDSC from WT and SA EL4 TB mice. The most prominent differences in the expression of specific transcripts are shown in Fig. 4c. Pathway analysis revealed that stabilization of IFNAR1 in PMN-MDSC in TB mice resulted in significant inhibition of glycolysis in these cells, as well as several pathways associated with cell movement and chemokine signaling (Fig. 4d). Thus, unabated IFNAR1 signaling was associated with the marked reduction of several major mediators of PMN-MDSC suppressive activity.

Mechanisms regulating IFNAR1 expression in MDSC. We next sought to identify the mechanisms regulating IFNAR1 expression on MDSC. It is known that the IFNAR1 level is regulated by phosphorylation-dependent ubiquitination and degradation of the IFNAR1 chain. This proteolytic inactivation of IFNAR1 is mediated by either protein kinase D2 in response to IFN1 itself or by p38 protein kinase in response to the non-ligand stimuli that are present in the tumor microenvironment including pro-inflammatory cytokines, tumor-derived vesicles, and the deficit of oxygen/nutrients leading to the integrated stress response. Therefore, we tested the effect of tumor-derived factors on IFNAR1 expression by exposing mouse PMN or Mon to the tumor explant supernatant (TES) obtained from different EL-4 tumors. We observed marked downregulation of IFNAR1 in PMN and Mon treated with TES (Fig. 5a), which contained negligible (if any) amounts of IFNα and IFNβ (Fig. 5b) suggesting the role of non-ligand factors in decrease in IFNAR1 levels. Similar experiments were performed with PMN isolated from healthy donors. TES from different primary human tumors, as
stress inducer thapsigargin (THG), which caused marked a
MDSC. To model the intratumoral stress, we used a potent ER
caused potent but similar levels of suppressive activity in PMN
induced suppression can be mediated via IFNAR1. PMN were
activity in PMN. Therefore, we tested the possibility that THG-
heatmap of signi
caused downregulation of IFNAR1 in PMN, but not Mon
peripheral lymphoid organs. However, since the downregulation
This may explain the decrease of IFNAR1 in PMN-MDSC from
independent IFNAR1 ubiquitination and degradation35,37,38, we
The immune-suppressive activity of MDSC56,57. Therefore, we
inflammation in tumors than in spleens, we
IFNAR2 in the conversion of PMN to PMN-MDSC. First, we
cells from
IFNAR1 was dramatically higher in PMN treated with TES alone
in the absence of p38 (in p38 KO mice), expression of
expression of IFNAR1 on MDSC.
well as supernatant from tumor cell line PCI-30, caused sub-
tenfold decreases in IFNAR1 expression (Fig. 5c). TES also-
caused downregulation of IFNAR1 in PMN, but not Mon
generated from CD34+ progenitor cells (Fig. 5d).
Thus, tumor-derived factors present in TES from both mouse
and human tumor cells caused downregulation of the receptor.
This may explain the decrease of IFNAR1 in PMN-MDSC from
peripheral lymphoid organs. However, since the downregulation
of IFNAR1 was markedly stronger in tumors than in spleens, we
asked whether anything could further downregulate IFNAR1 in
MDSC in the tumor site. Hypoxic conditions downregulated
IFNAR1 in melanoma cells37 and hypoxic areas of solid tumors
lacked IFNAR1 expression33. Exposure of mouse PMN to
hypoxia or lactic acid (major contributors to endoplasmic
reticulum (ER) stress response) notably decreased the receptor
levels (Fig. 5e). We and others previously implicated ER stress in
the immune-suppressive activity of MDSC56,57. Therefore, we
explored the role of ER stress in the regulation of IFNAR1 in
MDSC. To model the intratumoral stress, we used a potent ER
stress inducer thapsigargin (THG), which caused marked a
reduction in IFNAR1 expression in mouse PMN and Mon
(Fig. 5f). Previously, we showed that THG induces suppressive
activity in PMN. Therefore, we tested the possibility that THG-
duced suppression can be mediated via IFNAR1. PMN were
isolated from WT and SA mice and then treated with THG. THG
caus40ed potent but similar levels of suppressive activity in PMN
from WT and SA mice (Fig. 5f). In WT mice pre-treatment of
PMN with INFβ did not prevent induction of suppressive activity
by THG (Fig. 5i). Thus, IFNAR1 expression was not sufficient to
control potent ER stress induction by THG.
Although IFN signaling can be mediated by IFNAR2, the
current paradigm indicates that IFN signaling is impossible
without IFNAR158–64. Nevertheless, we tested the possible role of
IFNAR2 in the conversion of PMN to PMN-MDSC. First, we
quantified IFNAR2 expression on human PMN after the
treatment with TES. In contrast to IFNAR1, which was down-
regulated by TES (Fig. 5c) IFNAR2 expression was not affected
(Supplementary Fig. 4A). Furthermore, we obtained bone marrow
cells from Ifnar1 deficient mice65 and performed the experiments
to determine the possibility that lack of IFNAR2 alone could drive
the conversion of PMN in PMN-MDSC. No suppression was
detected (Supplementary Fig. 4B).
We focused on the mechanisms underlying IFNAR1 down-
regulation in MDSC. Since p38 was implicated in the ligand-
dependent IFNAR1 ubiquitination and degradation35,37,38, we
investigated the role of this kinase. Deletion of p38 completely
prevented downregulation of IFNAR1 in spleen PMN-MDSC
(Fig. 6a) suggesting an important role of p38 kinase in regulation
of IFNAR1 on MDSC.
Treatment of mouse PMN with TES activated p38 (as evident
by its increased phosphorylation) (Fig. 6b). Tumor PMN-MDSC
had higher levels of phospho-p38 than spleen PMN-MDSC
(Fig. 6c). As expected, hypoxia increased phospho-p38 in PMN
(Fig. 6d). In the absence of p38 (in p38 KO mice), expression of
IFNAR1 was dramatically higher in PMN treated with TES alone
or in combination with hypoxia (Fig. 6e). Since PMN-MDSC

