Type I IFNs induce anti-tumor polarization of tumor associated neutrophils in mice and human

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Novelty:

The significant role of tumor-associated neutrophils (TANs) in tumorigenesis is generally recognized. However, less is known about their polarization and the cytokines responsible for this process. Here, the authors report for the first time that type I interferons activate both mouse and human neutrophils and polarize them into pro-inflammatory anti-tumor phenotype with strong tumor cell killing capacities, facilitated partly by NETs formation. These findings reveal the potential of type I IFNs for an anti-cancer treatment.

Tumor Immunology

Keywords: Neutrophils, tumor, IFN-β, polarization, melanoma

Abstract

The importance of tumor associated neutrophils (TANs) in cancer development is in the meantime well established. Numerous of clinical data document the adverse prognostic effects of neutrophil infiltration in solid tumors. However, certain tumor therapies need functional neutrophils to be effective, suggesting altered neutrophil polarization associated with different outcomes for cancer patients. Therefore, modulation of neutrophilic phenotypes represents a potent therapeutic option, but factors mediating neutrophil polarization are still poorly defined. In this manuscript we provide evidence that type I IFNs alter neutrophilic phenotype into anti-tumor, both in mice and human. In the absence of IFN-β, pro-tumor properties, such as reduced tumor cytotoxicity with low neutrophil extracellular traps (NETs) expression, low ICAM1 and TNF-α expression, dominated neutrophil phenotypes in primary lesion and pre-metastatic lung. Interestingly, such neutrophils have significantly prolonged life-span. Notably, interferon therapy in mice altered TAN polarization towards anti-tumor N1. Similar changes in neutrophil activation could be observed in melanoma patients undergoing type I IFN therapy. Altogether, these data highlight the therapeutic potential of interferons, suggesting optimization of its clinical use as potent anti-tumor agent.

Introduction

The tumor microenvironment represents a special niche that massively influences invading immune cells. The concept of immune cell polarization, known already for macrophages (M1/M2) has been recently extended on neutrophils (TANs) (1). We and others could previously demonstrate that TANs with pro-tumor phenotype (N2) accumulate during tumor progression and contribute to tumor growth by a plethora of mechanisms (2,3,4) e.g. via strong up-regulation of pro-angiogenic genes (VEGF, MMP9) possibly through activation of STAT3 and c-myc transcription factors. Additionally, we could show that pro-tumor neutrophils efficiently support metastatic processes, due to up-regulation of pro-metastatic proteins, like Bv8, MMP9, S100A8 and S100A9 (5). Anti-tumor N1 neutrophils, in contrast, inhibit tumor growth (6) and are able to inhibit tumor seeding in the premetastatic lung via the generation of H_2O_2 (9) or increase immune recognition of tumor cells through neutrophil elastase-dependent up-regulation of self-antigen presentation (10). Furthermore, direct killing of tumor cells by such neutrophils, via the secretion of ROS has been demonstrated (11, own observation). Such TANs can also support adaptive anti-tumor immune responses by recruiting T cells to tumor sites via the secretion of chemokines like CXCL9 or CXCL10 (1,12). Interestingly, antigen-pulsed neutrophils have also been shown to cross-present antigens *in vitro* to activate cytotoxic T lymphocytes (CTLs) (13).

Strict classification of TANs into N1 or N2 phenotypes is surely an oversimplification. More likely, these two immune-phenotypes mark the end points of a continuum of functional states that neutrophils exhibit. Our previous data revealed IFN-β as N1 promoting cytokine (4,15,16) and TGF-β has been suggested to be a N2 inducer (14). In this manuscript, we provide further evidence emphasizing the involvement of type I IFNs in neutrophil polarization in tumor microenvironment. We demonstrate here for the first time the significant down-regulation of anti-tumor neutrophil markers, like ICAM1 and TNF-α in *Ifnb1*$^{-/-}$ mice. In

agreement, we could also show the increase of ICAM1 expression on the neutrophils isolated from melanoma patients undergoing adjuvant type I IFN therapy. Type I interferon deficient neutrophils showed reduced formation of NETs, accompanied by lower tumor cell killing capacity. Under these conditions, massively enhanced neutrophil turnover in combination with accumulation of immature neutrophils was observed. Importantly, therapeutic intervention in mice using low dose IFN-β induced anti-tumor activation of neutrophils in tumors and in pre-metastatic lungs. Correspondingly, in human melanoma patients, neutrophil anti-tumor characteristics were augmented upon type I IFN treatment and their migratory capacities reduced, suggesting an effective outcome of type I IFN therapy that should be further optimized in order to improve its therapeutic effect.

