

**Type I interferon-mediated polarization of neutrophils
and its impact on cancer progression and bacterial infections**

Inaugural Dissertation
for the doctoral degree of
Dr. rer. nat.

from the Faculty of Biology
University of Duisburg-Essen
Germany

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Date of submission December 2020

The experiments underlying the present work were conducted at the ENT Department of the University Hospital Essen, at the University of Duisburg-Essen.

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Date of the oral examination: 18.02.2021

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DOI: 10.17185/duepublico/74078

URN: urn:nbn:de:hbz:464-20210322-110137-6

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Abbreviations

CCL - C-C motif chemokine ligand
CD - cluster of differentiation
CXCL - chemokine (C-X-C motif) ligand
CXCR - chemokine (C-X-C motif) receptor
DNase - deoxyribonuclease
eDNA - extracellular deoxyribonucleic acid
G-CSF - granulocyte colony-stimulating factor
ICAM - intercellular adhesion molecule
IFN - interferon
IFNAR - receptor of type I IFNs
Ly6G - lymphocyte antigen 6 complex locus G6D
MMP - matrix metalloproteinase
NAC - N-acetylcysteine
NAMPT - nicotinamide phosphoribosyltransferase
NADPH - nicotinamide adenine dinucleotide phosphate
NETs - neutrophil extracellular traps
PAMPs - pathogen-associated molecular patterns
pelA - pellicle polysaccharide A
P. aeruginosa - *Pseudomonas aeruginosa*
ROS - reactive oxygen species
TNF - tumor necrosis factor
SIRT - sirtuin
TAN - tumor-associated neutrophil
VEGF - vascular endothelial growth factor
WT - wild type

Summary (English)

Neutrophils constitute the main leukocyte population in the human blood. Due to their abundance, rapid mobilization and potent cytotoxic properties (release of reactive oxygen species (ROS), bactericidal proteins and neutrophil extracellular traps (NETs), neutrophils become the first line of immune response during infection and inflammation. In tumor situation, neutrophils gain new properties due to the influence of specific tumor-microenvironment, and regulate tumor angiogenesis and metastasizing, as well as anti-tumor immune responses.

Neutrophil functionality is regulated by many factors, but type I interferons (IFNs) are one of the most potent cytokines in this context. It could be shown here that the presence of IFNs stimulates pro-inflammatory activity of neutrophils, while in the absence of interferons neutrophils develop anti-inflammatory, regenerative capacity. In the context of tumor, type I IFNs stimulate anti-tumor (N1) polarization of neutrophils. It was recently shown that during cancer progression, the signaling via type I IFNs receptor (IFNAR) is suppressed, due to the degradation of IFNAR in tumor tissue. . This results in the establishment of immune suppressive microenvironment in tumor and supports tumor progression. Here, the effect of such disbalance of IFN availability on neutrophil pro-tumor functions was analyzed. It could be demonstrated that the absence of type I IFNs signaling at the tumor site triggers pro-tumor polarization and nicotinamide phosphoribosyltransferase (NAMPT)-dependent proangiogenic switch of tumor-associated neutrophils, resulting in elevated tumor angiogenesis and growth. Inhibition of NAMPT signaling in TANs leads to their anti-tumor activation with decreased angiogenic activity. Transfer of such anti-tumor neutrophils into tumor-bearing mice was demonstrated to have a therapeutic suppressive effect on tumor growth.

In the context of bacterial infections, the upregulation of IFN signaling at the periphery results in the overactivation of neutrophils and their elevated release of Neutrophil Extracellular Traps (NETs). Accumulation of NETs in lung tissue during infection with *Pseudomonas (P.) aeruginosa* supports survival of bacteria, which could use such NETs as a scaffold for creating biofilms that protect them from the immune system and antibiotics. Prevention of NETs formation by activated lung neutrophils using N-acetylcysteine (NAC) or deoxyribonuclease (DNase) limits tissue damage and reduces bacterial persistence in the lung.

In summary, the essential role of type IFNs in the regulation of neutrophil activity could be here demonstrated, both during bacterial infections as well as during cancer progression. Changes of neutrophil functions may serve as potential diagnostic marker for disease progression or development of complications. Based on the results of the study, the new potential therapeutic targets can be identified to improve the disease treatment.

Summary (Deutsch)

Neutrophile stellen die Haupt-Leukozytenpopulation im menschlichen Blut dar. Aufgrund ihrer Häufigkeit, ihrer raschen Mobilisierung und Migration sowie ihrer starken zytotoxischen Eigenschaften (Freisetzung reaktiver Sauerstoffspezies (ROS), bakterizider Proteine und extrazellulärer Neutrophil-Fallen (NETs)) sind sie die erste Reaktionslinie bei Infektion und Entzündung. Nach der Migration in Tumorgewebe erhalten Neutrophile in dieser spezifischen Mikroumgebung neue Eigenschaften und regulieren die Tumorangiogenese und -metastasierung sowie die anti-tumoralen Immunreaktionen.

Die Funktionalität der Neutrophile wird durch viele Faktoren reguliert, dabei sind Typ-I-Interferone (IFN) eines der wirksamsten Zytokine. In Abwesenheit von Interferonen zeigen Neutrophile eine entzündungshemmende, regenerative Kapazität, während die Anwesenheit dieser Zytokine die pro-inflammatorische Aktivität von Neutrophilen stimuliert. Im Tumor-Zusammenhang regulieren Typ-I-IFNs den Übergang zwischen anti-tumoraler (N1) und pro-tumoraler (N2) Polarisation. Kürzlich konnte gezeigt werden, dass während der Krebsprogression die Signalübertragung über die Rezeptoren der Typ-I-IFNs (IFNAR) systemisch hochreguliert wird, im Tumorgewebe jedoch aufgrund des Abbaus von IFNAR unterdrückt wird. Dies führt zur Etablierung einer suppressiven Mikroumgebung im Tumor und unterstützt die Tumorprogression. Der Effekt eines solchen Ungleichgewichts der IFN-Verfügbarkeit auf die pro-tumorale Neutrophil-Funktionen wurde hier analysiert. Es konnte gezeigt werden, dass die Abwesenheit von Typ-I-IFN-Signalübertragung an der Tumorstelle zu einer pro-tumoralen Polarisation und einem Nicotinamid-Phosphoribosyltransferase (NAMPT)-abhängigen pro-angiogenen Wechsel der Tumor-assoziierten Neutrophile (TANs) führt, welches eine erhöhte Tumorangiogenese und -wachstum zur Folge hat.

Die Hemmung der NAMPT-Signalübertragung in TANs führt zu deren anti-tumoralen Aktivierung mit verminderter angiogenetischer Aktivität. Der Transfer solcher Neutrophile in tumortragende Mäuse zeigte eine therapeutische Wirkung und unterdrückte das Tumorwachstum.

Im Infektion-Zusammenhang führt die Hochregulation der IFN-Signalübertragung in der Peripherie zu einer Überaktivierung der Neutrophile und zu einer erhöhten Freisetzung von NETs. Die Anhäufung von NETs in Geweben unterstützt das Überleben pathogener Bakterien, die solche NETs als Gerüst für die Bildung von Biofilmen nutzen könnten und somit vor dem Immunsystem und Antibiotika geschützt werden. Verhinderung der NET-Bildung von aktivierten Lungen-Neutrophilen mit N-Acetylcystein (NAC) oder DNase begrenzt die Gewebeschädigung und die bakterielle Persistenz, die mit einer Typ-I-IFN-abhängigen Neutrophil-Überaktivierung verbunden sind.

Zusammenfassend konnte die zentrale Rolle von Typ-I-IFNs bei der Regulation der Neutrophil-Aktivität sowohl bei bakteriellen Infektionen als auch während der Krebsprogression nachgewiesen werden. Veränderungen der Neutrophil-Funktionen können als potentielle diagnostische Marker bei dem Fortschreiten der Krankheit und bei der Entwicklung von Komplikationen dienen. Basierend auf den Ergebnissen der Studie können neue potenzielle therapeutische Ziele identifiziert werden, um die Behandlung der Krankheit zu verbessern.

Introduction

Neutrophils constitute the main leukocyte population in human blood and are the first cells responding to inflammatory signals. The wide spectrum of neutrophil functions include (1) release of factors (tumor necrosis factor (TNF) - α , ROS, proteins, NETs) that are cytotoxic for pathogens, but also for tumor cells and host tissues, (2) support of angiogenesis by release of pro-angiogenic factors, (3) matrix degradation facilitating migration of endothelial, immune and tumor cells, and contributing to tissue damage, (4) support and shape of inflammation (release of chemotactic molecules and cytokines), (5) regulation of adaptive immune responses (antigen presentation, expression of immune checkpoint molecules as well as release metabolic factors and ROS, which regulate the lymphocyte response) and (6) dissemination of pathogens and tumor cells (adhesion and trapping with NETs, intracellular transport of microbes) [reviewed by Granot & Jablonska, 2015, Jablonska et al, 2017].

These functions can be both beneficial and unfavorable for the host, depending on the duration and severity of the inflammation, the possibility to eliminate the pathogen, and modulating microenvironment. The activity of neutrophils is regulated on many levels, but one of the most potent activators are cytokines, such as type I IFNs and granulocyte colony-stimulating factor (G-CSF) (**Figure 1**).

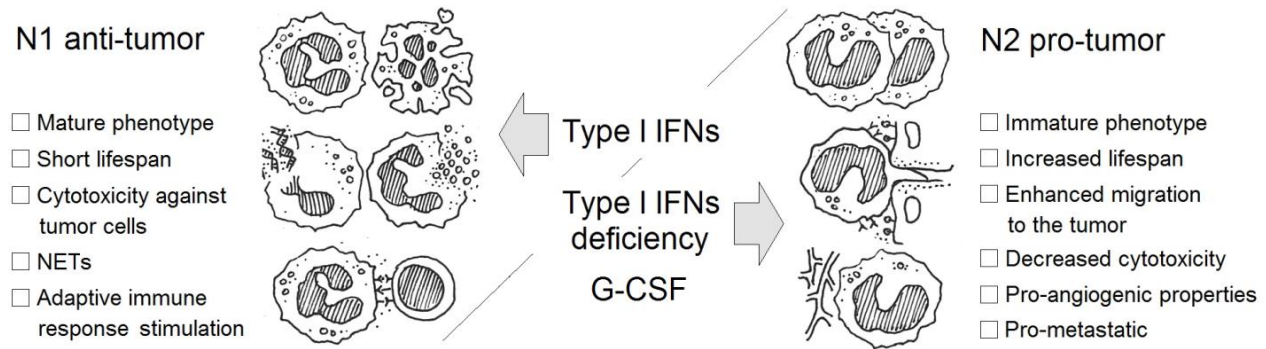


Figure 1. Type I IFN dependent regulation of the major neutrophil properties. Adapted from Pylaeva et al, 2016 with modifications. IFNs – interferons, G-CSF – granulocyte colony stimulating factor, NETs – neutrophil extracellular traps.

Type I IFNs system

One of the potent regulators of neutrophil activity are type I interferons. In the mouse, type I IFNs comprise a large family of cytokines with at least 12 IFN- α , IFN- β , IFN- ϵ and IFN- κ [Pestka et al, 2004, Chen et al, 2004]. All of them signal via a common receptor IFNAR, and induce the expression of multiple IFN-inducible genes with a broad range of biological functions [Pestka et al, 2004]. Within the type I IFNs, IFN- α and IFN- β are the best characterized. Importantly, a hierarchy of expression has been shown to exist for these cytokines [Erlandsson et al, 1998, Lienenklaus et al, 2009], where IFN- β is induced first. When it binds to IFNAR, IFN- β in a paracrine and autocrine fashion triggers a cascade of type I IFNs, including IFN- α and IFN- β . The only exception to this rule are plasmacytoid dendritic cells, which can start immediately with the secretion of IFN- α [Solodova et al, 2011]. Besides, its importance for the induction of the IFN cascade, IFN- β is also

constitutively expressed in low amounts under normal non-inflammatory conditions [Koerner et al, 2007, Lienenklaus et al, 2009]. The reason for such constitutive expression of IFN- β might be the priming of the immune system to persist in a preactivated state that guarantees a faster and stronger type I IFNs response when necessary. The systemic up-regulation of type I IFN signaling is observed in various clinical conditions, including viral infections and cancer [Musella et al, 2017]. At the same time, local loss of IFNAR due to its degradation was reported for tumor tissue [Katlinski et al, 2017], which results in diminished anti-cancer immune responses.

Type I IFNs are strong activators of the immune system [Gonzalez-Navajas et al, 2012]. Concerning neutrophils, type I IFNs modulate the lifespan, phenotype and functions of these cells. IFNAR signaling is critically important for short-term hematopoietic stem cells and for differentiation of late progenitors into mature neutrophils [Siakaeva et al, 2019], and further, their anti-tumor polarization. Type I IFN deficient neutrophils are characterized with prolonged lifespan due to apoptosis suppression, higher expression of chemokine (C-X-C motif) receptors (CXCR2, CXCR4) responsible for their migration to the tumor site, reduced cytotoxicity (decreased ROS and NETs release) and reduced potential to stimulate adaptive immune responses (decreased expression of intercellular adhesion molecule (ICAM)-1 and TNF- α). At the same time, IFN-deficient TANs possess the prominent pro-angiogenic and pro-metastatic activity (namely, elevated expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP) 9, Bv8 and S100A8,9 molecules) [Jablonska et al, 2010, Andzinski et al, 2015, Andzinski et al, 2016].

The up-regulation of G-CSF and its downstream molecules (NAMPT, SIRT1) (**Figure 2**) was observed in type I IFN deficiency [Andzinski et al, 2015, Siakaeva et al, 2019]. The role of G-CSF pathway in the survival and function of neutrophils is well recognized. NAMPT is essential for the

G-CSF-induced differentiation of hematopoietic precursors into myeloid-lineage cells [Skokowa et al, 2009], but also delays the maturation of differentiated neutrophils in bone marrow [Siakaeva et al, 2019]. It serves as a potent inhibitor of apoptosis of myeloid cells and was shown to promote pro-tumor polarization of macrophages stimulate expression of cytokines, matrix-degrading enzymes and chemokines in macrophages [Audrito et al, 2015].

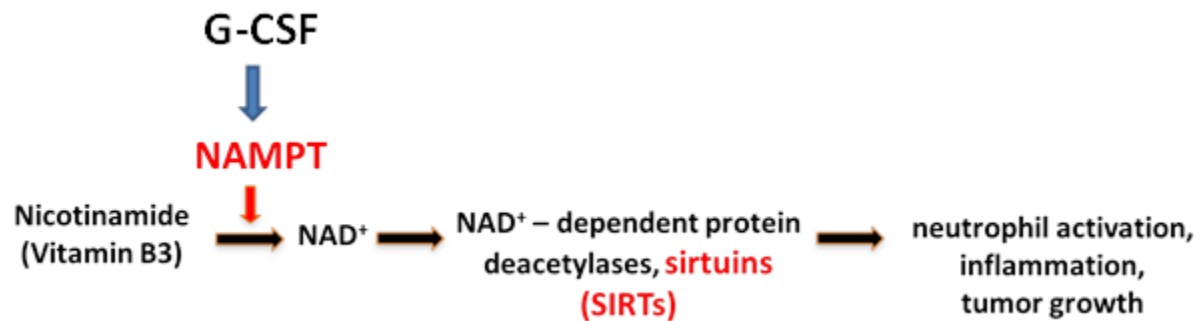


Figure 2. G-CSF signaling. Adapted from Skokowa et al, 2009. G-CSF – granulocyte colony stimulating factor, NAD - nicotinamide , NAMPT – nicotinamide phosphoribosyltransferase , SIRTs - sirtuins

Regulatory role of neutrophils in cancer

Despite the significant progress in instrumental and laboratory diagnostics, as well as treatment methods, the global cancer burden increased to 18.1 million new cases with 9.6 million deaths in 2018 [Bray et al, 2018]. Nowadays, the attention of the scientists and clinicians is focused on the role of immune system in the regulation of tumor growth. In the recent years it has become apparent that tumor development is determined not only by the intrinsic tumor cell features, but also by the infiltrating immune cells, such as neutrophils, whose functions are modified by the tissue

microenvironment [Jablonska et al, 2010, Fridlender et al, 2009]. Owing to their plasticity, neutrophils play diverse roles during cancer development and metastasis since they possess both tumor-promoting (N2) and tumor-limiting (N1) properties.

Anti-tumor properties of neutrophils include antibody-dependent or direct cytotoxicity mediated by ROS release and production of NETs. Moreover, neutrophils potentiate anti-tumor immune responses by the recruitment of other immune cells to the tumor site. Recently, the role of neutrophils as possible antigen-presenting cells was suggested. These cells were shown to modulate activation of cluster of differentiation (CD) 4⁺ and CD8⁺ T cells via expression of co-stimulatory molecules like CD86, ICAM-1, OX40L, and 4-1BBL [Andzinski et al, 2016, Otten et al, 2005, Granot et al, 2011, Scapini et al, 2001, Beauvillain et al, 2007, Eruslanov et al, 2014, Singhal et al, 2016].

Tumor promoting N2 neutrophils are characterized with immature nucleus shape and reduced tumor cell killing capacity [Andzinski et al, 2015]. Tumor-supporting properties of such cells include stimulation of tumor angiogenesis via secretion of VEGF and MMP9 [Jablonska et al, 2010]. This leads to the better vascularization of the primary tumor and its growth. Of note, not only primary tumor growth is maintained by neutrophils, but also the formation of metastases can be enhanced by these cells. Pro-tumor neutrophils upregulate the expression of pro-metastatic proteins, e.g., Bv8, S100A8, and S100A9, but also VEGF and MMP9. Moreover, in pro-tumor polarizing conditions (type I IFNs deficiency), higher metastatic burden is observed [Wu et al, 2015]. Several neutrophil-related mechanisms of metastasizing are described. Tumor-derived factors mobilize neutrophils and facilitate their homing at distant organs even before the arrival of tumor cells. Neutrophil integrins and released extracellular traps (NETs) support early adhesion of circulating tumor cells to

endothelium, secreted enzymes break down endothelial barriers and loosen extracellular matrix, and pro-angiogenic or pro-metastatic molecules support vascular growth and proliferation of tumor cells. Moreover, neutrophils are known to suppress adaptive immune responses to facilitate metastasis [reviewed by Jablonska et al, 2017, Cools-Lartique et al, 2013]. Importantly, neutrophils are also able to suppress adaptive immune responses. The expression of lectin-like oxidized low-density lipoprotein receptor-1 and arginase by neutrophils was reported to be associated with decreased activity and proliferation of effector T cells in tumor tissue [Si et al, 2019]. N2 neutrophils were also shown to recruit regulatory T cells in tumors by expression of C-C motif chemokine ligand (CCL) 17 [Mishalian et al, 2014].

Experimental and clinical data reveal significant negative influence of high neutrophil counts in blood and tumor tissue in cancer [Donskov, 2013, Sumner et al, 2017] on overall tumor patient prognosis. A high density of immunosuppressive TAN in tumor tissue, as well as high proportion of their conjugated with T cells, were significantly associated with reduced overall survival in head and neck cancer [Si et al, 2019].

The role of neutrophils during infections

Bacterial hospital-borne (nosocomial) infections remain a major cause of mortality and morbidity worldwide and account for 7% in developed and 10% in developing countries [Khan et al, 2017]. Nosocomial infections cause prolonged hospital stay, disability, and economic burden. The number of nosocomial infections is steadily growing due to aging of society, but also prolonged stay in the hospitals, invasive procedures and immunosuppressive treatments [Parkins et al, 2010]. One of the most common bacterial strains involved in such infections is *Pseudomonas (P.) aeruginosa*, that is a gram-negative nosocomial pathogen, forming structured multicellular communities on surfaces,

called biofilms. One of the components of biofilms is extracellular deoxyribonucleic acid (eDNA), which is attached by bacterial cationic exopolysaccharides due to its negative charge [Okshevsky et al, 2015]. Biofilm eDNA could be of bacterial or host cell origin [Walker et al, 2005, Parks et al, 2009, Caceres et al, 2014]. Hidden within self-produced matrix, bacteria in biofilms are protected from the host immune defense, antibiotics, or chemotherapy [Mulcahy et al, 2014]. Other mechanisms, allowing *P. aeruginosa* escape host immune response, include release of cytotoxic molecules (alginate, rhamnolipids) as well as contact-depending killing of immune cells (type III secretion system), high rate of mutations, loss of pathogen-associated molecular patterns (e.g. flagella, potent stimulators of toll-like receptor 5) and quorum sensing (communication allowing bacteria to adjust gene expression) [Veesenmeyer et al, 2009].