Fig. 4 Effect of Ifnar1SA on mRNA expression gene expression by PMN-MDSCs. a mRNA expression of genes associated with immunosuppressive activity in PMN-MDSCs. Analyzed by RT-qPCR. b Reactive oxygen species (ROS) levels by PMN-MDSCs from spleen and tumors of WT and Ifnar1SA tumor-bearing mice (n = 4). Data are expressed as mean ± SEM and the p values were calculated using unpaired two-sided Student’s t test. c Expression heatmap of significantly affected genes (FDR < 5%) for different fold change thresholds. d List of canonical pathways downregulated that were determined by Ingenuity Pathway Analysis (IPA) as significantly enriched pathways affected by Ifnar1SA. N = number of genes; p value = enrichment; FDR = false discovery rate of significant genes (FDR < 10%) for different fold change thresholds; Z = activation z-scores calculated by IPA represent predicted canonical pathway decreasing (Z threshold of 2 is used to call the state). A panel of top genes affected by Ifnar1SA for each pathway. Fisher exact test was used to calculate FDR.
demonstrated lower downregulation of IFNAR1 than M-MDSC we asked if p38 was activated similarly in these cells. PMN and monocytes were isolated from naïve tumor-free mice and treated with TES. Monocytes had a higher basal level of p-p38 than neutrophils and it is only slightly increased by TES treatment as compared to neutrophils where TES caused substantial upregulation of p-p38 (Fig. 6f). These results implicate p38 kinase in the regulation of myeloid cell responses to the tumor-derived factors.

We tested the effect of THG and tumor cell-conditioned medium on ubiquitination and degradation of IFNAR1 in THP-1 myeloid cell line. Both, THG and tumor cell-conditioned medium triggered phosphorylation and ubiquitination of IFNAR1 while decreasing total levels of IFNAR1 protein. Inhibitor of p38 kinase LY2228820 (Ralimetinib) abrogated those effects (Fig. 6g) confirming the role of p38 in ubiquitination and degradation of IFNAR1 caused by tumor-derived factors and ER stress in myeloid cells.