Material and Methods

Animals

8 to 12 week old female mice were used. *Ifnb1*$^{-/-}$ mice were backcrossed onto the C57BL/6 or BALB/c background for >15 generations. All mice were bred and kept under SPF conditions in the animal facility of the HZI (Braunschweig, Germany).

Cell lines

Tumor cell lines were cultivated in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL; Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 250µmol/l β-mercaptoethanol, and 1% (v/v) penicillin/streptomycin. B16F10 (C57BL/6), MCA205 (C57BL/6), LLC1 (C57BL/6), 4T1 (BALB/c) or CT26 (BALB/c) tumor cells were used in all experiments.

Antibodies

Anti-CD16/32 (clone 2.4G2, BD Parmingen), anti-CD11b (clone M1/70, eBioscience, PE or APC), anti-Ly6G (clone 1A8, Biolegend, PE-Cy7 or A647), anti-Ly6C (clone AL-21, BD Pharmigen, A700), anti-TNFα (clone MP6-XT22, eBioscience, FITC), anti-F4/80 (clone BM8, eBioscience, PerCP-Cy5.5), anti-ICAM1 (clone YN1/1.7.4., eBioscience, PE), anti-CD62L (clone MEL-14, eBioscience, APC), anti-DNA-histone H1-complex (Merck Millipore), anti-MPO (Dako), anti-active Caspase 3 (C92-605, BD Pharmingen, PE).

Murine tumor models

Exponentially growing tumor cells were harvested and injected subcutaneously (*s.c*). Tumor sizes were evaluated by caliper and the volume was calculated:

$V = 4/3 \times \pi \times (h \times w\ 2) / 8$ (h = height and w = width).

Tumor depth= width.

Low dose IFN-β therapy of tumor-bearing mice

From day 3 after tumor cell inoculation, *i.v.* treatment with 1000 IU of recombinant mouse IFN-β (rmIFN-β) was performed every other day. After two weeks, mice were sacrificed and cells from blood, tumor and lung analyzed by flow cytometry.

Flow cytometry of cells from tumor, lung, blood and bone marrow

Tumor tissue was digested using dispase/collagenaseA/DNase suspension (0.2mg/ml 0.2mg/ml 100mg/ml). Cells were meshed through 50µm filters (Cell Trics, Partec), erythrocytes removed and single cell suspensions prepared.

Lungs were perfused through the right heart chamber using PBS, cut into pieces and digested as described above.

Erythrocytes from blood and bone marrow samples were removed.

Single cell suspensions were stained with antibodies listed above. To avoid unspecific binding of antibodies, cells were treated with anti-mouse CD16/CD32 antibodies.

For intracellular staining, cells were fixed/permeabilized using BD^{TM} Cytofix-Cytoperm buffer. Flow cytometry was performed using BD LSRII system (BD Bioscience) and data analyzed using BD FACSDiva software (BD Bioscience).

Cell sorting

Single cell suspensions were prepared and stained. CD11b^{+}Gr1^{high} or CD11b^{+}Ly6G^{+} neutrophils were sorted using a FACSAria^{TM} cell sorter (BD Bioscience) and the purity of cells was assessed by reanalysis. Gating strategy for neutrophil staining and sorting is shown in Figure S3

BrdU labeling of bone marrow cells

On day 7 after tumor inoculation, 1 mg BrdU was injected *i.v.* and after certain time blood was taken. Cells were stained for Ly6G, CD11b and BrdU (BD BrdU Flow Kit, BD Bioscience). $BrdU^+$ neutrophils were assessed via flow cytometry.

***Ex vivo* tumor killing assay**

Neutrophils were sorted, distributed into 96-well cell-culture dish and co-cultivated overnight with Luciferase-expressing 4T1 cells (2:1). Substrate solution (Luciferin, Calipers) was added and luciferase activity measured by IVIS-200 (Calipers). Emitted light could be correlated to the amount of living tumor cells/well.

Cytospin analysis

Neutrophils were sorted, centrifuged onto Superfrost™ slides (Thermo Scientific), dried and stained with Giemsa solution (Sigma Aldrich). Nuclear morphology was assessed using light microscope (Zeiss). At least 10 fields of view were counted and percentage of immature ring shaped nuclei calculated. For human material ficoll purified blood neutrophils were treated as mentioned above and percentage of immature band shaped neutrophils was evaluated.