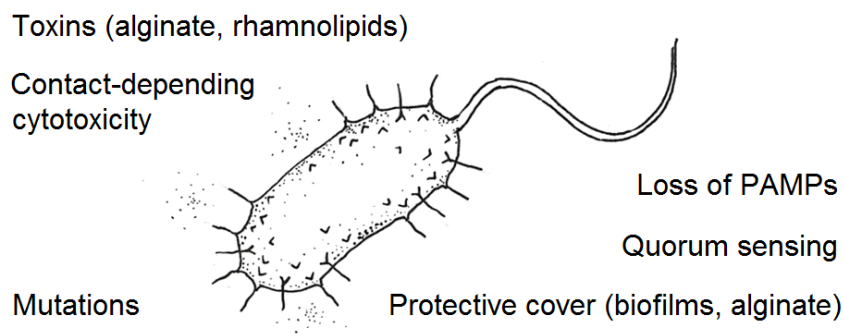


Figure 3. Mechanisms allowing *P. aeruginosa* to escape the immune system. PAMPs - pathogen-associated molecular patterns.

The cogent role of neutrophil responses in acute *P. aeruginosa* infection of respiratory tract was proven. Neutropenic mice as well as animals, which cannot recruit neutrophils to the lungs, were

highly susceptible to fatal *P. aeruginosa* lung infection, with bacterial doses 10^5 times lower than non-neutropenic mice, while mice lacking mature lymphocytes or alveolar macrophages demonstrated only mild changes. [Koh et al, 2008]. Antibacterial properties of neutrophils include release of ROS, production of bactericidal proteins, phagocytosis and formation of NETs [Lavoie et al, 2011].

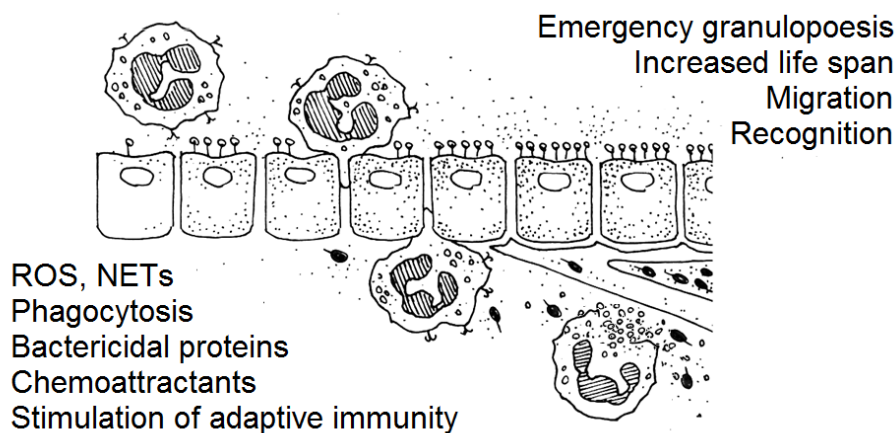


Figure 4. Neutrophil antibacterial responses. ROS – reactive oxygen species, NETs – neutrophil extracellular traps.

Impaired functions of neutrophils have been shown to be responsible for bacterial persistence in certain clinical situations. As an example, mutations in genes encoding components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex lead to a defect in ROS production and NETs formation by neutrophils, what results in susceptibility of such patients to recurrent and life-threatening bacterial and fungal infections [O'Neill et al, 2015, Bianchi et al,

2009]. Changes in the immune system, that are induced by cancer, may have an additional impact on antibacterial properties of neutrophils.

As prominent effect of type I IFN system and G-CSF pathway on the activity of neutrophils was observed, this work focuses on the delineation of the role that factors play during cancer progression and nosocomial bacterial infection with *P. aeruginosa*.

Results

All results included in this dissertation were published and are described as follows:

1. **Pylaeva E**, Harati MD, Spyra I, Bordbari S, Strachan S, Thakur BK, Höing B, Franklin C, Skokowa J, Welte K, Schadendorf D, Bankfalvi A, Brandau S, Lang S, Jablonska J. *NAMPT signaling is critical for the proangiogenic activity of tumor-associated neutrophils*. Int J Cancer. 2019 Jan 1;144(1):136-149. doi: 10.1002/ijc.31808.
2. **Pylaeva E**, Spyra I, Bordbari S, Lang S, Jablonska J. *Transfer of Manipulated Tumor-associated Neutrophils into Tumor-Bearing Mice to Study their Angiogenic Potential In Vivo*. J Vis Exp. 2019 Jul 20;(149). doi: 10.3791/59807.
3. **Pylaeva E**, Bordbari S, Spyra I, Decker AS, Häussler S, Vybornov V, Lang S, Jablonska J. *Detrimental Effect of Type I IFNs During Acute Lung Infection With Pseudomonas aeruginosa Is Mediated Through the Stimulation of Neutrophil NETosis*. Front Immunol. 2019 Sep 11;10:2190. doi: 10.3389/fimmu.2019.02190.

Discussion

NAMPT up-regulation in type I IFN deficiency is responsible for the pro-angiogenic activity of tumor-associated neutrophils

Neutrophils are traditionally described as short-living cells with a potent antibacterial activity. In the recent decade, the accumulating facts about cancer and its interaction with immune system changed the knowledge about neutrophil biology dramatically. Tumor and stromal cells release high concentrations of various chemokines and cytokines, including type I IFNs and growth factors (G-CSF), which have a significant impact on neutrophil phenotype and functions. Support of angiogenesis and metastasizing, as well as regulation of adaptive immune responses depending on the availability of different tumor-derived factors was reported for neutrophils.

As angiogenic capacity of neutrophils in tumors was shown to be dependent on type I IFN availability [Jablonska et al, 2010], we were interested how this process is regulated. Mice deficient in type I IFN signaling (*Ifnb1*^{-/-} lacking IFN- β and *Ifnar1*^{-/-} lacking the receptor of IFN- α and IFN- β), as well as wild type (WT) mice with sufficient type I IFN signaling, were used to reveal the regulation of signaling pathways responsible for pro-tumor polarization of neutrophils in type I IFN deficiency. Inhibition of these molecules would polarize neutrophils into N1 and would have beneficial effects on the course of disease.

In vitro inhibition of pro-angiogenic G-CSF/NAMPT pathway in isolated tumor-associated neutrophils was used to demonstrate the effect of suppressed pro-angiogenic capacity. Systemic administration of NAMPT inhibitor allowed to estimate the effect on tumor growth and neutrophil phenotype. As the last step, *in vivo* re-injection of neutrophils isolated and treated *in vitro* with

NAMPT inhibitor was used to prove the involvement of neutrophils in tumor angiogenesis and growth.

Elevated G-CSF/NAMPT pathway signaling (NAMPT, SIRT1, Cebpa, Cebpb) that was observed in pro-tumor TANs isolated from IFN-deficient mice was found to be responsible for the angiogenic switch of neutrophils. In agreement, *in vitro* inhibition of NAMPT with its molecular inhibitor FK866 reduced neutrophil pro-angiogenic potential (expression of pro-angiogenic molecules, proven in aortic ring assay mimicking all steps of angiogenesis) of *Ifnar1*^{-/-} neutrophils.

Systemic inhibition of NAMPT and its downstream molecule SIRT1 via injecting FK866 25 mg/kg, (Axon Medchem, the Netherlands) and (EX527 10 mg/kg, Cayman Chemical, US), respectively, suppressed neutrophil-mediated tumor angiogenesis. To prove the significant impact of neutrophils, adoptive transfer of FK866-treated neutrophils to the tumor-bearing IFN-deficient mice was performed, resulted in significantly suppressed tumor vascularization and growth in mice (**Figure 5**).

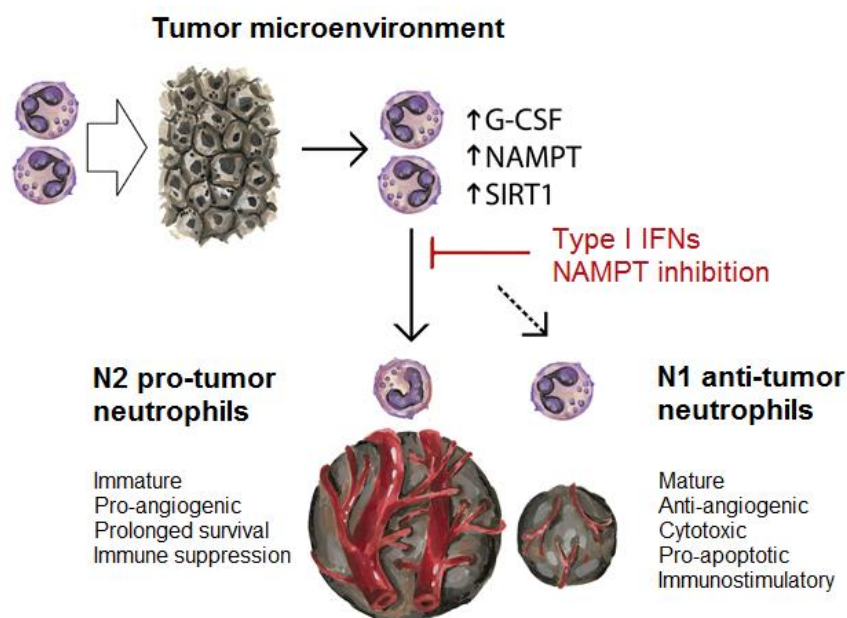


Figure 5. Targeting pro-angiogenic G-CSF/NAMPT signaling in tumor-associated neutrophils, with type I IFNs or NAMPT inhibitor shifts the neutrophil polarization into N1 anti-tumor. IFNs – interferons, G-CSF – granulocyte colony stimulating factor, NAMPT – nicotinamide phosphoribosyltransferase, SIRT – sirtuin.

The mechanisms of TAN-mediated support of tumor vessel growth include release of vascular endothelial growth factor as well as MMPs other enzymes [Jablonska et al, 2010], providing degradation of extracellular matrix and facilitate vessel growth [Bausch et al, 2011]. Certain chemokine (C-X-C motif) ligands (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8) are known to mediate angiogenic processes through direct activation of endothelial cells via CXCR2 receptor or recruit pro-angiogenic immune cells and endothelial progenitors to the neovascular niche [Dimberg, 2010, Teicher et al, 2010, Burger et al, 2006]. All these molecules are reported to be upregulated in TANs in type I IFN deficiency, which could serve as an additional anti-angiogenic mechanism during tumorigenesis [Jablonska et al, 2013, Jablonska et al, 2010]. Inhibition of NAMPT in tumor-associated neutrophils results in significant decrease of the expression of VEGF, MMP9 and chemokine receptors on neutrophils. Similarly, selective inhibition of NAMPT using FK866 resulted in maturation and higher CD11b expression on bone marrow neutrophils, as well as in murine and human neutrophil cell lines, suggesting similar regulation of neutrophil development by NAMPT in different species [Siakaeva et al, 2019].

***Ex vivo* NAMPT targeting in neutrophils impairs their pro-tumor and pro-angiogenic activity**

Concerning tumor-supporting N2 polarization of TANs, we could validate that *ex vivo* NAMPT targeting in neutrophils supports their anti-tumor features and impairs their pro-angiogenic activity.

Such neutrophils, when adoptively transferred into tumor-bearing mice efficiently suppress tumor vascularization and growth in these animals.

Different treatment modalities were suggested to escape pro-tumor activation of neutrophils and enhance their anti-tumor properties, but did not reach the complete efficiency. Immunotherapy with IFN- α is approved for the treatment of multiple hematologic malignancies and solid cancers, including melanoma, renal carcinoma and vascular neoplasias [Arico et al, 2019]. Nevertheless, its efficacy is limited and only a small proportion of patients benefit from such treatment. Notably, the level of responsiveness to IFN treatment varies among individuals. This might be due to genetic polymorphism in type I IFN-related genes that have been shown to exert a significant impact on survival and therapy outcome in melanoma patients [Lenci et al, 2012]. Another reason for impaired therapy response could be a suppression of pathways involved in IFN signal transduction in different microenvironment conditions [Zheng et al, 2011], or the cleavage of IFNAR in tumor tissue [Katlinski et al, 2017]. Suppression type 1 IFNs in cancer to dampen neutrophil activation will results in dramatically enhanced tumor growth [Jablonska et al, 2010]. Suppression of downstream molecules directly responsible for unfavorable changes in neutrophil functions in N1 as well as in N2 polarization demonstrated the efficiency in our experiments.

Depletion of neutrophils using lymphocyte antigen 6 complex locus G6D (Ly6G) antibody or inhibition of their migration with CXCR2 antibody results in decreased tumor angiogenesis, growth, and metastasis [Jablonska et al, 2010, Jablonska et al, 2013]. Nevertheless, generated monoclonal antibodies are immunogenic, and their administration is associated with a range of life-threatening side effects [Hansel et al, 2010]. Treatment with small molecules, such as NAMPT inhibitor FK866, that modulate neutrophil tumorigenicity, could help to avoid such complications.

Unfortunately, pharmacological systemic inhibition of NAMPT, next to its therapeutic effect on tumor growth, leads to severe side effects including gastrointestinal toxicity and thrombocytopenia. Therefore, the systemic application of NAMPT inhibitors is not feasible [Hasmann et al, 2003, Holen et al, 2008, von Heideman et al, 2010]. For this reason, we suggest here the protocol where NAMPT activity is blocked directly in isolated TANs. Such anti-tumor neutrophils are then adoptively transferred into tumor-bearing host. This protocol will allow avoiding systemic toxic side-effects of the compounds, while its effect on the target cells will be sustained.

Stimulation of NET formation by type I IFNs is responsible for persistence of *P. aeruginosa*

Changes in neutrophil activity due to availability of certain cytokines or growth factors, such as IFNs or G-CSF, play a role not only during tumor progression, but also in the susceptibility of the host to nosocomial bacterial infections with pathogens, such as *P. aeruginosa*. To study the regulation of antibacterial properties of neutrophils by type I IFNs, we used mice deficient in type I IFN signaling (*Ifnb1*^{-/-} lacking IFN- β or *Ifnar1*^{-/-} lacking the receptor for all type I IFNs), as well as control WT mice with sufficient type I IFN signaling. Mice were infected intratracheally (*i.t.*) with *P. aeruginosa* and the course of infection was monitored. Further, isolated WT, *Ifnb1*^{-/-} and *Ifnar1*^{-/-} neutrophils were challenged with *P. aeruginosa* *in vitro*, to confirm the input of neutrophils in the disease progression. Neutrophil extracellular traps released in response to bacteria were visualized after staining with 4',6-diamidino-2-phenylindole (DAPI) and anti-histone-antibodies, and their quantity and size were estimated microscopically [de Buhr N. and von Köckritz-Blickwede M., 2016]. The necessity of NETs as a scaffold for bacterial biofilms was proven *in vitro* and *in vivo* using the *P. aeruginosa* strain that is unable to bind eDNA (pellicle polysaccharide A (pelA) mutant 24480) [Jennings et al, 2015] and therefore cannot efficiently produce biofilms. Last, different

treatment modalities were used to modulate neutrophil antibacterial functions. Recombinant mouse IFN- β was used to enhance neutrophil activation, and DNase or NAC were used to abrogate NET release.

We demonstrated, that systemic type I IFN activation during acute lung infection with *P. aeruginosa* leads to the overactivation of neutrophils and tissue damage in response to bacterial invasion. While under normal conditions the release of NETs is a mechanism of killing of extracellular pathogens with high local concentration of effector components [Brinkmann et al, 2004], certain bacteria (e.g. *P. aeruginosa* used in the study) are able to utilize the components of NETs, for their biofilms [Parks et al, 2009], and survive there protected from the immune system and eventual antibiotic treatment [Walker et al, 2005, Caceres et al, 2014]. This leads to the high bacterial persistence in patients. In agreement, we observe the accumulation of green fluorescent protein-expressing (alive) bacteria in histone-rich areas (NETs) in the model of biofilm formation *in vitro*. ROS produced by neutrophils is suggested to trigger mutations in *P. aeruginosa*, including those responsible for exopolysaccharide production and biofilm formation [Mathee et al, 1999]. This may promote further adaptation and survival of bacteria. Persistence of the pathogen in organs leads to elevated immune responses and severe cytotoxicity. Excessive activation of neutrophils, or their prolonged survival in tissues during chronic inflammation, is associated with a high tissue damage due to release of proteases, such as neutrophil elastase [Venaille et al, 1998] or MMP9 [Sagel et al, 2005]. Interferons seem to influence the process of NET formation. Priming with IFN- α or IFN- γ with subsequent complement activation triggers release of NETs in mature human neutrophils. Notably, immature neutrophils are not able to release NETs in this condition, probably due to the lack of IFN signaling pathway mediators [Martinelli et al, 2004]. We could demonstrate

that type I IFNs excessively activate neutrophils and trigger their ROS production and NETs release in response to *P. aeruginosa* infection. This in turn supports biofilm formation by the bacteria and their survival in lungs.

Binding to eDNA in NETs is a crucial step facilitating biofilm formation by *Pseudomonas*. Here we demonstrated, that bacteria, which are unable to bind eDNA and therefore cannot efficiently produce biofilms, were efficiently cleared in both mouse strains, IFN-deficient and –sufficient. This was independent of the various amounts of NETs measured in these mice, as neutrophil ability to release NETs in response to PA24480 strain was comparable to wild type bacterial strain. It proves the essential role of NETs in the stimulation of biofilm formation.

The survival of bacteria in biofilms is one of the possible mechanisms associated with the inappropriate regulation of neutrophil functions. NETs released by neutrophils represent in addition the source of various biologically active components, which induce inflammation or tissue damage through multiple mechanisms. NET-derived histones are cytotoxic themselves and were shown to induce epithelial and endothelial cell death [Saffarzadeh et al, 2012] or act as damage-associated molecular pattern proteins, activating the immune system and leading [Allam et al, 2012], and their levels correlate with lung tissue damage [Abrams et al, 2013]. Moreover, certain bacterial species, including *Actinobacillus pleuropneumoniae* or *Haemophilus influenzae* were also proven to benefit from the presence of NETs, as they utilize eDNA material degraded by host nucleases to support their growth [de Buhr et al, 2019]. NET components increase also the vascular permeability and induce vascular occlusions [Sung et al et al, 2019, Schulz et al, 2020], which may additionally lead to the damage of the lung tissue. All observed mechanisms can be involved in the elevated lung tissue damage observed in our study in WT animals. To the opposite, limited type I IFN responses

in *Ifnb1*^{-/-} and *Ifnar1*^{-/-} mouse strains was associated with lower bacterial load and decreased damage of the lung tissue, corresponding with reduced NET release and biofilm formation by *Pseudomonas* in presence of such neutrophils (**Figure 6**).

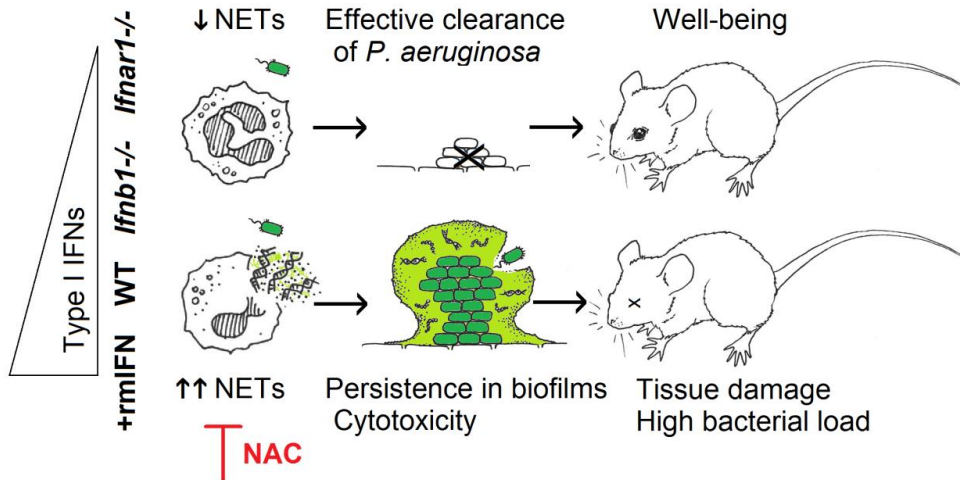


Figure 6. Boosted NET formation by type I IFN-activated neutrophils triggers biofilm formation by *P. aeruginosa* and supports its persistence in the infected organs. rmIFN – recombinant murine interferon, WT – wild type, NETs – neutrophil extracellular traps, NAC – N-acetylcysteine.