Therapeutic regulation of p38 abrogated suppressive activity of MDSC and elicits the antitumor effects. These results suggest that inhibition of p38 kinase may prevent downregulation of IFNAR1 expression and ensuing acquisition of suppressive activities by MDSC. To test this possibility, we used the p38 kinase inhibitor LY2228820. In healthy donor PMN, treatment with p38 kinase inhibitor upregulated expression of IFNAR1 and downregulation of IFNAR1 caused by TES was abrogated by inhibition of p38 kinase (Fig. 7a). Treatment with p38 kinase inhibitor caused upregulation of IFNAR1 in mouse PMN but not Mon (Fig. 7b). TES-induced downregulation of IFNAR1 expression was abrogated by inhibition of p38 kinase (Fig. 7a). Administration of LY2228820 inhibited the suppressive activity of PMN from control mice treated with TES (Fig. 7c) as well as spleen PMN-MDSC from EL4 TB mice (Fig. 7d). In contrast, treatment of control PMN with LY2228820 alone did not affect their ability to regulate T cell proliferation (Fig. 7e). These combined results suggest that a p38 kinase inhibitor may show an enhanced antitumor effect when combined with an IFN1 inducer. To test this hypothesis, TB mice were treated with Poly:IC (inducer of IFN1) and LY2228820. Treatment with Poly:IC alone or p38 inhibitor alone did not have a substantial effect on the proportion or an absolute number of myeloid cells, PMN-MDSC, M-MDSC, macrophages, or DCs in the spleen. Combination therapy caused only a modest increase in the number of DCs but had minimal effect on other myeloid cell populations.
Experiments were performed twice with the same result. Marrows from naïve mice, treated for 18 h in vitro with 20% of TES. Cells were then lysed and phospho-p38 and total p38 measured by western blot.

Phosphorylation and total p38 protein in BM PMNs treated with 20% TES for 18 h in vitro. A typical example of two performed experiments is shown. Histogram (left) of phosphorylation of p38 (p-p38) in spleen (red) and tumor (blue) PMN-MDSCs compared to isotype (gray)

Phosphorylated and total p38 protein in HD PMNs treated with 20% TES and vehicle (DMSO) or p38 inhibitor LY2228820 (15 μM)

Discussion

In this study, we identified the role of IFN1 in restricting the acquisition of immune-suppressive activity by MDSC. Furthermore, our data indicated that tumor-derived factors-driven p38 kinase-dependent downregulation of IFNAR1 represents a mechanism, by which this role of the IFN1 pathway in myeloid cells is overwhelmed during tumorigenesis leading to the acquiescence of immune-suppressive activities.

Fig. 6 Downregulation of IFNAR1 in MDSCs is mediated by p38. a IFNAR1 levels on PMN-MDSCs from tumor-free or WT and p38 KO (Mapk14Δ/Δ) TB mice (n = 3 for tumor-free and WT groups, n = 6 for p38 KO group). Data are expressed as mean ± SEM and the p values were calculated using unpaired two-sided Student’s t test. b Phosphorylation and total p38 protein in BM PMNs treated with 20% TES for 18 h in vitro. A typical example of two performed experiments is shown. c Histogram (left) of phosphorylation of p38 (p-p38) in spleen (red) and tumor (blue) PMN-MDSCs compared to isotype (gray) (n = 4; right). p Value was calculated in unpaired two-sided Student’s t test. d BM PMNs were cultured in 0.5% O2 for 2 h and p-p38 was measured by flow cytometry (n = 2). e IFNAR1 levels on BM PMNs from WT or p38 KO cultured with 30% TES in normoxia or hypoxia for 16–18 h (n = 3). Data are expressed as mean ± SEM and the p values were calculated using unpaired two-sided Student’s t test. f Monocytes and PMN were isolated from bone marrows from naïve mice, treated for 18 h in vitro with 20% of TES. Cells were then lysed and phospho-p38 and total p38 measured by western blot. Experiments were performed twice with the same result. g Tumor-free WT p38 KO Ctrl p38 KO Ctrl p38 KO Normoxia Hypoxia

Phosphorylation and total p38 protein in HD PMNs treated with different TES (30%) or THG (1 μM) for 16–18 h in vitro.