Visualization of NETs

Blood neutrophils were sorted as described above, distributed into Glass Bottom 96-well plates (MatTek Corporation, Ashland, USA) and incubated 3h with PMA (100 nM/well). Subsequently, cells were fixed (4% paraformaldehyde, PFA), NETs stained using DNA-histone-H1-complex (Alexa 488) and MPO (Alexa 633), as previously described (51). Slides were embedded in ProLong®Gold anti-fade reagent with Dapi (Invitrogen) to stain nuclei. Samples were visualized using Leica TCS-SP5 confocal inverted-base microscope with a HCX PL APO 40×0.75–1.25 oil immersion objective. To avoid overlapping emissions, fluorescent dyes were sequentially excited and fluorescence of single channels measured by

photon counting. Images were processed and evaluated by Adobe Photoshop 7. At least 8 randomly selected vision fields in each setting were analyzed.

Human studies

Peripheral blood from melanoma patients was drawn into citrate tubes after written local ethics committee approval. All patients had no evidence of disease at the time of phlebotomy. Patients from adjuvant interferon treatment group received recombinant interferon-alfa2 (3 million IU) three times/week. In all patients of the IFN group, blood was drawn during an ongoing adjuvant IFN treatment. Clinicopathological characteristics of patients enrolled in this study are listed in Supplementary Table 1.

Isolation of human neutrophils

Peripheral blood from melanoma patients was drawn into 3.8% sodium citrate anticoagulant monovettes (Sarstedt), mixed with PBS (1:1, v/v) before separation by density gradient centrifugation (Biocoll density 1,077g/mL, Biochrom). The mononuclear cell fraction was discarded and neutrophils (purity > 98%) isolated by sedimentation over 1% polyvinyl alcohol followed by hypotonic lysis (0.2% NaCl) of erythrocytes.

Apoptosis assay

Spontaneous apoptosis of human neutrophils was determined by AnnexinV-PE apoptosis detection kit (BD Biosciences) after 24 hours culture in RPMI-1640 (Invitrogen) supplemented with 10% FCS (Biochrom) and 1% (v/v) penicillin/streptomycin (Invitrogen). Cells were analyzed on a BD FACSCanto II using DIVA 6.0 software.

Flow cytometry of human neutrophils

Neutrophils were stained using antibodies: anti-CD66b FITC (clone 802H3), anti-CD49d APC-Alexa 750 (clone HP2/1, Beckmann coulter), anti-CXCR1 PE (clone 8F1/CXCR1,

BioLegend), anti-CXCR2 PerCP-eFluor 710 (clone 5E8-C7-F10, eBioscience) and anti-CD54APC (clone HA58, eBioscience). To determine nonspecific signals, isotype controls were used at the same concentration as for the specific antibody.

Statistics

Statistical analyses were performed using a 1-tailed Student's t-test for single-value comparisons and one-way ANOVA to compare three or more different data sets. $P < 0.05$ was considered significant. Values are expressed as mean ± SEM.

Study approval

Mice studies have been approved by the regulatory authorities LAVES (33.9-42502-04-13/1122 permission number).

Human studies have been approved by the appropriate institutional review board at the University of Duisburg-Essen, Essen, Germany. Written informed consent was received from participants prior to inclusion in the study.

Results

Endogenous IFN-β controls neutrophil proliferation and maturation in tumor bearing mice

Under homeostatic conditions neutrophil maturation, turnover and apoptosis are tightly controlled. Circulation time as well as life span of neutrophilic granulocytes are relatively short (17), but are prolonged under inflammatory conditions (17–19). Consequently, altered neutrophil kinetics are observed in tumor bearing hosts and seem to be associated with adverse prognosis for the respective patients (14). Still, investigation of the complex regulation underlying neutrophil kinetics in tumor conditions is just emerging and little is known about the role of type I IFNs in this scenario. We analyzed neutrophil turnover and maturation in tumor bearing mice, sufficient or deficient for endogenous IFN-β. Mice bearing the melanoma B16F10 were injected *i.v.* with BrdU to monitor proliferation of neutrophils. Additionally, the maturation status of circulating blood neutrophils was assessed using Giemsa staining. Interestingly, turnover and mobilization of $Gr1^{+}CD11b^{+}BrdU^{+}$ neutrophils was significantly faster in the absence of endogenous IFN-β (Fig.1). A higher number of neutrophils entered the blood stream under such conditions (Fig.1 A). This was most likely due to the release of immature neutrophils into circulation in *Ifnb1*$^{-/-}$ mice, since elevated numbers of neutrophils with a ring-shaped nucleus could be detected (Fig.1 B-D).