Multiple treatment modalities are insufficient in infectious complications with *P. aeruginosa*. The reasons for that are on the one hand a high intrinsic heterogeneity of the bacteria, and on the other hand the ability of *Pseudomonas* to form protecting biofilms. Therefore, treatment with factors able to disturb biofilm formation should be considered as a strategy to prevent or treat *P. aeruginosa* complications. This is particularly important in inflammatory conditions associated with augmented

neutrophil activation. We could show here that NAC, which is a reducing agent and ROS scavenger, decreases biofilm formation by *Pseudomonas*. Besides its capacity to directly disturb bacterial biofilms by reducing disulfide bonds in mucopolysaccharides [Choi et al, 2018], NAC has an immunotropic activity by suppressing NETs formation by neutrophils [Zawrotniak et al, 2015]. Here, we demonstrated promising therapeutic effect of NAC in *P. aeruginosa* infected mice. NAC treatment significantly improved the clearance of bacteria from lungs, diminished lung tissue damage, and decreased amounts of NETs in lungs. *In vitro* experiments corroborated these results, showing a direct inhibitory effect of NAC on NETs release by neutrophils and, later, on the NET-dependent biofilm production by *Pseudomonas*. In addition, we could validate the efficiency of DNase in the prevention of NET-mediated biofilm formation. This is in agreement with data showing improved clearance of *P. aeruginosa* from the lungs of cystic fibrosis patients after the treatment with DNase [Yang et al, 2018]. Therefore, we suggest that NET-disrupting agents should be considered as a treatment or prophylaxis in clinical conditions associated with elevated type I IFNs levels, such as cancer or viral infections.

Conclusion

The view on neutrophils is recently radically changing. Initially considered as simple killers, neutrophils were shown to participate in multiple biological processes and to orchestrate complex immune responses, covering all stages of inflammation from pathogen killing and tissue damage to the angiogenesis, spread of pathogens and regulation of adaptive immunity.

Overall, in here presented research results the central role of neutrophils in the regulation of carcinogenesis and nosocomial infections is obvious. Alterations of neutrophil activation due to the

availability of cytokines or growth factors could be responsible for the changed host predisposition to develop tumors or succumb infections.

While the up-regulation of IFNAR expression takes place systemically in cancer, cells in cancer tissue exhibit IFNAR degradation in comparison to healthy tissues due to exposure to tumor-derived factors. This disbalance results in the significant dysregulation of neutrophils activity, with pro-tumor N2 polarization at the tumor site (angiogenesis) and at the same time anti-tumor N1 polarization and overactivation at the periphery (tissue damage with ROS and NETs). This work describes the detrimental effects of systemic type I IFN activation and local suppression in tumor for neutrophil functions, taking place during cancer progression, and suggests the therapeutic approaches to compensate the neutrophil dysfunction (**Figure 7**).

Despite of the progress in surgical and pharmacological treatment of cancer and its complications, successful therapy remains a challenge. As dysregulation of immune functions, with tumor-supportive polarization in cancer tissue and overactivation on the periphery, takes place during cancer progression, novel methods modulating functions of immune cells should be established. Verification of the involvement of G-CSF/NAMPT signaling pathway in pro-tumor polarization of neutrophils allowed us to therapeutically suppress tumor growth via selective targeting of pro-angiogenic NAMPT signaling in TANs. Targeting of NET release and associated with this biofilm formation by *P. aeruginosa*, which is boosted by type I IFNs could offer a new therapeutic approach to prevent persistent bacterial infections in patients with diseases associated with the up-regulation of type I IFNs.

Systemic type I IFNs activation
promotes persistence of *P. aeruginosa* in biofilms

Local IFNAR suppression
supports tumor growth

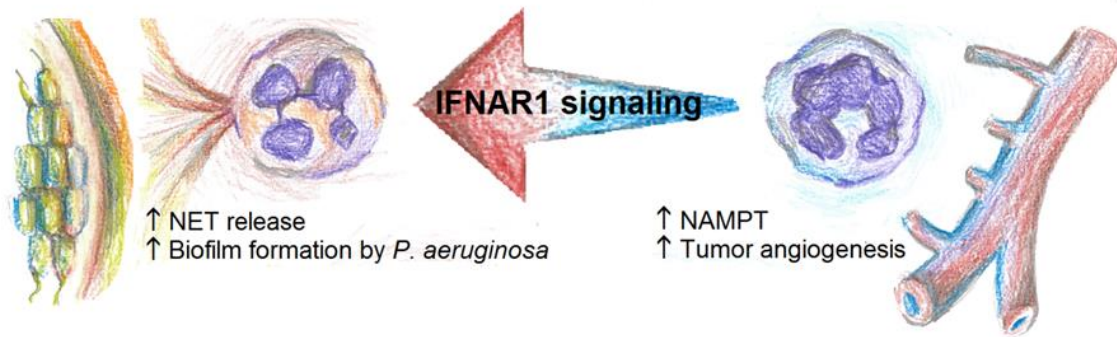


Figure 7. Detrimental effect of dysregulated Type I IFN signaling on neutrophil functions. IFNs – interferons, IFNAR – receptor of type I IFNs, NET – neutrophil extracellular traps, NAMPT - nicotinamide phosphoribosyltransferase.

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Cumulative Thesis/Extent of Contribution

Cumulative thesis of Ms Ekaterina Pylaeva

Author contributions

NAMPT signaling is critical for the proangiogenic activity of tumor-associated neutrophils.

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Contributions:

- Conception - 50%: Focus at the pro-angiogenic potential of tumor-associated neutrophils in type I IFN deficiency and after NAMPT inhibition, estimated *in vitro* and *in vivo*.
- Conduction of experimental work - 50%: *In vivo* experiments, flow cytometry and cell sorting, sample preparation for the methods of molecular biology.
- Statistical analysis - 90%: Test for normality of the data distribution, selection of the appropriate statistical tests, statistical analysis and the way of data presentation.
- Writing the manuscript - 50%: Materials and methods, results, figures; partially introduction and discussion
- Revision of the manuscript - 50%: Revising of the text, Additional experiments suggested by reviewers, Response to the Reviewer's Comments.

Transfer of Manipulated Tumor-associated Neutrophils into Tumor-Bearing Mice to Study their Angiogenic Potential In Vivo.

Pylaeva E, Spyra I, Bordbari S, Lang S, Jablonska J.

Contributions:

- Conception - 50%: Focus at the targeted treatment of tumor-associated neutrophils to reduce their proangiogenic properties as a potential anti-cancer approach.
- Conduction of experimental work - 50%: *In vivo* experiments, flow cytometry and cell sorting, sample preparation for the methods of molecular biology.
- Statistical analysis - 100%: Test for normality of the data distribution, selection of the appropriate statistical tests, statistical analysis and the way of data presentation.
- Writing the manuscript - 100%: All parts of the manuscript, figures.
- Revision of the manuscript - 50%: Revising of the text, Response to the Reviewer's Comments.

Detrimental Effect of Type I IFNs During Acute Lung Infection With *Pseudomonas aeruginosa* Is Mediated Through the Stimulation of Neutrophil NETosis.

Pylaeva E, Bordbari S, Spyra I, Decker AS, Häussler S, Vybornov V, Lang S, Jablonska J.

Contributions:

- Conception - 90%: Focus at the dysregulated neutrophil extracellular trap formation of IFN-stimulated neutrophils as a supportive mechanism for bacterial survival, estimated *in vitro* and *in vivo*.
- Conduction of experimental work - 90%: *In vivo* experiments, flow cytometry and cell sorting, *in vitro* experiments, histological examination.
- Statistical analysis - 100%: Test for normality of the data distribution, selection of the appropriate statistical tests, statistical analysis and the way of data presentation.
- Writing the manuscript - 100%: All parts of the manuscript, figures.
- Revision of the manuscript - 100%: Revising of the text, Response to the Reviewer's Comments.

Signature of the Doctoral Candidate



Signature of the Doctoral Supervisor



NAMPT signaling is critical for the proangiogenic activity of tumor-associated neutrophils

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Tumor-associated neutrophils (TANs) regulate many processes associated with tumor progression, and depending on the microenvironment, they can exhibit pro- or antitumor functions. However, the molecular mechanisms regulating their tumorigenicity are not clear. Using transplantable tumor models, we showed here that nicotinamide phosphoribosyltransferase (NAMPT), a molecule involved in CSF3R downstream signaling, is essential for tumorigenic conversion of TANs and their pro-angiogenic switch. As a result tumor vascularization and growth are strongly supported by these cells. Inhibition of NAMPT in TANs leads to their antitumor conversion. Adoptive transfer of such TANs into B16F10-tumor bearing mice attenuates tumor angiogenesis and growth. Of note, we observe that the regulation of NAMPT signaling in TANs, and its effect on the neutrophil tumorigenicity, are analogous in mice and human. NAMPT is up-regulated in TANs from melanoma and head-and-neck tumor patients, and its expression positively correlates with tumor stage. Mechanistically, we found that targeting of NAMPT suppresses neutrophil tumorigenicity by inhibiting SIRT1 signaling, thereby blocking transcription of pro-angiogenic genes. Based on these results, we propose that NAMPT regulatory axis is important for neutrophils to activate angiogenic switch during early stages of tumorigenesis. Thus, identification of NAMPT as the critical molecule priming protumor functions of neutrophils provides not only mechanistic insight into the regulation of neutrophil tumorigenicity, but also identifies a potential pathway that may be targeted therapeutically in neutrophils. This, in turn, may be utilized as a novel mode of cancer immunotherapy.

In recent years it has become apparent that tumor development is determined not only by the intrinsic tumor cell features but also by the infiltrating immune cells, such as neutrophils, whose

Key words: tumor-associated neutrophils, neutrophil polarization, NAMPT, angiogenesis

Additional Supporting Information may be found in the online version of this article.

E.P. and M.D.H. contributed equally to this work

Conflict of interest: The authors declare no potential conflicts of interest.

Grant sponsor: Deutsche Forschungsgemeinschaft; **Grant numbers:** JA 2461/2-1; **Grant sponsor:** Deutsche Krebshilfe; **Grant numbers:** 111647

DOI: 10.1002/ijc.31808

History: Received 17 Feb 2018; Accepted 16 Jul 2018; Online 19 Aug 2018

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functions are modified by the tumor microenvironment. Tumor-associated neutrophils (TANs) accumulate in cancer patients and represent an important prognostic marker in a broad variety of neoplasias. In accordance, TANs represent a highly potent therapeutic target. Neutrophils are recognized for their functional complexity,¹ as they can be primed into either tumor-promoting or inhibiting phenotype.^{1,2} Protumor neutrophils significantly support tumor angiogenesis and growth,^{3–5} and maintain metastatic dissemination of tumor *via* establishment of pre-metastatic niche.^{6–8} Depletion of such neutrophils, or suppression of their migration, leads to significant abrogation of tumorigenesis in mice.^{3,4} On the other hand, tumor microenvironment can also prime antitumor properties of neutrophils, leading to suppression of tumor growth and metastasis by these cells.^{6,9} Such plasticity of neutrophils provides a potential target for therapies that enhance their antitumor activity while limiting protumor functions.

Neutrophil function in cancer is modulated by environmental cues (reviewed by Granot and Jablonska²). Type I interferons (IFNs) have been shown to prime antitumor activity of neutrophils, and the absence of IFNs supports

What's new?

Depending on the microenvironment, tumor-associated neutrophils (TANs) can exhibit pro- or antitumor functions. The molecular mechanisms regulating such polarization remain unclear, however. Here, the authors demonstrate the contribution of NAMPT, a molecule involved in CSF3R downstream signaling, to the tumorigenic conversion of TANs. They found that pro-tumor TANs are characterized with high NAMPT expression, and that targeting of NAMPT suppresses neutrophil tumorigenicity by inhibiting SIRT1 signaling, thereby blocking transcription of pro-angiogenic genes. Identification of NAMPT as a critical molecule priming pro-tumor functions not only provides mechanistic insight into the regulation of neutrophil tumorigenicity but also represents a promising immunotherapy approach.

tumorigenic activation of these cells. In agreement, mice deficient in IFNs develop significantly larger and better vascularized tumors, which are strongly infiltrated with protumor neutrophils.^{3–5,8} However, molecular mechanisms behind tumorigenic conversion of TANs are not clear to date.

In the absence of endogenous IFN- β , the systemic granulocyte colony-stimulating factor (G-CSF) production, as well as expression in neutrophils isolated from different anatomical compartments, is markedly up-regulated.¹⁰ G-CSF (CSF3) is a potent regulator of neutrophil activity and was shown to signal through nicotinamide phosphoribosyltransferase (NAMPT).^{11,12} NAMPT is a unique enzyme with cytokine-like features. Intracellularly, it is a rate-limiting enzyme converting nicotinamide (NA) into NAD⁺, which in turn activates NAD⁺-dependent protein deacetylases—sirtuins (SIRTs).¹³ In addition, when released extracellularly, NAMPT exhibits cytokine-like functions (eNAMPT)¹⁴ leading to activation of STAT3.¹⁵ NAMPT was shown to be overexpressed in a number of cancer types, such as colorectal, ovarian, breast, gastric, prostate and gliomas.¹⁶ NAMPT is essential for the G-CSF-induced differentiation of hematopoietic precursors into myeloid-lineage cells.¹² It serves as an inhibitor of apoptosis of myeloid cells and was shown to stimulate expression of cytokines, matrix-degrading enzymes and chemokines in macrophages.¹⁷

Since we observed elevated G-CSF levels in fast growing tumors of IFN-deficient mice, which are strongly infiltrated by neutrophils, in this study we set out to determine whether NAMPT is involved in tumorigenic conversion of high density blood neutrophils and TANs.

Material and Methods**Animals**

Eight to twelve week-old female C56BL/6J, *Ifnar1*^{−/−} and *Ifnb1*^{−/−} mice were used. Mice were bred and kept under SPF conditions in the animal facility of the University Hospital Essen (Germany) and University Hospital Tuebingen (Germany).

Cell lines

B16F10 melanoma cells were purchased from DSMZ, Braunschweig, Germany, MCA205 fibrosarcoma was a kind gift from Prof. T. Blankenstein, Berlin. Cell lines were regularly tested for mycoplasma contamination, and were negative.

Murine tumor models

Tumor cells were injected subcutaneously (s.c). Tumor size was evaluated by caliper and the volume calculated:

$$V = 4/3 \times \pi \times (h \times w^2) / 8 (h = \text{height}, w = \text{width}, \text{depth} = \text{width}).$$

Flow cytometry analysis and TANs sorting

Tumor tissue was digested using dispase/collagenaseA/DNase I solution (0.2 mg/0.2 mg/100 mg in mL), cells meshed through 50- μ m filters (Cell Trics, Partec, Sysmex Europe GmbH, Goerlitz, Germany) and erythrocytes lysed. Single-cell suspensions were stained with antibodies listed below. For intracellular staining Cytofix-Cytoperm buffer was used, for TNF- α staining, Monensin (BD Bioscience, BD, New Jersey, USA).

Flow cytometry was performed using BD FACSCanto system and data was analyzed using BD FACSDiva software.

CD11b⁺ Ly6G⁺ neutrophils were sorted using a FACSaria cell sorter (BD Bioscience, BD, New Jersey, USA), and the purity of cells was assessed ($\geq 95\%$).

Gating strategy for neutrophil staining and sorting is depicted in Supporting Information Figure S1.

Antibodies and viability dyes for flow cytometry and cell sorting

Anti-mouse CD16/32 (clone 2.4G2, BD Pharmingen, BD, New Jersey, USA), anti-CD11b (clone M1/70, eBioscience, Affymetrix, California, USA), anti-Ly6G (clone 1A8, BD Pharmingen, BD, New Jersey, USA), anti-Ly6C (clone NK 1.4, eBioscience, Affymetrix, California, USA), anti-tumor necrosis factor alpha (TNF α) (clone MP6-XT22, eBioscience, Affymetrix, California, USA), anti-MHCII (clone M5/114.15.2, eBioscience, Affymetrix, California, USA), anti-ICAM1 (clone eBiokat-1, Affymetrix, California, USA), anti-active Caspase 3 (C92-605, BD Pharmingen, BD, New Jersey, USA), anti-CXCR2 (clone #866614, R&D Systems, Minnesota, USA) were used.

Anti-human anti-CD45 (clone 5B1, Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD16 (clone 3G8, BD Bioscience, BD, New Jersey, USA), anti-CD66b (clone 80H3, Beckman Coulter, California, USA), anti-CD11b (clone ICRF44, BD Bioscience, BD, New Jersey, USA) were used.

To determine nonspecific signals, appropriate isotype controls were used. Viability Dye eFluor™ 780 (eBioscience, Affymetrix,

California, USA) or 4',6-Diamidin-2-phenylindol (DAPI) (BioLegend, California, USA) were used to determine viable cells.

In vitro treatment of neutrophils

Neutrophils were treated with 100 nM FK866 (NAMPT inhibitor),¹⁶ 1 μ M EX527 (SIRT1 inhibitor),^{17,18} 1 μ M LLL12 (STAT3 inhibitor), 10 μ M P7C3 (NAMPT activator) or 2.5 U/mL recombinant murine (rm) Ifnb.

Mouse treatment with G-CSF or NAMPT/SIRT1 inhibitors

Tumor-bearing mice were treated i.p. every second day using G-CSF (300 μ g/kg), FK866 (25 mg/kg)¹⁹ or EX527 (10 mg/kg).

Tumor killing assay

Neutrophils were sorted, treated with FK866 for 2 hr, washed and cultivated for 48 hr with B16F10 cells (1:2). Viability of neutrophils and proliferation and viability of B16F10 cells was assessed using Annexin-7AAD kit (BD Biosciences, BD, New Jersey, USA).

Aortic ring assay

Thoracic aortae were dissected from WT mice, cleaned and cut into 0.5 mm width rings. They were serum-starved overnight and embedded in Matrigel (Corning, New York, USA) in 96-well plate. Once embedded, the rings were fed with endothelial cell growth medium (Promocell GmbH, Heidelberg, Germany) and 20,000 TANs isolated from WT, *Ifnar*^{-/-} FK866-treated *Ifnar*^{-/-} mice were added. The plate was incubated for 12 days at 37°C and imaged using standard phase-contrast microscope (AMG EVOS digital inverted microscope).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed using primers listed in the Table 1. *Rps9* housekeeping gene was used for murine studies, *BACTIN* for human studies. The mRNA expression was measured using the SYBR green qPCR kit.

Western blot

Whole-cell lysates of cells were prepared, boiled and loaded on 4–12% NuPAGE Bis-Tris gels (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated, transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Illinois, USA), washed with TBS-T, blocked (5% skimmed milk +0.05% Tween) and incubated overnight in blocking buffer (5% BSA for anti-pSTAT3 or 5% skimmed milk others) containing primary antibodies: anti-SIRT1 (Sigma-Aldrich/Merck, Darmstadt, Germany); anti-NAMPT (Abcam, Cambridge, England), anti- β Actin (Santa Cruz Biotechnology, Inc, Texas, USA), anti-STAT3 (Cell Signaling Technology, Massachusetts, USA). The membranes were washed and stained with HRP-conjugated antibodies in 5% skimmed milk (Cell Signaling Technology, Massachusetts, USA). The signal was detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Illinois, USA), and developed using FusionFX imaging system and Fusion Capt Advance Software (Vilber Lourmat). The quantification was performed using ImageJ software and normalized to β -actin.

SIRT1 activity fluorimetric assay

Fluorimetric Sirt1 activity was measured using Sirt1 Activity Assay Kit according to the manufacturer's protocol.