(Supplementary Fig. S5). Treatment of TB mice with Poly:IC and LY2228820 alone had very minor effect on tumor growth. However, a combination of these compounds elicited a substantial antitumor activity (Fig. 7f). This effect was abrogated by treatment with an anti-CD8 antibody (Fig. 7g). When the suppressive activity of PMN-MDSC was compared in these mice (with the same tumor burden), only PMN-MDSC from untreated TB mice were able to inhibit T-cell proliferation, whereas no suppressive activity was observed in PMN-MDSC isolated from treated mice (Fig. 7h). To test the association of the therapeutic effect of p38 inhibitor and poly:IC with IFNAR1 expression, we performed treatment experiments in mice with targeted deletion of IFNAR1 in neutrophils and monocytes (IFNAR1β/βS100A8Cre). We observed no antitumor effect of poly:IC and LY2228820 treatment in these mice (Fig. 7i). These results clearly indicate that the effect of p38 inhibitor was indeed mediated in large part by IFNAR1 expression on MDSC. Thus, stabilization of IFNAR1 by inhibition of p38 kinase resulted in antitumor effect in combination with type I interferon induction.

Furthermore, our data indicated that tumor-derived factors-driven p38 kinase-dependent downregulation of IFNAR1 represents a mechanism, by which this role of the IFN1 pathway in myeloid cells is overwhelmed during tumorigenesis leading to the acquiescence of immune-suppressive activities.

Our recent work demonstrated that IFNAR1 was downregulated on all types of cells in the tumor microenvironment of colorectal cancers leading to inactivation of the IFN1 pathway and generation of immune-privileged niches. We found that downregulation of IFNAR1 on MDSC was not restricted to the tumor site, it was observed in PB of cancer patients and in spleens of TB mice. However, IFNAR1 downregulation was higher in tumor MDSC than in spleen MDSC. It was associated with the more
Tumor growth was measured every 2 days in mice treated with PBS or anti-CD8 antibody, Poly I:C, and p38i. *p < 0.05, **p < 0.01, ***p < 0.001. Values are calculated by two-way ANOVA test with correction for repeated measurements. **Fig. 7** Effect of pharmacological inhibition of p38 phosphorylation of MDSC function and tumor growth. a) Left panel. PMN were isolated from the blood of healthy volunteers and cultured for 16-18 h with medium containing 20 ng/ml of GM-CSF with or without p38i (LY2228829 1 μM). IFNAR1 level was measured by flow cytometry. N = 4. p Value was calculated in two-sided unpaired Student’s t test. Right panel. Healthy donors PMNs were pre-treated with 1 μM LY2228820 (p38 inhibitor; p38i) followed by 16-18 h incubation with 30% TES. IFNAR1 levels were measured by flow cytometry (n = 3) and expressed as fold changes over untreated cells. p Values were calculated in one-way ANOVA test with correction for multiple comparisons. b) PMN and Monocytes were isolated from the bone marrow of mice by cell sorting, pretreated with 1 μM p38i followed by incubation with 20% TES (n = 3) for 16-18 h. IFNAR1 was measured by flow cytometry. Geometric MFI is shown. Data are expressed as mean ± SEM. p Values were calculated in one-way ANOVA test with corrections for multiple comparisons. c) HPC-derived PMN-MDSCs were treated with 1 μM p38i before assessing their suppressive activity in triplicates. Two experiments with the same results were performed. Data are expressed as mean ± SEM (n = 3). p Values were calculated in two-sided unpaired Student’s t test. d) Suppressive function of PMN-MDSCs from the spleen of EL-4 TB mice after in vitro treatment for 3 h with 1 μM p38i before coculture with responder T cells. p Value was calculated in two-sided unpaired Student’s t test. e) BM PMNs from tumor-free mice were treated with 1 μM p38i for 3 h prior to the suppression assay. f) MC38 tumor growth in mice treated with Poly I:C and p38i (n = 4). p Values are calculated by two-way ANOVA test with correction for repeated measurements. g) MC38 tumor growth in mice treated with anti-CD8 antibody, Poly I:C and p38i (n = 5). Control is mice treated with PBS. p Values are calculated by two-way ANOVA test with correction for repeated measurements. h) Suppressive activity of spleen PMN-MDSCs from control or anti-CD8 antibody, Poly I:C and p38i treatment group (n = 4). p Values were calculated in unpaired, two-sided Student’s t test. *p < 0.05. I) Totally, 10^5 MC38 cells was injected s.c. to the mice lacking IFNAR1 in myeloid cells (Ifnar1<sup>−/−</sup> Cre<sup>+</sup>) or control mice (Ifnar1<sup>−/−</sup> Cre<sup>−</sup>), and tumor growth was measured every 2-4 days (n = 5 in Ifnar1<sup>−/−</sup> Cre<sup>−</sup> group, n = 4 in all other groups). Treatment started at day 12 with poly:IC in PBS (10 μg/mouse) i.p. daily and p38 inhibitor (LY2228829 1 mg/kg) prepared in methylcellulose administered by oral gavage every other day. Control mice received an equal amount of vehicle (PBS i.p. and methylcellulose by oral gavage). p Values are calculated by two-way ANOVA test with correction for repeated measurements.
in human and mouse PMN and Mon. Since neither IFNa nor IFNb was detectable in those TES, ligand-independent downregulation of IFNAR1 was more likely. TES contains multiple cytokines and tumor-derived extracellular vesicles that can cause pathological MDSC activation74–76. In addition, cells from SA mice were shown to be relatively deficient in the uptake of the tumor-derived extracellular vesicles78, which may partially explain the lack of suppressive activity. However, it is unlikely that one or even a few of these mostly redundant factors are solely responsible for this phenomenon. We found that deletion of p38 completely abrogated downregulation of IFNAR1 in MDSC. Similar results were obtained with selective p38 inhibitor, strongly suggesting that p38 activation in response to diverse tumor-derived factors may be a major mechanism that regulates IFNAR1 expression in MDSC.