Altered expression of co-stimulatory molecules and activation markers on *Ifnb1*$^{-/-}$ TANs

One functional ability of neutrophils is the induction of an adaptive immune response (14). To this end, neutrophils express different co-stimulatory molecules such as CD80, CD86 or ICAM1 (20), with ICAM1 being down-regulated on N2 pro-tumor neutrophils (21). To elucidate the influence of endogenous IFN-β on the phenotype of TANs, we analyzed surface expression of ICAM1 on day 14 after *s.c.* inoculation of C57BL/6 mice with B16F10 melanoma cells or Balb/c mice using 4T1 breast carcinoma cells. *Ifnb1*$^{-/-}$ versus WT animals

were compared (Fig. 2 A and B). For blood neutrophils no significant differences were observed between the groups. In contrast, after neutrophil transmigration into the target tissue (tumor and lung) expression of ICAM1 was up-regulated. This up-regulation was reduced in the *Ifnb1*$^{-/-}$ mice.

Besides ICAM1 up-regulation (22) the shedding of L-selectin CD62L is associated with neutrophil maturation and activation (23). Therefore, CD62L expression on blood and tumor neutrophils was measured (Fig.2 C and D). Consistent with the observed accumulation of immature neutrophils in the circulation of tumor bearing *Ifnb1*$^{-/-}$ animals (Fig.1), a significantly increased percentage of CD62L^{+} neutrophils was detected in the blood of tumor bearing BALB/c *Ifnb1*$^{-/-}$ mice (Fig.2 D). In C57BL/6 mice, similar tendency could be observed (Fig. 2C). Once neutrophils had migrated into the tumor tissue, CD62L expression seems not to depend on the presence or absence of endogenous IFN-β anymore (Fig.2C and D).

Ifnb1$^{-/-}$ neutrophils are clearly no myeloid derived suppressor cells (MDSCs)

In contrast to the immune stimulatory capabilities of N1 anti-tumor TANs, N2 pro-tumor TAN are reported to exert immunosuppressive function towards T cells (18) via inhibition of T cell responses by secretion of ROS (23). To test whether the capability of blood and tumor neutrophils to suppress T cell proliferation is influenced by IFN-β, C57BL/6 and syngeneic *Ifnb1*$^{-/-}$ mice were challenged with B16F10 melanoma. After 14 days, neutrophils from different compartments were sorted out and incubated with CD4^{+}CD25^{-} T cells for 72h. After this time T cell proliferation was assessed. It could be shown that neutrophils sorted from different anatomical compartments of wild type or syngeneic *Ifnb1*$^{-/-}$ tumor bearing mice stimulate rather than inhibit CD3 mediated T cell proliferation (Fig. S1).

Reduced tumor cell killing capacity of neutrophils isolated from *Ifnb1*$^{-/-}$ mice

Besides their immunoregulatory functions, N1 neutrophils have the capacity to directly kill tumor cells (6). This ability is reduced in N2 TAN (14). To reveal the IFN-β impact on this phenomenon, neutrophil cytotoxicity from blood and tumors of BALB/c (Fig. 3A) or C57BL/6 (Fig. 3B), and syngeneic *Ifnb1*$^{-/-}$ animals was tested. Cells were sorted and co-cultivated with luciferase expressing 4T1 tumor cells (effector:target ratio 2:1). To some cultures rmIFN-β was added. Luciferase activity, developing only in living cells, was measured after 18 hours of co-incubation, using IVIS200. Importantly, cytotoxicity of *Ifnb1*$^{-/-}$ tumor neutrophils was significantly reduced, compared to WT (Fig. 3A and B), and could be restored by treatment with low amounts of rmIFN-β. The same was true for Balb/c, but not C57BL/6 blood neutrophils (Fig. 3A). Moreover, TANs showed generally reduction of cytotoxicity in comparison to blood derived neutrophils, indicating further influence of the tumor microenvironment on neutrophil function.