Table 1. Primers for qRT-PCR

Gene	Forward primer	Reverse primer
Murine		
Sirt1	5'-GTCTCCT GTGGGATTCTGAC-3'	5'-ACACAGAGACGGCTGGAAC-3'
Nampt	5'-TAC AGTGGCCACAAATTC-3'	5'-CAATCCCAGGCA CAGTATCT-3'
Mmp9	5'-TGTCTGGAGATTCGACTTGAAGTC-3'	5'-TGAGTTCCAGGGC ACACCA-3'
Vegf	5'-GGAGATCCTTCGAGGAGCACTT-3'	5'-GGCGATTAGCAGCAGATATAAGAA-3'
Gcsf	5'-CCAGAGGCGCATGAAGCTAAT-3'	5'-CG GCCTCTCGTCTGACCAT-3'
Bv8	5'-GCATGACAGGAGTCATCATTTT-3'	5'-AAATGGCAGGATATCAGG-AAA-3'
S100a8	5'-TGCGATGGTGATAAAA GTGG-3'	5'-GGCCAGAAGCTCTGCTACTC-3'
Stat3	5'-TGCCCATGGCT ACCTGTT-3'	5'-GAACCTCTGGGCTTAGTCC-3'
Rps9	5'-TTGACGCTAGACGAGAAGGAT-3'	5'-AATCCAGCTTCATCTTGCCCT-3'
Human		
CEBPA	5'-CGGTGGACAAGAAGCAGCAAC-3'	5'-CGG AATCTCTAGTCTGGC-3'
CEBPB	5'-CACAGCGACGACTGCAAGATCC-3'	5'-CTTGAACAAGTTCCGAGG-GTG-3'
NAMPT	5'-GCAGAAGCCGAG TTCAACATC-3'	5'-TGCTTGTGTTGGGTGGATATTG-3'
SIRT1	5'-TGC TGGCCTAATAGAGTGGCA-3'	5'-CTCAGCGCCATGGAAAATGT-3'
STAT3	5'-CCCCGCACTTTAGATTAT-3'	5'-GGTAGCGCCTCAGTCG TAT-3'
BACTIN	5'-AGCGGGAAATCGTGCCTG-3'	5'-GGGTACATGGTGGTGCCG-3'

Table 2. Clinicopathological characteristics of tumor patients enrolled in this study

(A) Melanoma patients				
	Healthy	Melanoma	Melanoma + IFN-α	p
	N = 5	N = 6	N = 8	
Age, years	49 (48–58)	43.4 (31–60)	45.6 (39–63)	ns
Gender, female	5 (100%)	3 (50%)	5 (62.5%)	ns
Stage (AJCC 2009)	–	Stage II: 4 Stage III: 2	Stage II: 4 Stage III: 4	
IFN- α 2 treatment duration, months –	–	–	7.7 (4–11)	
(B) HNC patients				
Total number of HNC patient N = 38				
Age, years	63.5 (interquartile range 58–68);			
Gender, female	n = 6, 15.8%;			
T stage	T1 (n = 7), T2 (n = 15), T3 (n = 8), T4 (n = 8);			
N stage	N0 (n = 12), N1 (n = 8), N2 (n = 18);			
M stage	M0 (n = 37), M1 (n = 1)			
Treatment	Samples were received during operative dissection, no previous radiotherapy or chemotherapy was used			

Extracellular NAMPT measurement

eNAMPT level in heparinized plasma was measured by using the Visfatin-Enzyme-Immunoassay (EIA) Kit (Sigma-Aldrich/Merck, Darmstadt, Germany) according to the manufacturer's protocol.

Immunofluorescent staining and fluorescent microscopy

Tumors were dissected and snap frozen in liquid nitrogen. About 7- μ m cryosections were fixed, stained with appropriate antibodies, dried and mounted with Neo-Mount.

Antibodies: Anti-mouse CD16/32 (clone 2.4G2, BD Pharmingen, BD, New Jersey, USA), anti-CD11b (clone M1/70, eBioscience, Affymetrix, California, USA), anti-Ly6G (clone 1A8, eBioscience, Affymetrix, California, USA), anti-SMA (clone 1A4, Sigma-Aldrich/MERCK, Darmstadt, Germany), anti-laminin (clone AP1001.1, Immunodiagnostik AG, Bensheim, Germany). DAPI staining of nucleus was used.

Microscopy was performed using Zeiss AxioObserver.Z1 Inverted Microscope with ApoTome Optical Sectioning equipped with filters for: DAPI, FITC, Alexa Fluor 488, GFP, DsRed, Cy3. Images were processed with ZEN Blue 2012 software and analyzed with ImageJ.

Quantification of vascularization (the number of Laminin⁺ and/or SMA⁺ vessels, the area covered by these vessels) and neutrophil infiltration in tumor was performed in 10 fields of view and calculated per tumor.

Isolation of human neutrophils

Peripheral blood was drawn from melanoma patients and healthy controls, after written local ethics committee approval. All patients had no evidence of disease at the time of phlebotomy. Patients from the adjuvant IFN-treatment group received recombinant IFN- α 2 (3 million IU) 3X/week. In all patients of the IFN- α 2

group blood was drawn during an ongoing adjuvant IFN treatment. Clinicopathological characteristics of healthy controls and melanoma patients enrolled in this study are listed in Table 2(A).

Blood and tumor tissue samples from head-and-neck carcinoma (HNC, squamous cell carcinoma) patients were obtained during the operation after written local ethics committee approval; no previous chemotherapy or radiotherapy was performed. Clinicopathological characteristics of HNC patients enrolled in this study are listed in Table 2(B).

Peripheral blood was drawn into 3.8% sodium citrate anticoagulant monovettes, and separated by density gradient centrifugation (Biocoll density 1,077 g/mL, Biochrom/Merck, Darmstadt, Germany). The mononuclear cell fraction was discarded and neutrophils (purity >95%) were isolated by sedimentation over 1% polyvinyl alcohol, followed by hypotonic lysis (0.2% NaCl) of erythrocytes. In view of the emerging diversity of circulating neutrophil subtypes in humans it has to be noted that high-density neutrophils have been investigated in this study.

Flow cytometry analysis

Percentage of neutrophils in the whole blood was assessed after erythrocyte lysis (BD PharmLyse, BD Biosciences, BD, New Jersey, USA) based on morphological gating, and CD16 expression. Single cell suspension of HNC tissue samples was prepared as described above; TANs were determined as viable CD66b⁺ cells (Supporting Information Fig. S2).

G-CSF and NAMPT measurements

G-CSF and eNAMPT content in plasma or tumor supernatants was analyzed with ELISA (R&D Systems, Minnesota, USA) or Visfatin-Enzyme-Immunoassay (EIA) Kit, according to the manufacturer's protocols.

Immunohistochemistry

Paraffin sections were stained with primary (rabbit-anti-NAMPT, mouse-anti-CD66b) and secondary antibodies (goat-anti-rabbit-PO, goat-anti-mouse-AP), followed by incubation with substrates (AEC Single Solution, Invitrogen and HighDef green, Enzo Lifesciences). For the nuclei visualization a short hematoxylin staining was performed.

Light microscopy

Microscopy was performed using Olympus BX51 upright epifluorescence microscope. Images were processed with CellSens Dimension software (Olympus).

Statistics

Statistical analyses were performed using Kruskal–Wallis ANOVA for multiple comparison and Mann–Whitney *U*-test for two independent samples; correlations were estimated with Spearman rank test. $p < 0.05$ was considered significant.

Study approval

Animal studies have been approved by the regulatory authorities: Regierungspräsidium Tuebingen and LANUV.

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

Type I IFN deficiency is associated with up-regulation of Nampt signaling pathway

TANs from IFN-deficient mice represent a model of a distinct neutrophil protumor phenotype.⁵ These cells accumulate in tumors and support tumor angiogenesis and growth. In agreement, their numbers correlate positively with tumor size (Supporting Information Fig. S3). Yet, the molecular mechanism behind tumorigenic activation of such neutrophils is not clear. Since we observe up-regulation of G-CSF in IFN-deficient animals, we set up to analyze the regulation of G-CSF receptor downstream signaling pathway in such TANs. While blood neutrophils represent a mixed population of differentially activated cells and there are only minor differences in genes expression, the results clearly demonstrate significant up-regulation of *Nampt*, *Sirt1*, *Cebpa* and *Cebpb* genes in IFN-deficient protumor TANs, as compared to WT (Fig. 1a). The expression of *Nampt*, *Sirt1*, *Cebpa* and *Cebpb* was significantly elevated, and enzymatic activity of Sirt1 was higher in these TANs (Fig. 1b). We also observed elevated eNampt in serum of IFN-deficient mice (Fig. 1c). Interestingly, rmlfmb

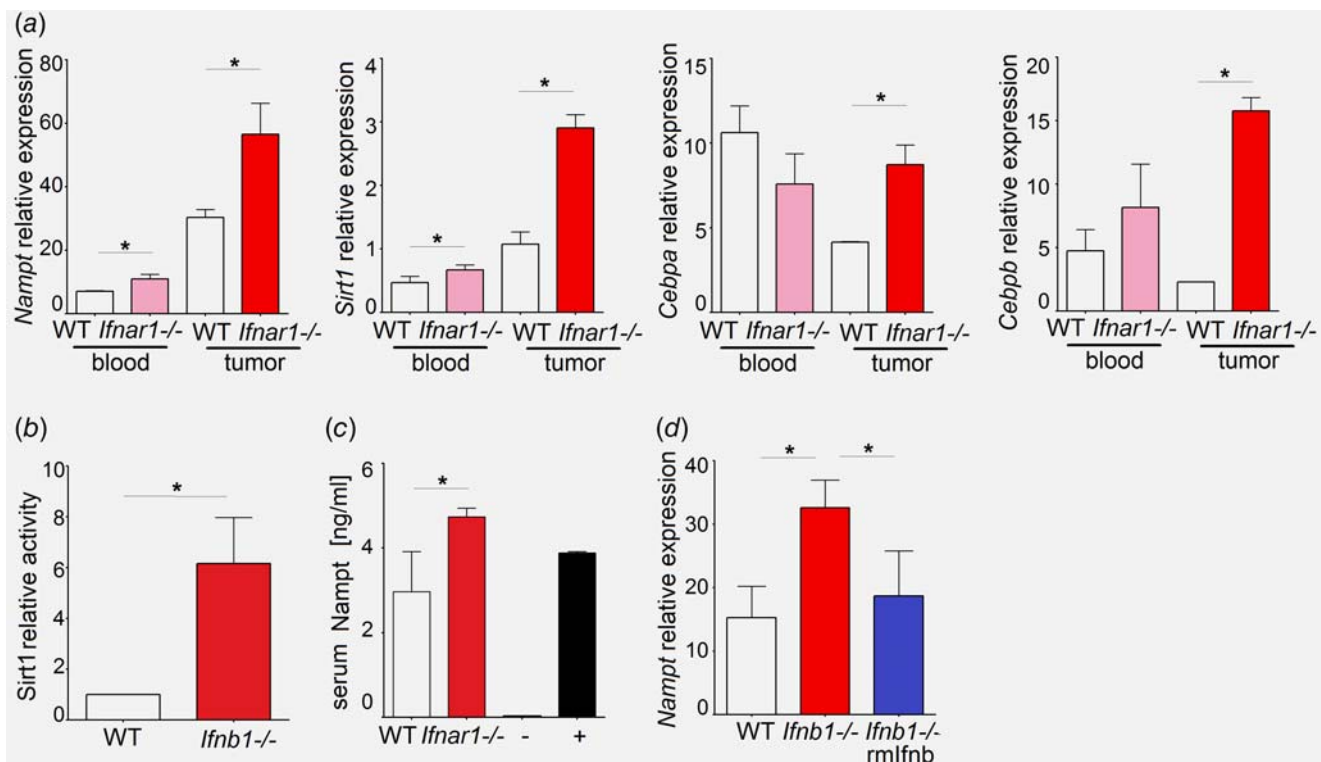
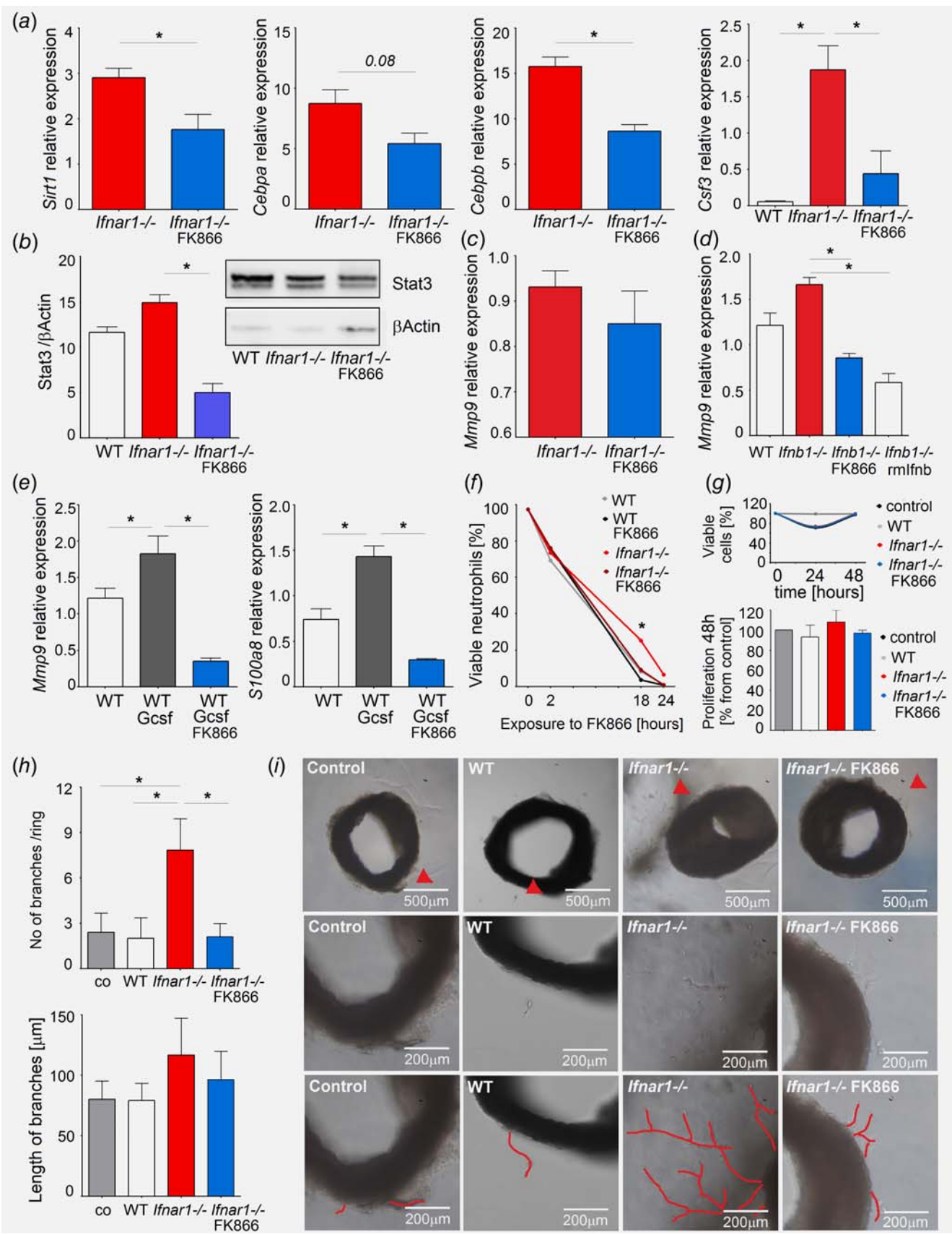


Figure 1. Nampt pathway is elevated in protumor TANs in mice. (a) Elevated intracellular Nampt pathway (*Nampt*, *Sirt1*, *Cebpa*, *Cebpb*) in protumor neutrophils from IFN-deficient mice. (b) Elevated Sirt1 activity in IFN-deficient TANs (SIRT1 Activity Assay Kit). (c) Elevated levels of eNampt in serum of IFN-deficient mice, negative control (-), positive control (+). (d) rmlfmb treatment down-regulates *Nampt* in *Ifnb1*^{-/-} TANs. Gene expression was assessed using qPCR. At least two independent experiments were performed, with five mice per group. Gene expression was assessed using qPCR and tested in triplicate, data are shown as mean \pm SEM. * $p < 0.05$.



treatment of *Ifnb1*^{-/-} neutrophils significantly down-regulated *Nampt* expression in these cells (Fig. 1d), being in agreement with our hypothesis concerning Nampt as an activator of protumor activity of TANs. Interestingly, IFN-deficient TANs show up-regulation of *Csf3*, suggesting the positive feedback loop (Fig. 2a). Hence, protumor shift of TANs correlates with elevated NAMPT signaling in these cells.

Inhibition of NAMPT signaling decreases proangiogenic properties of TANs

NAMPT, depending on its form can stimulate two different signaling pathways in neutrophils. Here, we could show that inhibition of Nampt using small molecular inhibitor FK866 significantly affects both these pathways. Intracellular Sirt1-mediated pathway was down-regulated, together with its target genes: *Cebpa* and *Cebpb* (Fig. 2a). Furthermore, Stat3, which is the key component of the extracellular pathway, was also suppressed (Fig. 2b). Interestingly, FK866 treatment diminished also *Csf3* expression in such TANs, possibly due to disturbed positive feedback loop (Fig. 2a).

To evaluate a direct effect of NAMPT inhibition on the tumorigenic capacity of neutrophils, we isolated protumor IFN-deficient (*Ifnar1*^{-/-} or *Ifnb1*^{-/-}) TANs and treated them with FK866. The expression of *Mmp9*, which is known to be one of the most potent proangiogenic molecules regulated during tumor growth, was down-regulated significantly in *Ifnb1*^{-/-} neutrophils and showed the down-regulation trend in *Ifnar1*^{-/-} neutrophils (Figs. 2c and d). Moreover, in agreement with our hypothesis that IFNs act as natural NAMPT inhibitors, we could observe that rmIfnb treatment similarly down-regulates *Mmp9* in IFN-deficient TANs (Fig. 2d). Direct activation of NAMPT in TANs, using G-CSF, up-regulated gene expression of their proangiogenic factors *i.e.* *Mmp9* and *S100A8* (Fig. 2e). Viability of TANs which is regulated by G-CSF was also decreased by long-term (18 hr) NAMPT inhibition, while short-term treatment did not have a significant effect (Fig. 2f).

To assess functionality of protumor TANs after Nampt inhibition, we evaluated two important features of these cells—their inability to kill tumor cells and their angiogenic activity. While tumor cell cytotoxicity was not significantly altered by Nampt inhibition (Fig. 2g), the proangiogenic functionality of IFN-deficient TANs was impaired, as evaluated using the aortic ring assay, reflecting all of the key steps in

angiogenesis (matrix degradation, migration, proliferation, reorganization). *Ifnar1*^{-/-} neutrophils showed the increased pro-angiogenic capacity—stimulating the formation of new endothelial branches. Inhibition of NAMPT in such neutrophils decreased this process to the control level. The similar trend is observed for the length of branches (Figs. 2h and i). Hence, Nampt activity seems to be crucial for the angiogenic activity of TANs.

Systemic inhibition of NAMPT impairs tumor angiogenesis and growth

We observed that elevated Nampt expression is characteristic for protumor neutrophils and Nampt suppression impairs their angiogenic functionality. Since angiogenesis is crucial for efficient tumor growth, we were interested whether Nampt inhibition will affect tumor angiogenesis and growth. Therefore, we treated B16F10 tumor-bearing *Ifnar1*^{-/-} mice with FK866 and monitored tumor growth. As a control we used WT animals that have intrinsically low Nampt expression in TANs. Importantly, FK866-treated IFN-deficient mice showed significantly reduced tumor growth, compared to untreated animals (Fig. 3a). This was associated with reduced neutrophil infiltration into tumors (Figs. 3b–3d). In agreement with *in vitro* data, neutrophils isolated from tumors of FK866-treated mice showed impaired angiogenic phenotype with down-regulated *Vegf*, *Mmp9*, *S100A8* and *Bv8* (Fig. 3e). IFN-deficient mice show distinct functional tumor vasculature with mature vessel phenotype, characterized by strong smooth muscle actin (SMA) staining (Figs. 3f and g).²⁰ In line with suggested role of Nampt in promoting neutrophil angiogenic switch, inhibition of Nampt impaired development of functional tumor vasculature. SMA staining in tumors, as well as total area covered by vessels, was decreased upon FK866 treatment (Figs. 3f and g), compared to untreated animals.