Our results suggest that the expression of IFNAR1 on MDSC may be a critical mechanism regulating their suppressive activity. Pharmacological regulation of the receptor by inhibiting p38 activation substantially enhances the antitumor effect of IFN1 inducers and thus may open therapeutic opportunities.

**Methods**

**Human samples.** PB was collected from untreated cancer patients at the Helen F. Graham Cancer Center. The study was approved by the institutional review boards (IRBs) of the Christiana Care Health System at Helen F. Graham Cancer Center and The Wistar Institute. All the patients and healthy donors signed IRB-approved consent forms. PB was collected from (i) 12 patients with non-small cell lung cancer and 5 with small cell lung cancer; (ii) 5 patients with breast cancer, (iii) 10 patients with colorectal cancer, (iv) 4 patients with pancreatic adenocarcinomas, (v) 3 patients with esophageal cancer; (vi) 3 patients with head and neck cancer, (vii) 1 patient had renal cancer. In some patients with lung, colorectal, renal, or head and neck cancer, tumor tissues (0.2–1 g) were surgically removed. The ages of cancer patients were between 46 and 92 years (median, 70 years), 24 males and 20 females. Peripheral sample of blood from 21 patients with esophageal cancer; (vi) 3 patients with head and neck cancer, (vii) 1 patient had renal cancer. In some patients with lung, colorectal, renal, or head and neck cancer, tumor tissues (0.2–1 g) were surgically removed. The ages of cancer patients were between 46 and 92 years (median, 70 years), 24 males and 20 females. Peripheral sample of blood from 21 healthy donors with ages 30–64 (median, 55 years), 7 males 14 females and were used as a control for cancer patients or for in vitro experiments.

**Mice.** All experiments with animals were approved by the IACUC of The Wistar Institute. Balb/c or C57BL/6 mice (female, 6–8 weeks old) were obtained from Charles River. OT-1 TCR-transgenic mice (C57BL/6-Tg(TCRα1TCRβ)1100mJf/f) (female, 6–8 week old) and Pmel1 mouse (B6.Cg-Tg(Ifnar1–/–)U1-129J scrJ/J) were purchased from Jackson Laboratory. Littermate C57BL/6 (“WT”), C57BL/6 Ifnar1fl/fl mice (“SA”), Ifnar1–/– and Ubc9-CreER:Mapk14/mice were described previously81. Ifnar1–/–S100A8–/– mice were generated by crossing Ifnar1–/– mice with S100A8–/– mice. Ifnar1fl/fl mice were obtained from Dr. Umansky (German Cancer Center, Heidelberg, Germany). KPC mice were obtained from Dr. Robert H. Abraham from University of Pennsylvania. Ifnar1–/– mice (Ifnar1–/–; Ifnar2fl/fl mice (KOMP)Vlcdn), which were originally purchased from UC Davis KOMP Repository, were bred and maintained at Montana State University (Bozeman, MT) according to the IACUC regulations. Bone marrow cells from these mice were provided by Dr. Rynda-Apple.