Reduced production of NETs by neutrophils from *Ifnb1*$^{-/-}$ mice

Neutrophils from tumor bearing WT mice show significantly higher tumor cell killing activities. One important mechanism involved in neutrophil cytotoxicity is the formation of extracellular neutrophil-derived DNA webs called neutrophil extracellular traps (NETs). NETs are released in response to inflammatory stimuli and were suggested to play a role in tumor cell killing (24). To assess regulation of NETs release in N1 (WT) versus N2 (*Ifnb1*$^{-/-}$) neutrophils, blood neutrophils were isolated from tumor bearing mice, stimulated with PMA or left in medium for 3h at 37^{o}C. PMA was chosen due to its established strong NETs induction capacity (25). Interestingly, in tumor bearing WT mice significantly more NETs were released by neutrophils, compared to *Ifnb1*$^{-/-}$. Thus, endogenous IFN-β appears to support formation of NETs (Fig. 4). These data are in agreement with the observed enhanced cytotoxicity of WT neutrophils against tumor cells.

Ifnb1$^{-/-}$ tissue neutrophils produce reduced amounts of TNF-α

TNF-α is now known as important regulator of inflammation that can exert both, pro-tumor as well as anti-tumor functions, depending on its concentration (26). The tumor toxic effect of TNF-α is not only due to direct cytotoxic or cytostatic effects on the tumor cells (27), but can also be attributed to its immunostimulatory and vessel disrupting functions. In accordance, important feature of N1 neutrophils is the local production of TNF-α at tumor sites (14). To elucidate the impact of endogenous IFN-β on neutrophilic TNF-α production, its expression in different anatomical compartments of WT and syngeneic *Ifnb1*$^{-/-}$ animals was assessed. No significant differences regarding the percentage of TNF-α producing neutrophils were detected in peripheral blood (Fig.S2 A and B), but neutrophilic TNF-α production was remarkably increased after transmigration into the target tissue (Fig.S2 C to F). This migration-associated polarization seemed to be strongly influenced by IFN-β, as neutrophils from tumor (Fig.S2 C and D) as well as lung (Fig.S2 E and F) of *Ifnb1*$^{-/-}$ mice displayed a significantly reduced expression of TNF-α, independently of mouse strain or tumor model.

Low dose type I IFNs therapy induces N1 neutrophil phenotype in mice

High dose type I IFN therapy is currently used in the clinics to treat tumors, infections or autoimmune diseases. Treatment causes severe side effects in a dose-dependent manner that negatively influences patient compliance and often leads to the discontinuation of treatment (28,29). Therefore, it is of special interest to determine the effects of low dose type I IFN therapy on disease modifying aspects, like immune surveillance. For that reason, *Ifnb1*$^{-/-}$ and WT mice were challenged with tumor cells and treated with low doses of IFN-β. On day 14 after tumor inoculation, neutrophilic phenotype was assessed by flow cytometry. IFN-β therapy indeed induced N1 neutrophil polarization. For C57BL/6 mice (Fig.5 A) this was most profound for the adhesion molecule ICAM1, but also other markers showed a similar tendency. In BALB/c mice (Fig.5 B), the N1 marker TNF-α and Caspase 3 were significantly

upregulated upon type I IFN treatment. Moreover, the extracellular N1 marker Fas as well as ICAM1 showed an increase, compared to untreated controls.

Metastasis i.e. the spread of tumor cells from the primary lesion to other non-adjacent organs is the fatal consequence of most malign tumors (30). Even though the molecular mechanisms underlying metastasis formation are largely elusive to date, it is in the meantime recognized that establishment of a pre-metastatic niche is critical for this process (31). Involvement of neutrophils in the formation of pre-metastatic niche has been suggested for lung metastasis (32) and we could show previously that type I IFNs were involved in the regulation of this processes (5). Therefore, we characterized the impact of type I IFNs on polarization of lung neutrophils. Mice were challenged with tumor cells that are known to form spontaneous lung metastases (33) and treated with low amounts of rmIFN-β for a period of 14 days. At given time points, polarization of lung neutrophils was analyzed (Fig. 5 A and B). In two mouse strains and both tumor models, low dose IFN-β therapy clearly induced an N1 polarization of neutrophils in the pre-metastatic lung (Fig. 5). The expression of TNF-α and the activity of effector caspase 3 were significantly up-regulated. Similarly, ICAM1 and Fas were increased.