Systemic inhibition of NAMPT is associated with antitumor polarization of TANs

Systemic application of FK866 into tumor-bearing IFN-deficient mice resulted in the regulation of factors responsible for tumorigenic activities of TANs, leading to their antitumor polarization (Supporting Information Fig. S4). CD11b expression was restored, and the percentage of cells expressing MHCII, ICAM-1 and TNF-α was significantly elevated

Figure 2. Inhibition of Nampt reduces neutrophil proangiogenic potential. (a) Nampt inhibitor (FK866) treatment suppresses Nampt downstream pathway (*Sirt1*, *Cebpa*, *Cebpb*) and *Gcsf* in TANs. TANs were sorted and expression of genes measured in triplicates. (b) Stat3 expression in *Ifnar1*^{-/-} TANs is diminished after Nampt suppression. TANs were sorted and Stat3 content estimated with Western blot. (c,d) *In vitro* treatment of TANs with FK866 (c) and rmIfnb (d) decreases *Mmp9* expression. (E) *In vitro* treatment of neutrophils with G-CSF increases expression of proangiogenic genes (*Mmp9*, *S100A8*, *Bv8*). (f) *In vitro* short-term (2 hr) treatment of TANs with FK866 does not influence their viability, while prolonged treatment (18 hr) decreases the survival of *Ifnar1*^{-/-} neutrophils to WT levels. (g) *In vitro* short-term (2 hr) treatment of TANs with FK866 does not significantly influence their killing capacity. (h,i) *In vitro* treatment of TANs with FK866 decreases their proangiogenic capacity in aortic ring assay. The number and length of endothelial branches were higher in the presence of *Ifnar1*^{-/-} neutrophils, inhibition of Nampt in neutrophils leads to reduced branch formation. Gene expression was measured in triplicate; data are shown as mean ± SEM, **p* < 0.05.

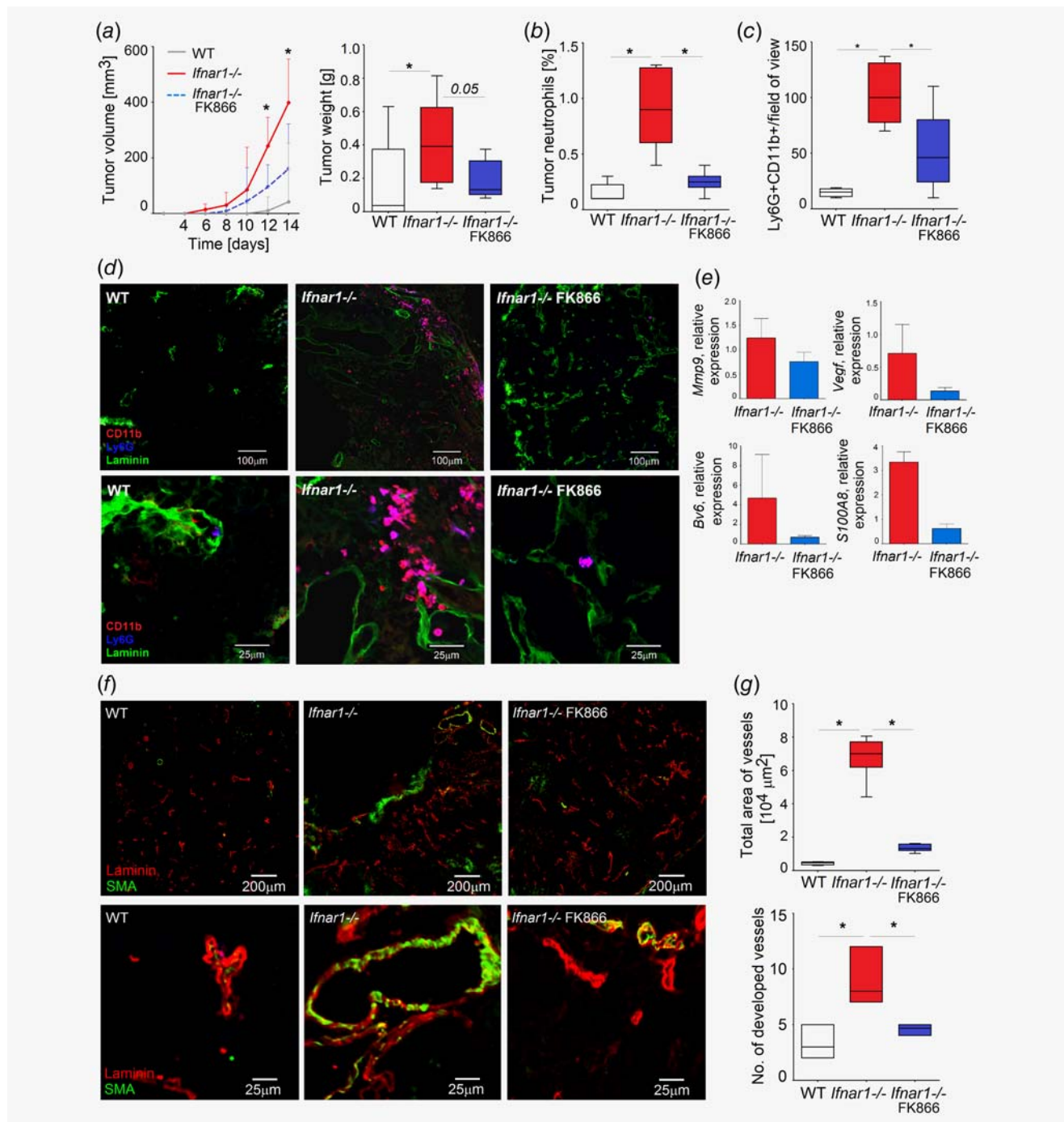


Figure 3. FK866 treatment suppresses neutrophil-mediated tumor angiogenesis. (a) B16F10 melanoma tumor growth. Cells were s.c. injected into the flank of mice, and one group of *Ifnar1*^{-/-} mice was treated *i.p.* with FK866. (b,c) Quantification of neutrophil infiltration in tumor single cell suspension (b) and sections, 10 fields of view were analyzed (c). (d) Exemplified TANS staining of tumor, CD11b (red), Ly6G (blue), laminin (green). Scale bars: 100, 25 μm. (e) Expression of proangiogenic factors in TANS. Gene expression was measured in triplicate, data are shown as mean ± SEM. (f) Representative image of decreased tumor vascularization after NAMPT inhibition. 7 μm cryosections were stained for laminin (endothelial cell marker, red) and SMA (smooth-muscle actin, green). Scale bars: 200 and 25 μm. (g) Quantification of vascularization of tumors (total area of vessels and number of developed vessels) presented in (f), calculated in 10 fields of view. Two independent experiments were performed, with at least five mice per group. For comparison of multiple and two independent groups Kruskal–Wallis test and Mann–Whitney *U* test were used, respectively. Data are shown as median, interquartile range, minimal and maximal values, *p < 0.05.

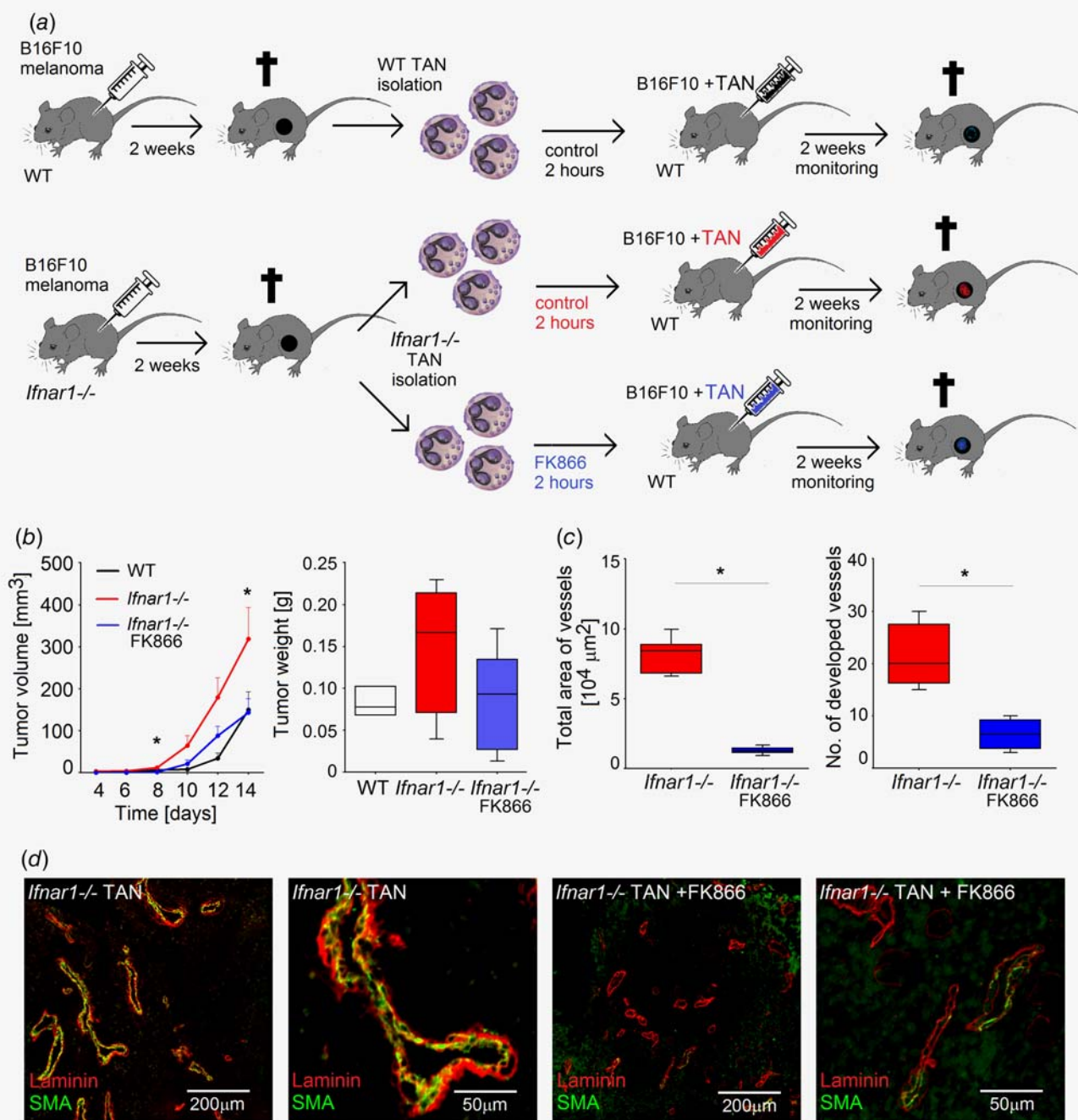


Figure 4. Adoptive transfer of FK866-treated neutrophils suppresses tumor vascularization and growth in mice. (a) The scheme of the experiment. Blood and tumor neutrophils were sorted from B16F10 bearing WT (antitumor) and *Ifnar1*^{-/-} mice (protumor), cultivated with FK866 (blue) or medium (black, WT; red, *Ifnar1*^{-/-}), washed, mixed with B16F10 melanoma cells (1:10) and s.c. injected into the flank of WT mice. At Day 3 tumor-bearing mice received FK866-treated or medium-treated neutrophils (*i.v.*), respectively. (b) Attenuation of tumor growth by FK866-treated neutrophils. (c) Quantification of vascularization of tumors after adoptive transfer of TANs. Vessels were stained as shown in (d) and their number/ area calculated in 10 fields of view. (d) Exemplified staining of vessels in tumor. Laminin (red), SMA (green). Scale bars: 200 μm, 50 μm. Experiments were repeated at least twice, with five animals per group. For comparison of two independent groups Mann–Whitney *U* test was used. Data are shown as median, interquartile range, minimal and maximal values, **p* < 0.05, # *p* = 0.05.

(Supporting Information Fig. S4A). *Cxcr2* expression was reduced on such TANs, which can explain their impaired tumor infiltration (Supporting Information Fig. S4B). Caspase

3 expression was elevated in TANs upon FK866 treatment (Supporting Information Fig. S4C), and their life-span after long exposure (18–24 hr) to FK866 was reduced (Fig. 2f). Of

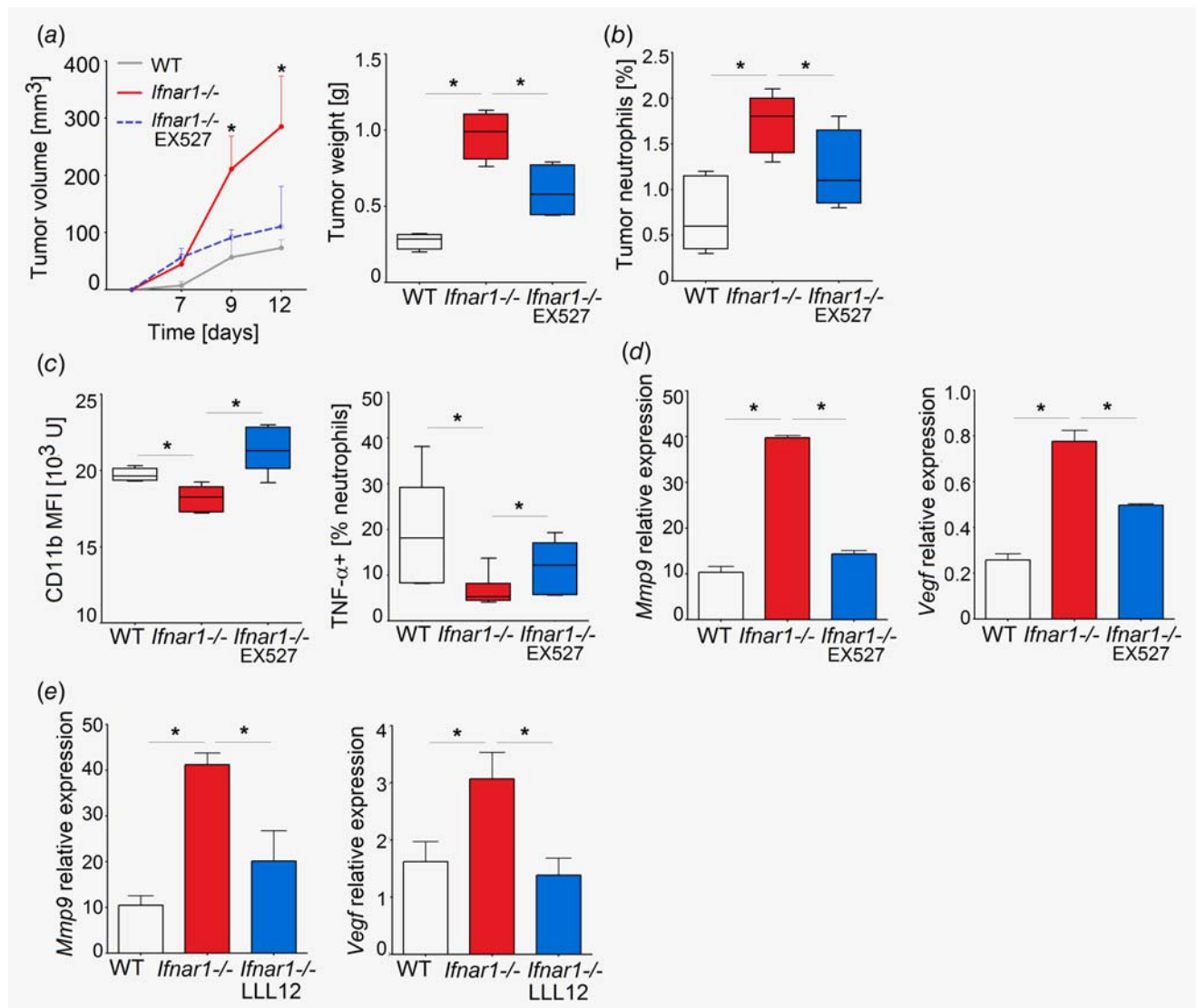


Figure 5. Inhibition of Nampt downstream signaling components resulted in decrease of protumor properties of TANs. (a) Tumors growth of B16F10. Cells were s.c. injected into the flank of mice, and one group of *Ifnar1*^{-/-} mice was treated *i.p.* with EX527. (b) Quantification of neutrophil infiltration in tumor single cell suspension. (c) Antitumor polarization of TANs after EX527 treatment. The expression of CD11b and TNF-α on TANs were assessed by flow cytometry (c). (d) Suppression of proangiogenic potential of TAN by Sirt1 inhibition. (e) Suppression of proangiogenic potential of TAN by Stat3 inhibition. Experiments were repeated twice, with at least five animals per group. For comparison of multiple and two independent groups Kruskal–Wallis test and Mann–Whitney *U* test were used, respectively. Data are shown as median, interquartile range, minimal and maximal values. Gene expression was measured in triplicate, data are shown as mean ± SEM. **p* < 0.05.

note, short-time exposure that we used for our *in vitro* experiments (2 hr) had no significant influence on neutrophil viability (Fig. 2i). Altogether, inhibition of NAMPT led not only to neutrophil antiangiogenic phenotype, but also to significant antitumor bias of these cells.

Adoptive transfer of FK866-treated antitumor TANs suppresses tumor angiogenesis and growth in mice

To validate therapeutic antitumor potential of FK866-treated neutrophils, we isolated blood and tumor neutrophils from WT and *Ifnar1*^{-/-} mice and incubated them with FK866 for 2 hr. Untreated neutrophils were used as a control. After

incubation neutrophils were washed, mixed with B16F10 cells and immediately injected s.c. into WT mice, as we published before.³ (Experimental schema Fig. 4a). At Day 3, these mice received similarly prepared neutrophils *i.v.* Tumor growth was monitored for 14 days; then tumors were removed and analyzed. In mice that received FK866-treated TANs, a significant attenuation of tumor growth (Fig. 4b) and vascularization (Figs. 4c and d) was observed. In contrast, mice injected with untreated *Ifnar1*^{-/-} neutrophils show accelerated growth in comparison to mice that received WT neutrophils. This compellingly demonstrates the potential of neutrophil-specific Nampt targeting in order to functionally activate antitumor

phenotype of these cells. This could be therapeutically utilized to suppress tumor development.

Inhibition of downstream effectors of Nampt pathway has a similar effect on TAN characteristics as direct Nampt inhibition

To see how the inhibition of Sirt1, a downstream effector of Nampt, influences neutrophil tumorigenicity, we treated B16F10 tumor-bearing *Ifnar1*^{-/-} mice with Sirt1 inhibitor EX527. Similar to FK866 treatment, we observed a strong suppression of tumor growth (Fig. 5a) in EX527-treated animals, compared to untreated mice. EX527-treated animals showed also diminished TAN numbers (Fig. 5b) and typical antitumor characteristics of these cells, with elevated CD11b and increased TNF- α production (Fig. 5c). A direct inhibition of Sirt1 in isolated *Ifnar1*^{-/-} TANs reduced significantly their angiogenic potential (Fig. 5d).

While Sirt1 regulates intracellular arm of Nampt signaling, Stat3 facilitates signaling downstream of putative Nampt receptor.²¹ This extracellular pathway appears also to be involved in protumor priming of neutrophils, since inhibition of Stat3 phosphorylation in isolated TANs, using LLL12, down-regulated their angiogenic molecules (Fig. 5e).

The antitumor effect of Nampt inhibition in neutrophils is not limited to B16F10 melanoma

To test the uniformity of the obtained data in other tumor models, we injected MCA fibrosarcoma cells s.c. into mice and treated one group of *Ifnar1*^{-/-} animals with FK866, as described above. Due to their low Nampt expression WT animals were used as a control. Comparably to B16F10 data, inhibition of Nampt significantly suppressed growth of MCA205 (Supporting Information Fig. S5A), reduced neutrophil infiltration into tumors (Supporting Information Figs. S5B–S5D) and impaired tumor angiogenesis (Supporting Information Figs. S5E and S5F). Moreover, TANs exhibited significant antitumor bias (Supporting Information Fig. S5G). Thus, FK866-mediated antitumor activation of neutrophils and resulting retardation of tumor growth appears to be a common phenomenon, not restricted to one tumor model.