**Bone marrow chimeras.** Mixed bone marrow (BM) chimeric mice were obtained as described previously72. Briefly, pooled tibial and femoral BM cells from donor mice were lysed with ACK buffer. BM cells from SA mice transferred to lethally irradiated syngeneic (CD45.1+ mice) recipients obtained from Charles River. In control BM from WT mice was transferred to lethally irradiated recipients. Totally, 8–10 weeks after BM reconstitution mice were injected s.c. with 5 × 106 LLC cells and measured tumor growth.

**Cell lines and tumor models.** Mouse cell lines: EL4 lymphoma, LLC (Lewis lung carcinoma), CT-26 colon carcinoma, B16F10 melanoma were purchased from ATCC. MC38 colon carcinoma (provided by I. Turkova, University of Pittsburgh, Pittsburgh, PA). MethA (methylcholanthrene induced) sarcoma cell line was originally obtained from Dr. Lloyd J. Old (Cancer Research Institute, New York, NY). MethA tumor was established i.p. in Balb/c mice and passed in vivo as an ascitic tumor. LLC, B16F10 and CT-26 TB mice were generated by injecting 5 × 105 tumor s.c. into C57BL/6 or Balb/c mice, respectively. EL-4 and MethA TB mice were prepared, and red cells were removed using ACK lysing buffer. In other experiments, cells were cultured in vitro before flow cytometry analysis was performed. All antibody incubations were performed for 15 min at 4 °C in dark and all centrifugation was done at 1500 r.p.m. at 4 °C for 5 min. Usually, up to 1 × 106 cells were incubated with Fc-block (BD Biosciences) for 10 min and surface staining was performed at 4 °C for 15 min. Cells were run on an LSR II flow cytometer (BD Biosciences) and data were analyzed by FlowJo (Treestar).

**Human.** Single-cell suspensions from peripheral blood were incubated with Fc-block (Miltenyi) for 10 min and surface staining was performed at 4 °C for 30 min. Cells analyzed as described above. A list of antibodies used is in Supplementary Table 1.

**Isolation of PMN-MDSCs and M-MDSCs.** Single-cell suspensions were prepared from the spleen followed by red blood cell removal using ACK buffer. Single-cell suspensions from tumor tissues were prepared using Mouse Tumor Dissection Kit according to the manufacturer’s recommendation (Miltenyi). For PMN-MDSCs isolation, cells were labeled with biotinylated anti-Ly6G antibody (Miltenyi Biotech), incubated with streptavidin–coated microbeads (Miltenyi Biotech), and separated using MACS columns (Miltenyi Biotech). M-MDSCs were sorted by using either FACS Aria II (BD Biosciences) and MoFlo Astrios EQ (Beckman Coulter) cell sorters. The antibodies for M-MDSCs isolation are described in Supplementary Table 1.

**Suppression assay.** Mouse. After isolation of Ly6G+ or Ly6C+ cells as described above, cells were plated in U-bottom 96-well plates (duplicates or triplicates) in complete RPMI. Cells were co-cultured at various ratios with total splenocytes from Pmel or OT-1 transgenic mice in the presence of cognate peptides: OT-1 (SINFFEKL; 0.05 nmol/ml) and Pmel (EGSRGNQDWL; 0.1 µg/ml). After 48 h, cells were incubated with [3H]-thymidine (PerkinElmer) for 16–18 h. Proliferation was measured by using TopCount NXT instrument (PerkinElmer). Human. In vitro treated PMNs from healthy donors were plated in U-bottom 96-well plates (triplicates) in complete RPMI. Concurrently, CD14+ T cells were isolated from PBMCs of the same donor using the EasySep Human T Cell Enrichment Kit (STEMCELL Technologies). PMNs were coculture at different ratios with 10⁵ T cells and 2.5 µl of Human T-Activator CD3/CD28 Dynabeads (Gibco). After 48 h, cells were incubated with [3H]-thymidine as described above.