Neutrophil characteristics in melanoma patients are influenced by adjuvant type I IFN therapy

Even though the mechanisms responsible for the therapeutic effects of type I IFNs are not clearly defined, these cytokines are in clinical use as adjuvant treatment e.g. in high-risk melanoma patients (29). Since type I IFN therapy in mice resulted in changes of neutrophil polarization, we wanted to corroborate these findings in the human system. Therefore, we analyzed neutrophil characteristics in human melanoma patients under type I IFN therapy and compared them to neutrophils from untreated control individuals. Importantly, spontaneous apoptosis of neutrophils isolated from type I IFN treated patients was increased (Fig. 6 A) and significantly more immature neutrophils were detected in the blood of these patients,

compared to controls (Fig 6 B). Interestingly, percentage of CXCR2 expressing PMNs was not influenced (Fig. 6 C), but chemotactic features of these cells, i.e. expression levels of the CXC chemokine receptors 1 and 2, were significantly reduced upon therapy (Fig. 6 D and E). Adjuvant type I IFN therapy seems also to influence ICAM1 expression levels (Fig.6 F), but has no significant impact on tumor killing capacities of neutrophils (data not shown). All in all, these data suggest an important role of type I IFNs in neutrophil modulation not only in mice but also in man.

Discussion

Contribution of TANs to tumor angiogenesis, immunosurveillance and invasive growth is already well established by us and others (1,4), even though there are still some controversies regarding the differences between granulocytic myeloid-derived suppressor cells and TANs (34,35). Neutrophils represent an independent prognostic marker in a broad variety of neoplasias e.g. high number of intra-tumoral neutrophils in localized as well as metastatic renal cell carcinoma, correlates with a negative prognosis (36). Therefore, TANs represent a highly potent therapeutic target and understanding the physiology of such cells can provide improvement of existing anti-cancer therapies.

Recently, we published that higher numbers of neutrophils accumulate in tumors of mice that lack endogenous IFN-β. Such TANs do not only efficiently support tumor angiogenesis and growth by up-regulating pro-angiogenic molecules, but also secrete higher amounts of neutrophil attracting chemokines and display prolonged survival, compared to their WT counterparts (4,15,16).

The present work adds important evidence to the major role of type I IFNs in the regulation of cancer immune surveillance via the polarization of neutrophils. In tumor-bearing *Ifnb1*$^{-/-}$ mice an enhanced mobilization of immature neutrophils into circulation was observed, accompanied by significantly faster turnover of these cells, compared to WT controls. The reason for it could be the increased G-CSF expression that we observed in *Ifnb1*$^{-/-}$ mice (16), since G-CSF stimulates neutrophil proliferation (18). One possible way how IFN-β could be involved in G-CSF regulation is via inhibition of IL-23 or IL-17 expression. IL-23 induces IL-17 expression and IL-17 in turn is known to induce G-CSF production by stromal cells (37). Moreover, IFN-β has been demonstrated to inhibit the production of IL-17 by $CD4^+$ T cells (38). The prolonged life span of neutrophils in the absence of endogenous IFN-β is probably

also responsible for an increased production of IL-23 by tissue macrophages, as the frequency of phagocytosis of dying neutrophils by macrophages limits the production of IL-23 (37).

Previously, we could show that IFN-β is involved in the regulation of the life span (16), mobilization and turnover of mouse neutrophils via a plethora of different mechanisms. Importantly, most of the pro-apoptotic neutrophil features (16) are likewise associated with anti-tumor features of TANs. As such, a pro-apoptotic gene expression pattern including up-regulation of the death receptor Fas (1), as well as an enhanced production of ROS (11), are important characteristics of anti-tumor neutrophils. Further evidence indicating IFN-β as a factor mediating anti-tumor polarization is given by its capacity to facilitate the expression of co-stimulatory molecules like ICAM1, that was shown to be up-regulated in anti-tumor neutrophils upon tissue migration and activation (21). Importantly, ICAM1 is crucial for T cell activation under conditions where co-stimulation by CD80 and CD86 is low (39). High ICAM1 expression can therefore induce the activation of cytotoxic $CD8^+$ T cells (40) as well as repress the secretion of immunosuppressive IL-10 by $CD4^+$ T cells (41). In this manuscript we demonstrate for the first time the interferon-mediated stimulation of ICAM1 expression on neutrophils from WT tumor bearing mice treated with rmIFN-β. In agreement, we could observe upregulation of ICAM1 on the blood neutrophils from melanoma patients undergoing adjuvant type I IFN therapy. This finding has very important implications for reconsidering type I interferons in anti-tumor therapies due to its role in tumor immunosurveillance.