NAMPT pathway is up-regulated in neutrophils isolated from melanoma and head-and-neck cancer patients

Prompted by the animal studies showing a robust up-regulation of NAMPT signaling in tumorigenic TANs, we assessed neutrophil characteristics and NAMPT regulation in TANs isolated from human specimen. In melanoma patients, we could observe elevated neutrophil counts in blood (M), compared to healthy controls (H). Interestingly, neutrophil numbers were significantly reduced after therapy with IFN- α 2 (M + IFNa) (Fig. 6a), while no other cells were affected (Supporting Information Fig. S2B). In agreement with mouse data, neutrophils from melanoma patients showed prominently up-regulated NAMPT and it was significantly down-regulated after IFN- α 2-treatment (Fig. 6b). Moreover, CD11b

expression on these cells was up-regulated upon therapy (Fig. 6c), suggesting neutrophil antitumor polarization. CEBPA gene expression was up-regulated, and NAMPT and SIRT1 expression showed a trend in the up-regulation. Missing significance in gene regulation is possibly due to the sample heterogeneity and sample limited number (Fig. 6d). Nevertheless, NAMPT downstream molecule STAT3 (Fig. 6e) was up-regulated on protein level. Histological staining of melanoma tumor sections confirmed also a high expression of NAMPT protein in TANs (Figs. 6f and g).

To provide broader insight into NAMPT regulation in human cancer, we analyzed the regulation of NAMPT signaling in neutrophils isolated from head-and-neck carcinoma (HNC) patients. Similarly to melanoma, we observed elevated neutrophil counts in blood and tumors of HNC patients, which correlated with tumor progression (Fig. 6j). Neutrophils from HNC individuals display elevated NAMPT and SIRT1 expression, compared to healthy donors (Fig. 6k). Immunohistochemistry analysis confirmed that HNC-associated tumor neutrophils express high levels of NAMPT (Figs. 6h and i).

These data show up-regulation of NAMPT signaling in blood and TANs of HNC patients. Moreover, IFN-mediated down-regulation of NAMPT pathway in human neutrophils could also been shown, correspondingly to the mouse system.

In sum, in this study we could demonstrate that NAMPT signaling is indispensable for tumorigenic activation of TANs in mice and human, and that inhibition of NAMPT leads to antitumor bias of such neutrophils that could be used therapeutically to suppress tumor growth.

Discussion

Here we demonstrate for the first time that NAMPT activity in TANs is crucial for the proangiogenic and tumorigenic activity of these cells. Inhibition of NAMPT reprograms neutrophils into antitumor effector cells that efficiently repress tumor growth. Elevated eNAMPT levels are found in many cancers,²² correlating with poor prognosis.²³ NAMPT, together with its downstream effector molecule SIRT1, orchestrates neutrophil differentiation *via* activation of CEBP α and CEBP β .¹² CEBP α and CEBP β regulate in turn expression of many myeloid-specific genes,²⁴ and are essential for steady-state or emergency granulopoiesis.^{25,26} Here, we observed a significant up-regulation of *Nampt* and *Sirt1* in protumor TANs from *Ifnar1*^{-/-} mice. This was accompanied by elevated *Cebpa* and *Cebpb* expression in these cells. This suggests the role tumor microenvironment in the transformation of mature neutrophils into “immature” protumor state in a manner that mimic processes triggered by developmental cues in hematopoietic progenitors.

Inhibition of Nampt in TANs leads to their antitumor and antiangiogenic polarization, mature phenotype, reduced migratory capacities and elevated apoptosis. In agreement, treatment of *Ifnar1*^{-/-} mice with FK866 reduces infiltration of neutrophils into tumors and significantly decreases their angiogenic activity. We observed decreased expression of

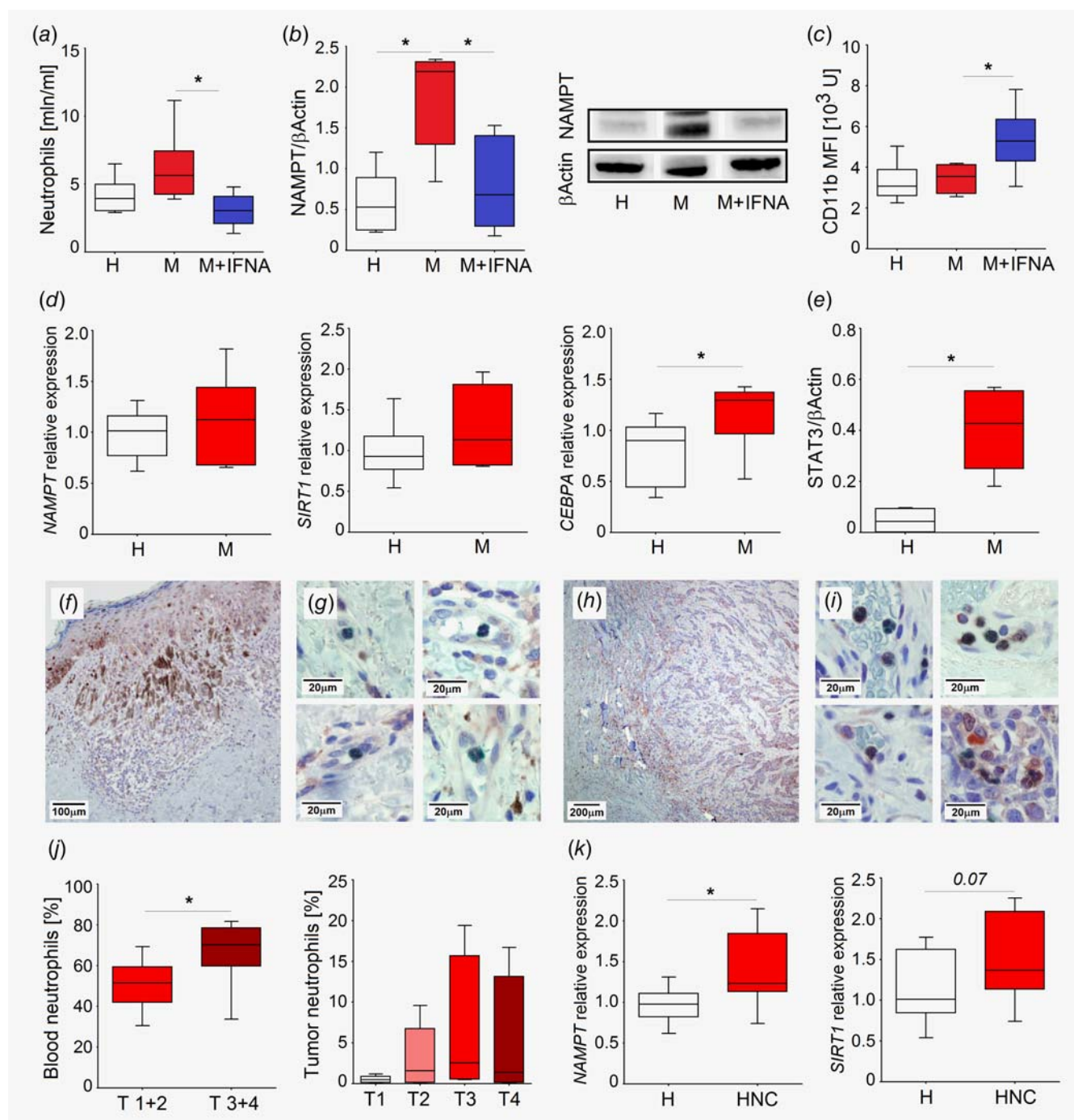


Figure 6. Elevated NAMPT signaling in neutrophils from melanoma and HNC patients A-B. IFN- α 2 therapy reduces neutrophil numbers (a) and their NAMPT expression (b) in melanoma patients. Healthy controls (H), melanoma patients (M), IFN- α 2 treated melanoma patients (M + IFN α). (c) Expression of CD11b on isolated high density neutrophils was assessed using flow cytometry. (d,e) Gene expression of NAMPT and downstream signaling molecules in blood neutrophils. (f,g) Elevated NAMPT expression in TANs of T1 melanoma sample. Paraffin sections were stained for CD66b (green), NAMPT (red), nuclei (violet). Scale bars: 100 μ m, 20 μ m. (h,i) Elevated NAMPT expression in TANs of T2 HNC. Scale bars: 200 μ m and 20 μ m. (j) The percentage of blood and tumor neutrophils positively correlates with HNC stage. (k) Elevated NAMPT pathway in HNC neutrophils, compared to healthy individuals. Comparison of multiple independent groups was performed with Kruskal-Wallis test, two independent groups were compared using Mann-Whitney *U* test. Data are shown as median, interquartile range, minimal and maximal values, **p* < 0.05.

Mmp9 by TANs isolated from such animals. It may be responsible for their diminished capacity to degrade extracellular matrix, leading to reduced tumor angiogenesis. Indeed, functional analysis (aortic ring assay) has shown that neutrophils are capable to stimulate all steps of angiogenesis, and that this activity depends on Nampt signaling. As a consequence of Nampt inhibition, tumor vascularization and growth are suppressed.

Other mechanisms of antitumor activity of FK866-treated neutrophils include stimulation of adaptive immune responses, as TANs from FK866-treated animals show elevated ICAM1, CD11b and MHCII expression. Apart from activating T cells *via* antigen presentation, neutrophils could also bolster T cell proliferation in a positive-feedback loop *via* up-regulation of *Icam1*. In addition, ICAM1 supports neutrophil interactions with T cells,²⁷ and boosts generation of reactive oxygen species (ROS).^{28,29} In addition, neutrophils produce cytokines stimulating T cell differentiation and activation *e.g.* TNF- α . We could show in this study that FK866 treatment up-regulates TNF- α expression in neutrophils, hence improving activation of adaptive immunity. This mechanism of neutrophil antitumor activity needs further evaluation.

FK866 treatment inhibits enzymatic and cytokine activities of Nampt, resulting in down-regulation of both Sirt1 and Stat3. Stat3 is considered an oncogene as *Stat3*^{-/-} mice develop smaller tumors³⁰ and inhibition of Stat3 phosphorylation reduces tumor growth in mice.³¹ Stat3 supports expression of antiapoptotic, proangiogenic and prometastatic factors in cancer cells.³² Our data suggest that in neutrophils this molecule has similar functions.

We observe that tumor-infiltrating neutrophils produce G-CSF. Since TAN numbers correlate with tumor size, their elevated numbers would lead to higher expression of G-CSF in the tumor and up-regulation of Nampt signaling. This phenomenon could be responsible for the effect described recently by Mishalian *et al.*,³³ showing that the size of tumor positively correlates with tumorigenicity of neutrophils. G-CSF is known to support the release of neutrophils from the bone marrow and to prolong their lifespan in tissues.^{34,35} For that reason cancer

patients are treated with G-CSF in order to accelerate recovery of neutrophils from chemotherapy-induced myelosuppression. However, the clinical effect of such treatment is controversial. Numerous studies demonstrate G-CSF-mediated stimulation of angiogenesis and cancer progression.^{36–38} G-CSF-stimulated neutrophils were shown to support metastasis formation in breast and lung tumor models.^{39–41} All these studies raise a central question with respect to the use of G-CSF as a supportive drug in neutropenic cancer patients.

We could previously show that depletion of neutrophils or inhibition of their migration results in significant abrogation of tumorigenesis in mice. These strategies in patients are not feasible or not yet successful.⁴² Our studies suggest an alternative therapeutic approach: using *ex vivo* activated antitumor neutrophils to suppress tumor growth. Here, we could validate that NAMPT targeting in neutrophils supports their antitumor features and impairs their proangiogenic activity. Such neutrophils, when adoptively transferred into tumor-bearing mice efficiently suppress tumor vascularization and growth in these animals. It is tempting to speculate that such approach in human neutrophils could provide an effective therapeutic alternative for cancer patients.

All in all, with this study we provide a mechanistic insight into the polarization of neutrophils that involves regulation of NAMPT signaling. NAMPT can be targeted in neutrophils, resulting in antitumor activation of these cells and tumor growth retardation. This has important clinical implications as TANs represent a highly potent therapeutic target. As clinical trials targeting neutrophils are not successful thus far, therapeutic strategies aimed at repolarizing neutrophils, or interfering with their tumorigenic activity, offer alternative opportunities for intervention.

Acknowledgements

This work was kindly backed by the COST Action BM1404 Mye-EUNITER (www.mye-euniter.eu). COST is supported by the EU Framework Program Horizon 2020. Authors thank the physicians within the Otorhinolaryngology and Dermatology Departments at University Hospital Essen for providing subject material. They also thank V. Heinrich for an excellent technician support.

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Video Article

Transfer of Manipulated Tumor-associated Neutrophils into Tumor-Bearing Mice to Study their Angiogenic Potential In Vivo

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URL: <https://www.jove.com/video/59807>

DOI: [doi:10.3791/59807](https://doi.org/10.3791/59807)

Keywords: Cancer Research, Issue 149, Tumor-associated neutrophils, neutrophil polarization, angiogenesis, neutrophil transfer, tumor growth, Nicotinamide Phosphoribosyltransferase (NAMPT) inhibitor

Date Published: 7/20/2019

Citation: Pylaeva, E., Spyra, I., Bordbari, S., Lang, S., Jablonska, J. Transfer of Manipulated Tumor-associated Neutrophils into Tumor-Bearing Mice to Study their Angiogenic Potential In Vivo. *J. Vis. Exp.* (149), e59807, doi:10.3791/59807 (2019).

Abstract

The contribution of neutrophils to the regulation of tumorigenesis is getting increased attention. These cells are heterogeneous, and depending on the tumor milieu can possess pro- or anti-tumor capacity. One of the important cytokines regulating neutrophil functions in a tumor context are type I interferons. In the presence of interferons, neutrophils gain anti-tumor properties, including cytotoxicity or stimulation of the immune system. Conversely, the absence of an interferon signaling results in prominent pro-tumor activity, characterized with strong stimulation of tumor angiogenesis. Recently, we could demonstrate that pro-angiogenic properties of neutrophils depend on the activation of nicotinamide phosphoribosyltransferase (NAMPT) signaling pathway in these cells. Inhibition of this pathway in tumor-associated neutrophils leads to their potent anti-angiogenic phenotype. Here, we demonstrate our newly established model allowing in vivo evaluation of tumorigenic potential of manipulated tumor-associated neutrophils (TANs). Shortly, pro-angiogenic tumor-associated neutrophils can be isolated from tumor-bearing interferon-deficient mice and repolarized into anti-angiogenic phenotype by blocking of NAMPT signaling. The angiogenic activity of these cells can be subsequently evaluated using an aortic ring assay. Anti-angiogenic TANs can be transferred into tumor-bearing wild type recipients and tumor growth should be monitored for 14 days. At day 14 mice are sacrificed, tumors removed and cut with their vascularization assessed. Overall, our protocol provides a novel tool to in vivo evaluate angiogenic capacity of primary cells, such as tumor-associated neutrophils, without a need to use artificial neutrophil cell line models. *vc*

Video Link

The video component of this article can be found at <https://www.jove.com/video/59807/>

Introduction

Type I Interferons (IFNs) play an important role in the stimulation of host responses to neoplasias, as the lack of type I IFN signaling results in significantly elevated tumor growth¹. One of the mechanisms involved in this process is the regulation of tumorigenic activity of tumor-associated neutrophils, which is controlled by colony-stimulating factor 3 receptor (CSF3R) downstream signaling². Colony-stimulating factor 3 (CSF3), or granulocyte colony-stimulating factor, was shown to activate signaling involving nicotinamide phosphoribosyltransferase (NAMPT)^{3,4}. NAMPT is a rate-limiting enzyme for nicotinamide adenine dinucleotide synthesis, which enhances glycolysis and regulates DNA repair, gene expression, and stress response promoting cancer cells survival and proliferation⁵. NAMPT is overexpressed in multiple cancer types, including colorectal, ovarian, breast, gastric, prostate cancer and gliomas⁶. NAMPT is essential not only for tumor cells, but also for a wide variety of other cell types that are present in tumors, such as myeloid cells - it drives their differentiation⁴, inhibits apoptosis and stimulate expression of multiple cytokines or matrix-degrading enzymes in macrophages⁷.

Tumor-associated neutrophils represent important modulators of tumor growth. TAN functions are strongly dependent on the type I IFN availability, as these cytokines prime anti-tumor activity of neutrophils. To the contrary, the absence of IFNs supports tumorigenic activation of these cells, especially their pro-angiogenic properties. In agreement with this, mice deficient in IFNs develop significantly larger and better vascularized tumors, which are strongly infiltrated with pro-tumoral/pro-angiogenic neutrophils^{1,2,8,9,10}. Importantly, such pro-angiogenic TANs show elevated activity of NAMPT, suggesting its essential role in pro-tumor polarization of neutrophils.

Depletion of neutrophils using Ly6G antibody or inhibition of their migration (CXCR2 antibody) results in decreased tumor angiogenesis, growth, and metastasis^{1,8}. Nevertheless, generated monoclonal antibodies are immunogenic, and their administration is associated with a range of life-threatening side effects¹¹. Treatment with small molecules, such as NAMPT inhibitor FK866, that modulate neutrophil tumorigenicity, could help to avoid such complications. Unfortunately, pharmacological systemic inhibition of NAMPT, next to its therapeutic effect on tumor growth, leads to severe side effects including gastrointestinal toxicity and thrombocytopenia. Therefore, the systemic application of NAMPT inhibitors is not feasible^{12,13,14}.

For this reason, we suggest here a protocol where NAMPT activity is blocked directly in isolated TANs. Such anti-tumor neutrophils are then adoptively transferred into a tumor-bearing host. This protocol will help avoid systemic toxic side-effects of the compounds, while its effect on the target cells will be sustained.

Protocol

All the procedures including animal subjects have been approved by the regulatory authorities: LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz NRW) and Regierungspräsidium Tübingen, Germany. All manipulations should be performed in sterile conditions (under laminar flow hood) using sterile reagents and instruments (syringes, scissors, forceps, disposable scalpels, Petri dishes).

NOTE: The overall scheme of the protocol is shown in the **Figure 1**.

1. Preparation of B16F10 melanoma cell line

1. Prepare mycoplasma-negative cells grown to a 90% confluent monolayer (approximately 10×10^6 cells/T75 flask) in complete Iscove's Modified Dulbecco's Medium (IMDMc: IMDM + 10% Fetal Bovine Serum (FBS) + 1% penicillin-streptomycin).
2. Remove the medium, and rinse the cells with phosphate buffered saline (PBS). Apply 6 mL of a cell detachment solution containing proteolytic and collagenolytic enzymes (see the **Table of Materials**), and incubate at 37 °C for 2 min.
3. Knock the flask gently to mobilize remaining adherent cells from the bottom. Collect the cell suspension in 15 mL tubes and centrifuge at 300 x g for 7 min and 20 °C.
4. Remove the supernatant, and resuspend the pellet well in 1 mL of PBS. Add 14 mL of PBS and centrifuge (300 x g and 20 °C for 7 min).
5. Remove the supernatant and resuspend the pellet in 1 mL of PBS.
6. Count the cells, and resuspend them to the concentration of 3×10^6 /mL PBS (for the step 2) or 6×10^6 /mL PBS (for the step 6) for injection. Keep cells on ice for a maximum of 30 min.

2. Allogenic tumor model in mice

1. Use 10 female *Ifnar1^{-/-}* mice 8-12 weeks old that are kept under specific-pathogen-free (SPF) conditions.
NOTE: Female mice are preferable in a subcutaneous model of tumor growth, since males are more aggressive and thus prone to infractions of the tumor site, which influences tumor growth.
2. Shave the skin of the mouse on the flank with an electrical shaver, and disinfect the skin with tissue wet with 70% ethanol.
3. Collect the prepared B16F10 melanoma cells at a concentration of 3×10^6 /mL PBS (see the step 1) in a 1 mL syringe and 0.4 x 19 mm needle. Inject 100 µL of the suspension subcutaneously.
 1. Mix the cells well before every injection. Use needles not less than 0.4 mm in diameter as to not disturb tumor cells.
4. Place up to 5 mice in one cage, and control tumor size (length, width and depth) with a caliper for 14 days.
NOTE: According to the animal regulations, the tumor size should not exceed 15 mm in diameter, mice with bigger or necrotic/open tumors should be sacrificed beforehand.
5. At day 14, sacrifice the mice in the CO₂ chamber.
6. Disinfect the skin with 70% ethanol and remove tumors with scissors and forceps in a sterile Petri dish. Keep tumors in a 50 mL tube in complete Dulbecco's Modified Eagle Medium (DMEMc: DMEM + 10% FBS + 1% penicillin-streptomycin) on ice.