**Generation of TES.** Mouse. TES were prepared from excised non-ulcerated EL4, LLC, MC38, Ret melanoma, or KPC pancreas tumors. A small tumor piece (0.5 g) was excised aseptically into pieces of 0.1 mm–0.5 mm in diameter in diaminopimelic acid (Sigma-Aldrich) RPMI. After 16–18 h of incubation at 37 °C with 5% CO2, the cell-free supernatant was collected using 0.22 µm filters (EMD Millipore) and kept at −80 °C. Human. TES was prepared from surgically removed tumors and a small tumor piece (0.1–0.5 g) was processed. After 16–18 h of incubation at 37 °C with 5% CO2, the cell-free supernatant was collected using 0.22 µm filters (EMD Millipore) and kept at −80 °C.

**ELISA.** Mouse interferon-alpha (IFNα) and beta (IFNβ) concentrations in TES were measured by using Mouse IFN Alpha ELISA Kit (TCA) (PBL Assay Science) and Mouse IFN-beta ELISA Kit (R&D Systems), according to manufacturer’s instructions.

**Western blot analysis.** Proteins were extracted from BM or HD PMNs in RIPA buffer followed by western blot staining with anti-p38 (Santa Cruz Biotech) and anti-phospho-p38 (Cell Signaling) followed by anti-rabbit-HRP conjugated secondary antibodies (Sigma Aldrich).

**MDSCs generated in vitro.** Mouse. Hematopoietic progenitor cells (HPCs) were isolated from mouse bone marrow by using a Lineage depletion kit (Miltenyi), according to the manufacturer’s instructions. Cells were seeded at 25,000 cell/ml in 24-well plates and recombiant GM-CSF (20 ng/ml; Invitrogen), 20% v/v TES were added on day 1 and day 3. At day 5, Ly6G positive neutrophils were isolated by using anti-Ly6G (Miltenyi) and streptavidin beads (Miltenyi), according to manufacturer’s followed by suppression assay. In addition, total cells were stained and analyzed for flow cytometry. In other experiments, Ly2288820 p38 inhibitor (1 µM; Selleckchem) was added to HPC culture on day 3.

**Isolation of human cells.** Human PMNs from healthy donors were isolated with two methods. (1) PMNs were isolated by centrifugation using double density gradients. Human PMNs were collected and CD14+ T cells s.c. were collected using 1.119. (2) Whole blood was enriched for PMNs using MACSExpress Neutrophil Isolation Kit (Miltenyi) following the protocol provided by the manufacturer.

**Flow cytometry.** Mouse. Single-cell suspensions of BM, spleen, and tumors were prepared, and red cells were removed using ACK lysis buffer. In other experiments, cells were cultured in vitro before flow cytometry analysis was performed. All antibody incubations were performed for 15 min at 4 °C in dark and all centrifugation was done at 1500 r.p.m. at 4 °C for 5 min. Usually, up to 1 × 10⁶ cells were incubated with Fc-block (BD Biosciences) for 10 min and surface staining was performed at 4 °C for 15 min. Cells were run on an LSR II flow cytometer (BD Biosciences) and data were analyzed by FlowJo (Treestar).

**Human.** Single-cell suspensions from peripheral blood were incubated with Fc-block (Miltenyi) for 10 min and surface staining was performed at 4 °C for 30 min. Cells analyzed as described above. A list of antibodies used is in Supplementary Table 1.
before assessing their suppressive activity. Total BM cells were treated with TES (10 ng/ml) for 16 h and recombinant GM-CSF (10 ng/ml) for 16–18 h followed by flow cytometry analysis. BM PMNs and Mon pretreated with LY2228820 p38 inhibitor (1 μM; Selleckchem) or vehicle (DMSO) for 2 h and then treated with TES (30% v/v) for an additional 2 h. Experiments with hypoxxia (0.5% O₂) for 16–18 h were maintained using a hypoxic chamber (BioSpherix).

Human HD PMNs were isolated as described above and treated with TES (30% v/v), TCM (30% v/v) or THG (1 μM; Sigma), and recombinant GM-CSF (20 ng/ml; PeproTech) for 16–18 h. PMNs were pretreated with human IFNγ (2000 units/ml; PBL Assay Science) for 2 h followed by THG (1 μM) for another 16–18 h before suppression assay as described previously.