The secretion of TNF-α is another anti-tumor feature of neutrophils (14). In this manuscript we show significant reduction of TNF-α expression in tumor and pre-metastatic lung neutrophils of *Ifnb1*$^{-/-}$ mice, once again demonstrating tumor supportive activity of neutrophils in such mice. Importantly, TNF-α has been also demonstrated to induce neutrophil apoptosis (42). Therefore, an elevated production of TNF-α by anti-tumor neutrophils observed in this manuscript is consistent with the shorter life span of such cells and its regulation by IFN-β.

The most prominent anti-tumor N1 neutrophil feature, i.e. their ability to directly kill tumor cells (2,14), seems also to be regulated by IFN-β. We observed that neutrophils isolated from blood or tumor of *Ifnb1*$^{-/-}$ mice display significantly reduced tumor killing capacity in comparison to WT. Addition of rmIFN-β into the culture completely rescued the N1 phenotype, indicating the possibility to re-polarize neutrophils by therapeutic intervention. This phenomenon was independent of mouse strain or tumor model used.

Formation of NETs is postulated to play a role in efficient tumor cell killing and cancer immunoediting (43, 24). It has recently been shown that neutrophils next to bacterial, fungal, and protozoan invaders are able to efficiently trap circulating tumor cells using NETs (44). Such trapped tumor cells could easily be destroyed with reactive oxygen species (ROS) or TNF-α (45). In this manuscript we demonstrate for the first time that NETs release is enhanced by endogenous IFN-β. Blood neutrophils from tumor bearing WT mice produce significantly higher amounts of NETs, compared to *Ifnb1*$^{-/-}$ animals. Importantly, tumor environment strongly stimulates neutrophils and initiates NETs release. This process is not further augmented by stimulation with PMA, however here NETs were formed slightly faster (data not shown) and MPO accumulated strongly in the cytoplasm. Thus, an additional molecular mechanisms involved in repressed neutrophil-mediated tumor cell killing in *Ifnb1*$^{-/-}$ mice could be the reduced release of NETs together with inhibited neutrophilic ROS production, as we have shown previously (16).

Some studies attribute T cell suppressive properties to tumor supportive N2 neutrophils (1,7), while other authors use this feature to identify granulocytic MDSCs (46). To address this question we assessed the ability of TANs to regulate T cell proliferation and could show that these cells efficiently stimulate T cell proliferation in both *Ifnb1*$^{-/-}$ and WT mice, and therefore could not be considered MDSCs. Remarkably, type I interferons seem to enhance blood-

derived neutrophil capacity to stimulate T-cell proliferation hence supporting their tumor supressory functions.

Besides influence on the neutrophil phenotype demonstrated in the present work, we could previously show that type I IFNs repress the pro-angiogenic activities of these cells (4) as well as their longevity (16). Importantly, here we could show that low doses of rmIFN-β are capable to augment anti-tumor N1 characteristic of neutrophils associated with the primary tumor and the pre-metastatic lung, independently of the mouse strain or tumor model used. Neutrophils infiltrating pre-metastatic niche were stronger influenced than TANs, probably due the comparably low amounts of IFN and the intravenous route of application used during the present work. Most likely, highly vascularized organs like lung are favorably supplied with therapeutic relevant concentrations of IFN (47). Agents abrogating the formation of a permissive pre-metastatic environment like IFN-β could be of substantial profit for the clinical management of high risk patients by inhibiting metastasis.

In this manuscript translating our findings to the human system revealed an influence of type I IFN therapy on several neutrophil features. For the first time we could show here that blood derived neutrophils from melanoma patients significantly down-regulate their IL-8 receptors - CXCR1 and CXCR2 expression upon adjuvant type I IFN treatment.. It results in interferon-dependent inhibition of neutrophil migration from the bone marrow that is of high importance since clinical studies have indicated poor prognosis for tumor patients showing enhanced numbers of blood or tumor infiltrating neutrophils. In line with the data generated in our mouse model, neutrophil amounts decrease in type I IFN treated patients confirming the influence of type I IFNs on neutrophil survival and apoptosis (16). In view of the emerging diversity of circulating neutrophil subtypes in mice and humans (48) it has to be noted that high-density neutrophils, but not low-density neutrophils have been investigated in this study. Low-density neutrophils are enriched in many human cancer types including melanoma (49), are related to granulocytic MDSC and include activated as well as immature functional

subpopulations (35, 36). High-density neutrophils mainly consist of mature differentiation stages, but increased proportions of immature cells have been reported in some types of cancer (50). Interestingly, interferon-induced features of activation have also been observed in the high-density fraction in this study. In summary, human data reveal a clear influence of type I IFN therapy on neutrophil polarization in melanoma patients that should be further investigated in detail.