3. TAN isolation

1. Place tumors into sterile 6-well plates, 5 tumors per well. Cut tumors into 2-3 mm pieces with sterile scissors.
 1. Digest with 1 mL of dispase/collagenase D/DNase I solution (0.2 mg/0.2 mg/100 mg in 1 mL of DMEMc) per tumor. Incubate at 37 °C, 5% CO₂ in a humid incubator, and mix with a 10 mL syringe without a needle every 15 min 3 times.
2. To remove undigested fibers, mesh cells through 100 µm filters into 15 mL tubes (one well per filter per tube). Add PBS to 15 mL, centrifuge tubes at 460 x g, 4 °C for 5 min, and remove the supernatant.
3. Lyse erythrocytes with a lysis buffer (NH₄Cl 150 mM, KHCO₃ 10 mM, EDTA 0.1 mM, pH 7.3, 20 °C) by adding 1 mL into each tube. Mix well, and combine the solution from all tubes into one. Stop the reaction after 2 minutes with 11 mL of ice-cold (4 °C) DMEMc.
4. Centrifuge at 460 x g, 4 °C for 5 min, and remove the supernatant. Resuspend the pellet with 15 mL of cold PBS. Centrifuge at 460 x g, 4 °C for 5 min, and remove the supernatant.
5. Resuspend the pellet in 1 mL of PBS. Add 3 mL of Fc-block antibodies (CD16/CD32, stock 0.5 mg/mL), and incubate on ice for 15 min.
6. Add antibodies: 10 mL of Ly6G-PE (stock 0.2 mg/mL) and 10 mL of CD11b-APC (stock 0.2 mg/mL). Add 20 mL of 6-Diamidin-2-phenylindol viability dye (DAPI, stock 5 mg/mL) and incubate on ice in darkness for 30 min.
NOTE: Another combination of viability dyes and fluorescent conjugates of antibodies can be used.
7. Add PBS up to 15 mL, centrifuge at 460 x g, 4 °C for 5 min, and remove the supernatant.
8. Resuspend the pellet in DMEMc to the concentration approximately 10×10^6 /mL, and keep on ice.
9. Sort CD11b⁺ Ly6G^{hi} alive (DAPI-negative) neutrophils with a fluorescence-activated cell sorter (gating strategy see **Figure 2**).
NOTE: Keep the tube with cell suspension and the tube with DMEMc for sorted cells at 4 °C. Use the following optimal sorting settings: a 70 mm nozzle, a threshold rate of maximal 22,000 events/second and a flow rate of 1-3.
10. Check the purity of the sorted neutrophils using a cytometer for a recommended purity of >95%.
11. Centrifuge sorted neutrophils at 460 x g, 4 °C for 5 min, and remove the supernatant. Resuspend the sorted cells in DMEMc to the concentration of 1×10^6 /mL.
NOTE: The expected number of neutrophils in one 14-day B16F10 tumor (10 mm diameter) is approximately 3×10^4 cells.

4. NAMPT inhibition in TANs in vitro

1. Prepare FK866 (NAMPT inhibitor) stock in dimethylsulfoxide (DMSO) at a final concentration of 100 mM.
2. Seed sorted neutrophils (step 3.11) into 2 wells of a 96-well U-bottom plate (1.5×10^5 neutrophils/well). Add FK866 into the intervention well (final concentration of 100 nM), and an equal amount of DMEMc with DMSO into the control well. Incubate for 2 h at 37 °C, 5% CO₂ in a humid incubator.
3. Centrifuge at 460 x g, 4 °C for 5 min, and remove the supernatant. Resuspend in 200 μL of PBS in each well. Repeat 2 times.
4. Centrifuge at 460 x g, 4 °C for 5 min, and remove the supernatant. Resuspend in commercial endothelial cell growth medium (supplemented with 4 μL/mL endothelial cell growth supplement, 0.1 ng/mL recombinant human epidermal growth factor, 1 ng/mL recombinant human basic fibroblast growth factor, 90 μg/mL heparin and 1 μg/mL hydrocortisone) to a final concentration of 0.2×10^6 cells/mL (in 0.75 mL) (for step 5) or in PBS to the final concentration of 0.6×10^6 cells/mL (in 0.25 mL) (for step 6).

5. Estimation of angiogenic properties of TANs using the aortic ring assay

1. Dissect the thoracic aorta from a male C57BL/6J (WT) mouse. Clean and cut into 0.5 mm width rings. Place all rings in a well of a 24-well plate with 1 mL of supplemented endothelial cell growth medium. Incubate overnight at 37 °C, 5% CO₂ in a humid incubator.
NOTE: The use of young (younger than 8 weeks) male mice for aorta dissection is preferable, since they give a more robust angiogenic response¹⁵.
2. Fill the wells of the 96-well flat-bottom plate with 50 μL of solubilized basement membrane matrix, let the gel set for 30 min in a 37 °C, 5% CO₂ humid incubator to allow the matrix to polymerize. Prepare at least 3 wells per condition.
3. Embed the rings in solubilized basement membrane matrix by placing an aortic ring on the top of the solid matrix layer, 1 ring in the center of each well. Add another 50 μL of solubilized basement membrane matrix to cover each ring.
 1. Place the plate in a 37 °C, 5% CO₂ humid incubator for another 30 min to allow the polymerization of the second matrix layer.
4. Add 150 μL/well of supplemented endothelial cell growth medium and 2×10^4 *Ifnar1*^{-/-} TANs (control and FK866-treated) (step 4.4).
5. Incubate the plate for 14 days at 37 °C, 5% CO₂ in a humid incubator.
6. Image using a standard phase-contrast microscope and estimate the endothelial branching. Quantitative assessment of vessel morphometric and spatial parameters including branching index can be performed automatically using the image processing program designed for scientific images.
NOTE: Representative results are depicted in the **Figure 3**.

6. Adoptive transfer of treated neutrophils in the allogeneic tumor model

1. Prepare B16F10 melanoma cells (step 1.6) in PBS at a concentration of 6×10^6 cells/mL.
2. Prepare 2 types of neutrophils: FK866-treated neutrophils and control untreated neutrophils (step 4.4) in PBS at a concentration 6×10^5 cells/mL. Mix neutrophils with B16F10 melanoma cells (the final neutrophil to tumor cells ratio 1:10) to have 2 types of cell mixtures.
3. Take 10 female WT mice 8-12 weeks old, 5 in each group. Shave the skin on the flank with an electrical shaver, and disinfect with 70% ethanol.
4. Inject 100 μL of the cell suspension (step 6.2) subcutaneously with an insulin syringe and a 0.4 mm diameter needle, to both groups of mice. Place 1-5 mice from the same group in one cage.
5. At day 2, prepare 2 types of neutrophils: FK866-treated neutrophils and control untreated neutrophils (step 4.4) in PBS at a concentration 6×10^5 cells/mL. Inject 100 μL of cell suspension (step 6.4) i.v. into the tail vein with an insulin syringe and a 0.4 mm diameter needle, to both groups of mice. Place mice back to the cage.

7. Tumor growth measurement, histological examination

1. Monitor tumor growth every other day. Evaluate the tumor size with calipers and calculate the tumor volume with the formula $V = 4/3 \times \pi \times (h \times w^2)/8$ (h=height, w=width, depth= width).
2. Sacrifice mice at the day 14 in the CO₂ chamber. Remove tumors and measure the tumor weights.
3. Freeze tumors in optimum cutting temperature compound in liquid nitrogen, and store at -80 °C.
4. Thaw the samples to -20 °C and prepare 5 mm sections using a cryotome. Let the cryocuts dry for 30 min at 20 °C. Fix the sections in -20 °C cold acetone for 2 min and let them dry for 30 min at 20 °C.
5. Block with Fc-block antibodies (CD16/CD32, stock 0.5 mg/mL 1:500) in PBS for 1 h at 20 °C.
6. Stain with rabbit anti mouse Laminin gamma antibody (1:1500 in PBS, 200 μL) for 1 h at 20 °C. Wash with PBS three times.
7. Stain with secondary goat anti-rabbit antibody (stock 0.5 mg/mL, 1:400 in PBS.), anti-mouse αSMA (1:500 in PBS) and 2 μL of DAPI (stock 5 mg/mL, 1:100 in PBS) in a final volume of 200 μL of antibody solution. Incubate for 1 h at 20 °C in darkness. Wash with PBS three times.
8. Dry slides for 20 minutes at 20 °C in darkness. Mount with anhydrous mounting medium for microscopy and cover with a coverslip. Let it dry 1 h in 37 °C.
9. Perform microscopical examination. Quantify the vascularization by counting the total number (optionally area) of Laminin⁺ vessels and the number (area) of SMA⁺ developed vessels.
NOTE: To perform image analysis, take all images under the same conditions (light, contrast, magnification). In this case, processing parameters are fixed, and image processing becomes completely automatic. Representative results are depicted in the **Figure 4** and **Figure 5**.

Representative Results

Using the procedure described here, *lfnar1*^{-/-} neutrophils were isolated from tumors and treated with NAMPT inhibitor FK866 for 2 h. Untreated *lfnar1*^{-/-} neutrophils were used as a control. The effectivity of the treatment was evaluated using the aortic ring assay, which reflects the key steps involved in angiogenesis (matrix degradation, migration, proliferation, reorganization). We could demonstrate that FK866-treated neutrophils have a significantly decreased capacity to stimulate aortic branch formation, as compared to untreated cells (**Figure 3A, 3B**). FK866-treated anti-angiogenic neutrophils were injected subcutaneously into tumor-bearing mice (at day 0 flank and day 2 i.v.). We could observe significantly impaired tumor growth, as compared to mice injected with untreated *lfnar1*^{-/-} neutrophils (**Figure 4A, 4B**). Histological examination of the extracted tumors proved the significant suppression of angiogenesis in tumors isolated from mice treated with FK866-treated TANs, as compared to those injected with untreated *lfnar1*^{-/-} neutrophils (**Figure 5A,B**).

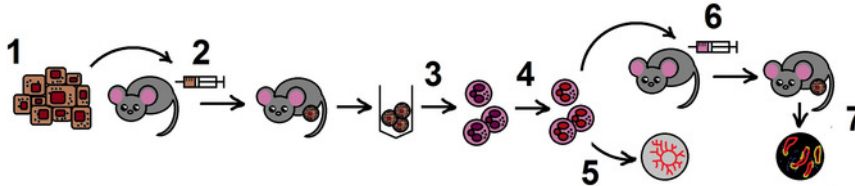


Figure 1. The scheme of the protocol. Step 1. Preparation of B16F10 melanoma cell line; 2. Allogenic tumor model in mice; 3. Isolation of TANs from the tumors; 4. Inhibition of NAMPT in TANs in vitro; 5. Estimation of angiogenic properties of TANs in the aortic ring assay; 6. Adoptive transfer of treated neutrophils in the allogenic tumor model; 7. Tumor growth monitoring, histological examination. [Please click here to view a larger version of this figure.](#)

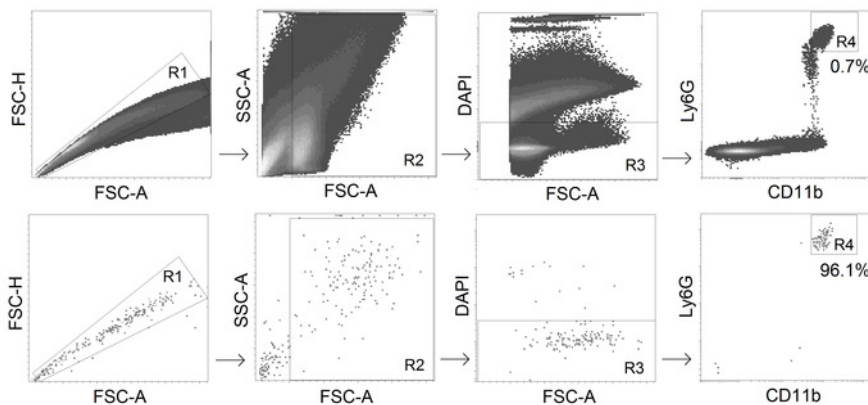


Figure 2. Gating strategy for TANs sorting. CD11b⁺ Ly6G^{hi} alive neutrophils are sorted from tumors with the purity ≥95%. [Please click here to view a larger version of this figure.](#)

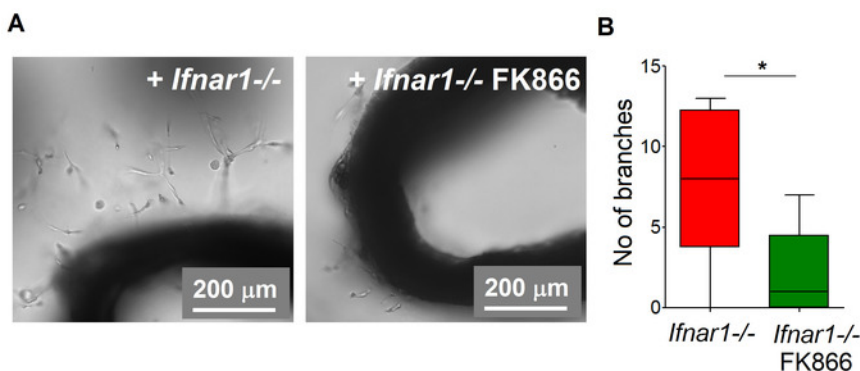


Figure 3. Suppression of angiogenic properties of TANs after FK866 treatment. Angiogenic properties of sorted *lfnar1*^{-/-} TANs treated with FK866 or with medium were estimated using aorta ring assay. Branch formation was monitored during 14 days, representative results at the day 14 are presented (**A**). Treatment with FK866 significantly decreased the number of endothelial branches (**B**). Data are shown as median, interquartile range and min-max, *p<0.05. [Please click here to view a larger version of this figure.](#)

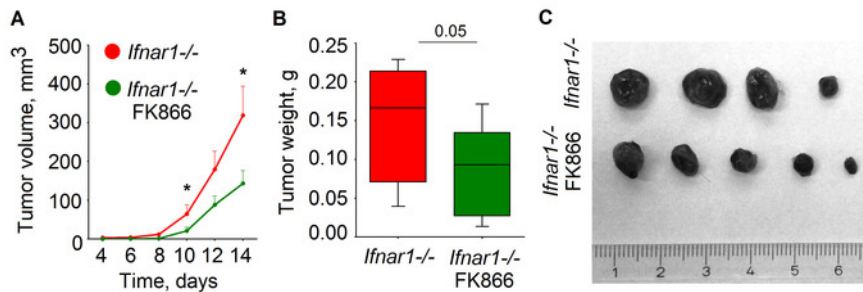


Figure 4. Retardation of tumor growth after adoptive transfer of FK866-treated neutrophils. The influence of TANs on the tumor growth was assessed. TANs were isolated, treated with FK866 and injected into tumor-bearing mice as described above. At day 14 mice were sacrificed, tumors removed and analyzed. *Ifnar1*^{-/-} TANs treated with FK866 versus controls were compared. (A) Tumor growth was measured, (B) tumor mass and (C) size were estimated. Data are shown as median, interquartile range and min-max, *p<0.05. [Please click here to view a larger version of this figure.](#)

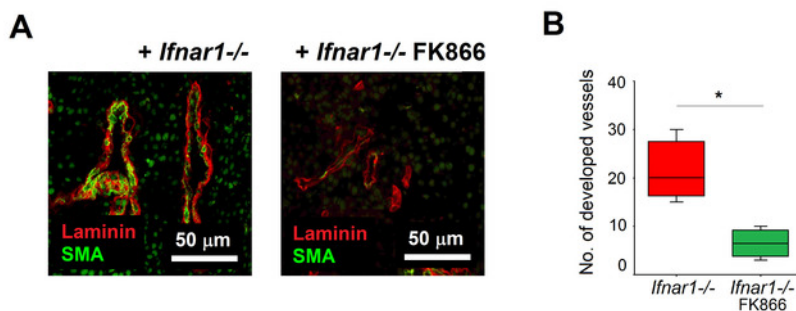


Figure 5. Suppressed tumor vascularization after adoptive transfer of FK866-treated neutrophils. Tumors were isolated as described above (Fig 4). Vessel maturation was assessed using anti-SMA antibodies (mature vessels) and anti-gamma laminin (endothelial cells). (A) Representative staining of tumors are shown: SMA (green), laminin (red). Scale bars: 50 μ m. (B) Quantification of tumor vascularization after adoptive transfer of TANs cultivated with FK866 (green) or medium (red) Data are shown as median, interquartile range and min-max, *p<0.05. [Please click here to view a larger version of this figure.](#)

Discussion

Despite progress in surgical and pharmacological cancer treatment, successful therapy remains a challenge. Since immune cells are known to play an important role in the regulation of tumor growth, novel methods inhibiting tumorigenicity of such cells should be established. Here we demonstrate a novel approach to suppress tumor growth via adoptive transfer of anti-angiogenic tumor-associated neutrophils. Selective targeting of pro-angiogenic NAMPT signaling in TANs, using FK866 inhibitor, prevents side effects, which are observed upon systemic FK866 treatment.

The most critical part of the protocol is the need to use freshly isolated primary neutrophils. Neutrophils are short-living cells, undergoing apoptosis or activated during the procedure of isolation. Murine neutrophils should be kept in 4 °C media during all steps of isolation, including cell sorting. Isolation of neutrophils should be performed as soon as possible and the experiment should not be paused. Usage of Fc-block allows reducing the unspecific staining of the cells with high Fc-receptor expression, like NK cells. We also recommend to minimize the number of fluorescent-conjugated antibodies to simplify the gating strategy and to avoid the activation of neutrophils due to antibody binding.

The limiting step of the protocol is the isolation of alive neutrophils from tumors due to a relatively low amount of these cells in tumors (not more than 1% of single alive cells in melanoma). This could only be possible using flow cytometry-based sorting. At the same time, the usage of blood neutrophils for this protocol should be avoided due to only minor regulation of NAMPT expression and their low functionality, which is altered upon tumor tissue arrival¹⁶. Possibly, in order to use blood neutrophils, they should be previously activated using tumor-derived growth factors.

To avoid neutrophil apoptosis, short treatment with FK866 (2-4 h) is suggested, as it has no influence on the viability of TANs, while prolonged treatment induces neutrophil apoptosis¹⁶. In sum, the protocol demonstrates the potential of ex vitro manipulated anti-angiogenic neutrophils to functionally suppress tumor growth in mouse melanoma tumor model.

Disclosures

The authors have nothing to disclose.

Acknowledgments

Our work was supported by grants from Deutsche Krebshilfe, Grant Number: 111647, and German Research Council (DFG), Grant Number: JA 2461/2-1.

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Detrimental Effect of Type I IFNs During Acute Lung Infection With *Pseudomonas aeruginosa* Is Mediated Through the Stimulation of Neutrophil NETosis

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Immunology

Received: 15 March 2019

Accepted: 30 August 2019

Published: 11 September 2019

Citation:

Pylaeva E, Bordbari S, Spyra I, Decker AS, Häussler S, Vybornov V, Lang S and Jablonska J (2019) Detrimental Effect of Type I IFNs During Acute Lung Infection With *Pseudomonas aeruginosa* Is Mediated Through the Stimulation of Neutrophil NETosis. *Front. Immunol.* 10:2190. doi: 10.3389/fimmu.2019.02190

Pseudomonas aeruginosa is an opportunistic multidrug-resistant pathogen, able to grow in biofilms. It causes life-threatening complications in diseases characterized by the up-regulation of type I interferon (IFN) signaling, such as cancer or viral infections. Since type I IFNs regulate multiple functions of neutrophils, which constitute the first line of anti-bacterial host defense, in this work we aimed to study how interferon-activated neutrophils influence the course of *P. aeruginosa* infection of the lung. In lungs of infected IFN-sufficient WT mice, significantly elevated bacteria load was observed, accompanied by the prominent lung tissue damage. At the same time IFN-deficient animals seem to be partly resistant to the infection. Lung neutrophils from such IFN-deficient animals release significantly lower amounts of neutrophil extracellular traps (NETs) and reactive oxygen species (ROS), as compared to WT neutrophils. Of note, such IFN-deficient neutrophils show significantly decreased capacity to stimulate biofilm formation by *P. aeruginosa*. Reduced biofilm production impairs in turn the survival of bacteria in a lung tissue. In line with that, treatment of neutrophils with recombinant IFN- β enhances their NETosis and stimulates biofilm formation by *Pseudomonas* after co-incubation with such neutrophils. Possibly, bacteria utilizes neutrophil-derived NETs as a scaffold for released biofilms. In agreement with this, *in vivo* treatment with ROS-scavengers, NETs disruption or usage of the bacterial strains unable to bind DNA, suppress neutrophil-mediated biofilm formation in the lungs. Together, our findings indicate that the excessive activation of neutrophils by type I IFNs leads to their boosted NETosis that in turn triggers biofilm formation by *P. aeruginosa* and supports its persistence in the infected lung. Targeting these mechanisms could offer a new therapeutic approach to prevent persistent bacterial infections in patients with diseases associated with the up-regulation of type I IFNs.