**In vivo treatment.** Depletion of CD8+ T cells. To deplete CD8+ T cells, 100 μg anti-CD8 (Bio-XCell) or control PBS per mouse was delivered by i.p. injection day −1, day 0, 4, 8, 12, 16, 18, 22. MC38 (1 × 10⁶) were injected s.c. into C57BL/6 mice. On day 12, TB mice were treated by gavage with p38 inhibitor LY2228829 (1 mg/kg; Selleckchem) prepared in methylcellulose. MC38 TB mice received p38 inhibitor for 16–18 h. PMNs and Mon were treated with LPS (0.1 mg/ml; PBL Assay Science) for 2 h for the THG assay (1 μM) for another 16–18 h before suppression assay as described previously.

**Quantitative real-time PCR.** Total RNA was extracted using the Quick-RNA MicroPrep Kit (Zymo Research) according to the manufacturer’s protocol. cDNA was generated with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) in 96-well plates. Plates were read with ABI 7500 Fast Real-Time PCR system (Applied Biosystems). Primers are described in Supplementary Table 2.

**RNA-sequencing.** Totally, 75 ng of DNAse I treated, total RNA extracted from PMN-MDSCs. RNA-sequencing (RNA-seq) analysis was performed using Illumina HiSeq 2500 (Illumina, San Diego, CA). Microarray data from previous study were tested for differences between PMN-MDSCs using t test and p values were adjusted for multiple testing by Bonferroni’s procedure. A list of 631 genes known to be induced by type I interferon at least tenfold in mice was derived from the interferome.org database.

**Detection of ubiquitinated INFAR1 in THP-1 cells.** Totally, 1 × 10⁷ of THP-1 cells were pretreated with vehicle (DMSO) or p38 inhibitor LY2228820 (2 μM; Selleckchem) 1 h prior to 30 min treatment with MC38 tumor conditioned media (TCM) (75%, v/v) or 1 h treatment with Thapsigargin (1 μM, Sigma). Serum-free medium (SFM) served as a negative control to MC38 TCM. Whole-cell lysates were obtained by using 1% NP40 Tris- HCl lysis buffer supplemented with 0.5 M NaF (Sigma), 100 mM Na₂VO₃ (Sigma), 1 μM β-glycerol phosphate (Sigma), and 10 mM N-Ethylmaleimide (Sigma). Totally, 1.5 mg of whole-cell lysates were mixed with 1.5 μg of anti-INFAR1 antibody (clone EA12Z1) and incubated in lysis buffer in a final volume of 500 μl at 4 °C under rotation for 4 h. Protein G agarose beads (Invitrogen) were then incubated with whole-cell lysates overnight at 4 °C with rotation. After washing the beads three times with lysis buffer, the proteins were eluted from the beads in a 4× SDS sample loading buffer. Anti-UB antibodies (clone FK2, Sigma) were used to detect ubiquitinated INFAR1 protein. Totally, 100 μg of cell lysates were processed to determine p-INFAR1 (anti-phospho-INFAR1 antibodies) and 60 μg of cell lysates were processed for INFAR1 (anti-human INFAR1 antibody, Abcam) and β-actin (clone AC-15, Sigma).

**Statistics.** Statistical analysis was performed using a two-tailed Student’s t test or Mann–Whitney test after the analysis of the distribution of variables. If the analysis included more than two groups, an ANOVA test with corrections for multiple comparisons was used. Significance was determined at p < 0.05. TwoWay ANOVA was performed using two-way ANOVA with adjustments for repeated measurements. All calculations were made using GraphPad Prism 8.4.2 (GraphPad Software Inc.).

**Reporting statement.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
RNAseq data generated in this study have been deposited in the GEO GenBank under accession code GSE166770. The remaining data are available within the Article, Supplementary Information or available from the authors upon request.

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**Authors contributions**

K.A-T. and E.S. conducted the experiments, analyzed the data, wrote the paper, J.G., F.V., Q. Yu, L.D., C.L., and S.F. conducted the experiments, A.K. analyzed the data, Y.N. provided the material, wrote the paper. C.M., B.N., G.M., F.D., J.B., N.H., A.R.-A. provided material, S.Y.F. and D.I.G. designed research studies, analyzed data, wrote the paper.

**Competing interests**

E.S. and D.I.G. are full-time employees of AstraZeneca. The remaining authors declare no competing interests.

**Additional information**

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