All in all, present data have important implications as they translate recently generated data from mouse tumor models into the clinical situation, thus highlighting the tremendous therapeutic potential of type I IFNs. Future research on the therapeutic use of type I IFNs should therefore carefully consider its effect on activation and migration of neutrophils.

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Figure legends

Figure 1. Altered mobilization and maturation of neutrophils in the absence of endogenous IFN-β. (**A**) To investigate neutrophil turn over in presence or absence of IFN-β, C57BL/6 and syngeneic *Ifnb1*$^{-/-}$ mice were challenged with B16F10 melanoma. On day 7 after tumor inoculation the mice were *i.v.* injected with BrdU (1 mg) and Gr1^{+} CD11b^{+} BrdU^{+} neutrophils were monitored in blood for 7 days via flow cytometry. (**B-D**) Blood neutrophils were sorted on day 14 and cytospin analysis, followed by Giemsa staining, were performed. Quantitative analysis (**B**) and representative staining for neutrophils from WT (**C**) and *Ifnb1*$^{-/-}$ (**D**) tumor-bearing mice is shown. Data were presented as mean ± SEM (**p≤0,005). The experiment was repeated twice with at least 5 mice per group.

Figure 2. Neutrophils polarize into N2 phenotype in the absence of IFN-β. To assess the polarization status of neutrophils from different tissues, WT and *Ifnb1*$^{-/-}$ animals were challenged s.c. with tumor cells. C57BL/6 mice with B16F10 melanoma cells and BALB/c mice with 4T1 breast carcinoma cell line. Single cell suspension of blood, tumor and lung were prepared at day 14 and neutrophils analyzed for ICAM1 (**A** to B) and L-selectin (CD62L) (**C** to **D**) expression. Data were presented as mean ± SEM (*p≤0,05). The experiment was repeated twice with at least five mice per group.

Figure 3. Reduced tumor killing capacity of neutrophils isolated from *Ifnb1*$^{-/-}$ mice. Mice were challenged with B16F10 or 4T1 tumor cells and on day 14 neutrophils from blood and tumors were sorted. Neutrophils were co-incubated 18h with luciferase expressing tumor cells. Luciferase activity of the residual living tumor cells was measured using IVIS-200. Data were presented as mean ± SEM (*p≤0,05, (***p≤0,005). The experiment was repeated twice with at least five mice per group.

Figure 4. Neutrophil extracellular traps (NETs) formation is induced in presence of IFN-β. Mice were challenged *s.c.* with B16F10 cells, on day 14 neutrophils were isolated, incubated in 37°C with medium (tumor) or stimulated with PMA to assess NETs formation. After 4h slides were fixed, stained for DNA (blue), histone H1-DNA-complexes (green) and MPO (red). Results show representative images (**A**) and quantification of neutrophils decondensating and releasing NETs (**B**). Size bars 50 and 25 μm, respectively. Error bars represent the mean ± SD. (*p≤0,05, (***p≤0,005). The experiment was repeated twice with at least five mice per group.

Figure 5. Low dose IFN-β therapy leads to N1 neutrophil polarization in mice. C57BL/6 (**A**) and BALB/c (**B**) mice were s.c. injected with B16F10 or 4T1 tumor cells, respectively. From day 3 low dose IFN-β therapy (1000 IU per injection) was applied *i.v.* every other day. On day 14 single cell suspensions from tumor or lung were analyzed for ICAM1, Fas, TNF-α and active Caspase 3 expression. Data were presented as mean ± SEM (*p≤0,05, ***p≤0,005). The experiment was repeated at least once with five mice per group.

Figure 6. N1 biased human neutrophils polarization after adjuvant type I IFN therapy of melanoma patients. Neutrophils were isolated from the peripheral blood of patients treated with type I IFN or from untreated patients. (**A**) Apoptosis was assessed after 24h cultivation *ex vivo*. (**B**) Maturation state was determined by cytospin. (**C-E**) Chemokine receptor expression and (**F**) expression of ICAM were analyzed. Data were presented as mean ± SEM (*p≤0,05). The experiment was done using min. 6 samples per group.