Keywords: *Pseudomonas aeruginosa*, neutrophils, IFN- β , IFNAR, innate immunity, bacterial infection, NETs, biofilms

INTRODUCTION

Pseudomonas aeruginosa is a gram-negative nosocomial pathogen. It shows relatively low virulence in healthy individuals, but can lead to life-threatening complications in hospitalized patients with cancer (1) or with virus infections (2). In such patients, *P. aeruginosa* infection often manifests as pneumonia, wound or implant infections, and sepsis. Besides the prolonged stay of such patients in the hospital, multiple invasive interventions, exposure to in-hospital microflora, declined tissue clearance, and immunological disorders may play a role in the pathogenesis of the infection (1). *P. aeruginosa* can form structured multicellular communities on surfaces, called biofilms. One of the components of biofilms is extracellular DNA (eDNA), which is attached by bacterial cationic exopolysaccharides due to its negative charge (3). Biofilm eDNA could be of bacterial or host cell origin (4–6). Hidden within self-produced matrix, bacteria in biofilms are protected from the host immune defense, antibiotics, or chemotherapy (7).

The host immune defense mechanisms against infection consist of structural barriers, soluble antimicrobial molecules, but also resident or recruited immune cells, such as neutrophils. The cogent role of neutrophil responses in acute *P. aeruginosa* infection of respiratory tract was proven by Koh et al. (8). Antibacterial properties of neutrophils include release of reactive oxygen species (ROS), production of bactericidal proteins, phagocytosis, and formation of neutrophil extracellular traps (NETosis) (9).

One of the potent regulators of neutrophil activity are type I interferons (IFNs) (10). These cytokines are released shortly after cell damage and are strong activators of the immune system (11). The up-regulation of type I IFN signaling is observed in various clinical conditions (12), including infections with *P. aeruginosa* (13). Type I IFNs are a large cytokine family including, among others, 14 IFN- α and a single IFN- β , all signaling through single receptor IFNAR (14). The master regulator of the whole IFN family is IFN- β (15). Type I IFNs modulate neutrophil phenotype and functions, e.g., by reducing their viability and migration, improving cytotoxicity and inhibiting their pro-angiogenic properties (16–18). During bacterial infections, the influence of type I IFNs on neutrophil functions appears to be controversial. In certain studies, the protective role of IFNs for the host was shown (19–24), while others revealed increased tissue damage and bacteria colonization in the presence of IFNs (25–29). At the same time, little is known about the influence of IFNs on neutrophil antibacterial functions during infection with *P. aeruginosa*.

In this manuscript, we set out to determine the role of type I IFNs in the modulation of neutrophil activity during acute lung infection with *P. aeruginosa* in mice and its impact on the course of infection. We have found that the absence of type I IFN signaling during acute phase of infection leads to reduced *Pseudomonas* persistence in the lung. Apparently, the absence of IFNs diminishes the capability of lung-associated neutrophils to release neutrophil extracellular traps (NETs). This in turn impairs biofilm formation by bacteria. Without protection that is provided by biofilms, *P. aeruginosa* is efficiently eliminated from

the lungs of infected IFN-deficient mice, leading to the reduced bacterial load.

MATERIALS AND METHODS

Bacteria

Pseudomonas aeruginosa strains that were used in this study: PA14 parental strain (wild-type serogroup O10 strain, cytotoxic ExoU+), *pelA* mutant PA14_24480 (*pelA* is the gene coding oligogalacturonide lyase related to exopolysaccharide production) and GFP PA14 *P. aeruginosa*. Bacteria have been cultured in Luria-Bertani (LB) broth for 3 h to reach the early exponential phase, washed twice in PBS, the optical density of 100 μ l suspension was measured in 96 well flat-bottom cell culture plates (Cellstar, Greiner Bio One International GmbH, Frickenhausen, Germany) at 600 nm using a microplate reader Synergy 2 (BioTek Instruments, Inc., Vermont, U.S.). OD 0.4 corresponds to a bacterial density of 5×10^9 /ml, as determined by serial dilutions and colony-forming unit (CFU) assays. Bacteria concentration was adjusted to the desired values and verified by plating on 2% LB agar plates.

Animals

Eight to twelve week-old mice of C56BL/6J wild type (WT), *Ifnar1*^{-/-} and *Ifnb1*^{-/-} strains were used in all experiments. Mice were bred and kept under SPF conditions in the animal facility of the University Hospital Essen (Germany).

Lower Respiratory Tract Infection in Mice

For intratracheal inoculation of *P. aeruginosa*, mice were anesthetized with Ketamin (bela-pharm GmbH & Co, Vechta, Germany) 100 mg/kg and Xylazin (Ceva Tiergesundheit GmbH, Düsseldorf, Germany) 10 mg/kg in 0.9% NaCl solution, intubated and 2×10^6 CFUs of *P. aeruginosa* in sterile PBS (50 μ l) was administered using the Minivent Mouse Ventilator type 845 (Harvard Apparatus, Massachusetts, U.S.) with stroke volume 150 μ l and frequency 150 breaths/min. The control of distribution of liquid in both lungs during intratracheal administration was performed prior to the experiments using Trypan blue (Sigma-Aldrich/Merck, Darmstadt, Germany). The adapted intratracheal method demonstrated accurate delivery and retention of *P. aeruginosa* in lungs. Animals were monitored post-operatively in a heated box until ambulant and clinically normal. Mice were transferred to a clean box with food and water *ad libitum* and monitored for 20 h. The duration of the experiment was chosen according to the published data confirming that biofilm formation by *P. aeruginosa* is a rapid process and takes place during first 10 h on plastic and 17 h in 3-D lung epithelial model (30, 31). After 20 h, mice were sacrificed. Heparinized blood was collected via heart puncture, plasma was prepared after centrifugation. Broncho-alveolar lavage (BAL) was collected after bronchial perfusion through the trachea with 1 ml of sterile PBS. Lung tissue was mechanically homogenized in 1 ml PBS. BAL and lung homogenates were plated in serial dilutions to estimate CFUs on 2% LB agar and examined after 24 h incubation.

Animal Treatment

I.p. injection of N-acetyl-L-cysteine (NAC) (Sigma-Aldrich/Merck, Darmstadt, Germany) treatment (dose 100 mg/kg) was performed 24 h and 1 h prior to *P. aeruginosa* inoculation.

I.p. treatment with rmIfnb (PBL assay science, Pestka Biomedical Laboratories, Inc, New Jersey, U.S.) in dose 1,000 U/mouse was performed 48 and 24 h before organs collection and neutrophil isolation.

Assessment of Neutrophil Infiltration in Lungs During *P. aeruginosa* Infection

Lungs were collected as described above; organs from non-infected animals were used as a control. Lung tissue was digested using dispase 0.2 µg/ml, collagenase A 0.2 µg/ml, DNase I 100 µg/ml (all Sigma-Aldrich/Merck, Darmstadt, Germany) solution in DMEM (Gibco, Life Technologies/Thermo Fisher Scientific, Massachusetts, U.S.) containing 10% FCS and 1% penicillin-streptomycin. Cells were meshed through 50 µm filters (Cell Trics, Partec, Sysmex Europe GmbH, Goerlitz, Germany) and erythrocytes lysed in ACK buffer containing NH₄Cl 150 mM, KHCO₃ 10 mM, Na₂EDTA 0.1 mM. BAL and single-cell suspensions were stained with antibodies listed below. The Ly6G⁺ neutrophil counts were assessed using BD FACS Canto system and data was analyzed using BD FACS Diva software (BD Biosciences, BD, New Jersey, U.S.).

Isolation of Lung Neutrophils

For estimation of neutrophil functions, neutrophils were isolated from lungs of non-infected WT, *Ifnar1*^{-/-} and *Ifnb1*^{-/-} mice. Lung tissue was harvested from each animal under aseptic conditions; single cell suspension was prepared as described above. Single-cell suspensions were stained with antibodies listed below, CD11b⁺ Ly6G⁺ alive neutrophils were sorted using a FACS Aria cell sorter (BD Biosciences, BD, New Jersey, U.S.), and the purity of cells was assessed (≥95%) (Figure S1). After sorting cells were resuspended in DMEM containing 10% FCS.

Antibodies

Anti-mouse CD16/32 (clone 2.4G2, BD Pharmingen, BD, New Jersey, U.S.), anti-Ly6G (clone 1A8, BD Pharmingen, BD, New Jersey, U.S.), anti-CD11b (clone M1/70, eBioscience, Affymetrix, California, U.S.). Viability Dye eFluorTM 780 (eBioscience, Affymetrix, California, U.S.) or DAPI (BioLegend, California, U.S.) were used to determine viable cells.

NETs Release

Isolated neutrophils 15,000/well were incubated with *P. aeruginosa* (MOI 10) in glass-bottom 96-well plate (MatTek Corporation, Massachusetts, U.S.) pre-coated with poly-D-lysine 1 mg/ml (Sigma-Aldrich/Merck, Darmstadt, Germany) for 4 h at +37°C, 5% CO₂, sterile medium was used as a negative control. Samples were fixed with paraformaldehyde (Thermo Fisher Scientific, Massachusetts, U.S.) to the final concentration 4%, permeabilized with Triton X-100 (Sigma-Aldrich/Merck, Darmstadt, Germany) 0.2% containing buffer. Since the visualization of NETs using DNA-intercalating

dyes alone has the risk of detection of necrotic cells or the generation of artificial results based on dye-blocking peptides associated with NETs, antibody-based techniques are required to visualize NETs. Antibodies for citrullinated histones detect PAD4-dependent NETosis, but not NETs released through other mechanisms (32). Therefore, anti-histone 1 antibodies (Merck Millipore, Darmstadt, Germany) were used to detect all NETs. Donkey-anti-mouse-AF564 (Invitrogen, Thermo Fisher Scientific, Massachusetts, U.S.) were used as secondary antibodies. Stainings were mounted with ProLong Gold Antifade Mountant with DAPI (Invitrogen, Thermo Fisher Scientific, Massachusetts, U.S.). Percent of NET-producing cells and NETs length were estimated by microscopy.

Reactive Oxygen Species

Lung tissue was harvested from non-infected WT, *Ifnb1*^{-/-} and *Ifnar1*^{-/-} animals under aseptic conditions; single cell suspensions were prepared and stained with antibodies as described above. Cells were washed and resuspended in DMEM containing 10% FCS, *P. aeruginosa* PA14 WT MOI 10 was added. Sterile medium was used as negative control. ROS production by Ly6G⁺ alive neutrophils was estimated after 60 min of exposure to *P. aeruginosa* using Dihydrorhodamine 123 (Sigma-Aldrich/Merck, Darmstadt, Germany) with flow cytometry.

Phagocytosis

Lung tissue was harvested from non-infected WT and *Ifnar1*^{-/-} animals under aseptic conditions; single cell suspension was prepared and stained with antibodies. Cells were then washed and resuspended in DMEM containing 10% FCS, *P. aeruginosa* PA14 WT-GFP (MOI 10) added. Phagocytosis of GFP-bacteria by Ly6G⁺ neutrophils was estimated after 60 min using flow cytometry.

Endocytosis and Procession in Phagolysosomes

Lung tissue was harvested from non-infected WT and *Ifnar1*^{-/-} animals; single cell suspension prepared and stained with antibodies. Cells were washed and resuspended in DMEM containing 10% FCS, DQ ovalbumin added (Molecular probes, Invitrogen, Thermo Fisher Scientific, Massachusetts, U.S.). After 30 min, the reaction was stopped by placing the plate on ice, green fluorescence of processed DQ ovalbumin in Ly6G⁺ neutrophils was estimated using flow cytometry.

Migration to Lipopolysaccharide (LPS)

Cell migration was evaluated using a two-chamber transwell system (3 µm pore size) cell culture inserts (Falcon, Corning, New York, U.S.). Seven hundred microliter of DMEM + 10% FCS containing LPS (Invitrogen, Thermo Fisher Scientific, Massachusetts, U.S.) in concentration 1 ng/ml or LPS-free medium, as a negative control for spontaneous migration, were added to the lower chamber. Isolated lung WT and *Ifnar1*^{-/-} neutrophils 3 × 10⁵ cells in 300 µl DMEM + 10% FCS were added to the upper chamber, then the chamber was placed into medium for 3 h in an incubator at 37°C and 5% CO₂. Cells transmigrated to the lower chamber were counted using

CASY (Innovatis, Roche Innovatis AG, Bielefeld, Deutschland). Measurements were performed in independent experiments and mean counts calculated.

Biofilms

96-well flat plastic-bottom plates were used, lung neutrophils in concentration 2×10^5 /well and *P. aeruginosa* (MOI 10) were added to the final volume 150 μ l. After 72 h wells were washed with deionized water twice and stained with 0.4% crystal violet (Sigma-Aldrich/Merck, Darmstadt, Germany), then washed with deionized water twice. Crystal violet is a basic protein dye that stains negatively charged surface molecules of viable and alive cells and extracellular matrix of polysaccharides (33). Microscopy photographs of dry wells were taken using AMD EVOS fl digital inverted microscope in brightfield. Crystal violet stain was measured after the addition of 30% acetic acid on a plate reader at OD600. All samples were tested at least in 3 independent wells.

For fluorescent staining biofilms of *P. aeruginosa*-GFP were similarly prepared in glass-bottom non-covered 96-well plates, permeabilized with TritonX 0.2%, stained with anti-histone 1 antibodies and donkey-anti-mouse-AF564 secondary antibodies, mounted with DAPI ProLong Gold Antifade Mountant. No DAPI signal from nuclei was detected, proving the absence of alive neutrophils with preserved nuclei in 72 h co-culture.

Concentration of DNase in *in vitro* studies: 100 μ g/ml, NAC concentration: 100 μ M.

Histology

For histological examination of lungs, WT, *Ifnb1*^{-/-}, and *Ifnar1*^{-/-} mice were infected *i.t.* with *P. aeruginosa*. At the certain time point mice were sacrificed, lungs perfused with Tissue-Tek O.C.T. Compound (Sakura Finetek, Japan) containing 5% paraformaldehyde, the lumen of the trachea was fixed with ligature; lungs were dissected and snap frozen at -80°C. 7- μ m cryosections were fixed with ice-cold acetone, stained with hematoxylin-eosin or anti-histone 1 and DAPI, dried and mounted with Neo-Mount (Merck, Darmstadt, Germany).

Microscopy

Microscopy was performed using Zeiss AxioObserver.Z1 Inverted Microscope with ApoTome Optical Sectioning equipped with filters for: DAPI, FITC, Alexa Fluor 488, GFP, DsRed, Cy3 or Olympus BX51 upright epifluorescence microscope. Images were processed with ZEN Blue 2012 software or CellSens Dimension software (Olympus), respectively, and analyzed with ImageJ.

ELISA

TNF- α in plasma samples were analyzed with ELISA (R&D Systems, Minnesota, U.S.) according to manufacturer protocols.

RT-qPCR

RNA was isolated from WT, *Ifnar1*^{-/-}, and *Ifnb1*^{-/-} neutrophils, using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and cDNA prepared. Real-time RT-PCR was performed using primers listed in Table 1.

TABLE 1 | Primers used for qRT-PCR.

Gene	Forward (5' to 3')	Reverse (5' to 3')
<i>Mpo</i>	CGTGTCAAGTGGCTGTGCCTAT	AACCAGCGTACAAAGGCACGGT
<i>Lyz2</i>	TGCCAGAACTCTGAAAAGGAATGG	CAGTGTCTTGGTCTCCACGGTT
<i>Def1</i>	AACTGAGGAGCAGCCAGGAGAA	CTTCCTTTGAGCCTCTTGATCT
<i>Nox2</i>	TGGCGATCTCAGCAAAAGGTGG	GTACTGTCCACCTCCATCTTG
<i>Rps9</i>	TTGACGCTAGACGAGAAGGAT	AATCCAGCTTCATCTTGCCCT

Expression of IFNAR Subunits on Circulating Leukocytes in Lung Cancer and Lower Respiratory Tract (LRT) Viral Infection

We used previously published microarray data deposited in the Gene Expression Omnibus database GSE42834 (34) and GSE60244 (35). Analyzed samples are listed in **Supplementary Materials**.

Statistics

Statistical analyses were performed using Kruskal-Wallis ANOVA for multiple comparisons with the Bonferroni correction, and Mann-Whitney U-test for two independent samples. $P < 0.05$ was considered significant.

Study Approval

The animal experiments have been approved by the regulatory authorities LANUV (Das Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen), Germany. Our animal care and use protocols adhere to the regulations of das Deutsche Tierschutzgesetz (TierSchG) and follow FELASA recommendations.

RESULTS

Elevated Expression of IFNAR on Leukocytes From Patients With Cancer and Viral Infections

Diseases such as cancer or viral infections are characterized with the enhanced production of type I IFNs (12). To study the regulation of type I IFN receptor (IFNAR) on immune cells, we performed the analysis of available Gene Expression Omnibus databases GSE60244 and GSE42834 (34, 35). We observed an up-regulation of IFNAR subunits 1 and 2 on circulating leukocytes during cancer and viral infections, giving the evidence of the activated interferon signaling in immune cells during the course of disease (Table 2).

Such a significant up-regulation of IFNAR on leukocytes in diseases commonly associated with *P. aeruginosa* infections (e.g., lung cancer, viral lower respiratory tract infections) (1, 2), prompted us to evaluate the role of type I IFNs in the regulation of antibacterial functions of neutrophils in the model of *P. aeruginosa* induced pneumonia.

TABLE 2 | Expression of IFNAR subunits on circulating leukocytes in lung cancer and lower respiratory tract (LRT) viral infection.

	LUNG CANCER Healthy vs. Disease	VIRAL LRT INFECTION Healthy vs. Disease
IFNAR1	−0.2 (−0.4; 0.0) vs. 0.6 (0.4; 0.8) $p < 0.0001$	278 (221; 347) vs. 382 (319; 534) $p < 0.0001$
IFNAR2	−0.2 (−0.5; 0.1) vs. 0.2 (−0.0; 0.4) $p < 0.0001$	354 (316; 383) vs. 381 (316; 480) $p < 0.05$

To study the regulation of type I IFN receptor (IFNAR) on immune cells, we performed the analysis of available Gene Expression Omnibus databases GSE60244 and GSE42834 (34, 35). Data are shown as median (interquartile range).

IFN-Deficient Mice Infected With *P. aeruginosa* Show Lower Lung Colonization and Reduced Tissue Damage, as Compared to WT Animals

P. aeruginosa often cause infections of lower respiratory tract and is associated with elevated type I IFN signaling in the host (13). Therefore, we were interested of how IFN signaling influence the course of *Pseudomonas* infection. To this end, we infected mice intratracheally with *P. aeruginosa* PA14, and compared the course of infection between WT and IFN-deficient (*Ifnar1*^{−/−} and *Ifnb1*^{−/−}) animals. We observed that after 20 h *P. aeruginosa* was efficiently cleared from the lung and BAL of IFN-deficient mice, while WT mice showed elevated bacteria counts (Figure 1A).

Since neutrophils are the first line of defense and play an important role in controlling bacterial infections, we were interested if the different susceptibility for *Pseudomonas* infection between WT and IFN-deficient animals could be due to altered neutrophil counts in infected lungs. Therefore, we evaluated numbers of Ly6G⁺ neutrophils in the lungs of WT and IFN-deficient animals (gating strategy see Figure S1), but no significant differences between the mouse strains were observed (Figure 1B).

As neutrophil counts obviously do not contribute to elevated susceptibility of WT mice to *Pseudomonas* infection, we were interested if the activation status of these cells could be different between tested mouse strains. Activated neutrophils may lead to tissue damage; therefore, we analyzed structural changes, such as swelling of alveoli walls or reduced aeration, in the lungs of mice infected with *P. aeruginosa*. Importantly, we could observe more prominent tissue damage in the lungs of WT mice, as compared to IFN-deficient animals (Figures 1C,D). In line with this, systemic level of TNF- α , which is the key component of inflammatory responses, was significantly elevated in WT animals (Figure 1E).

Together, these findings suggest that altered neutrophil activity, but not their counts, are responsible for the observed sensitivity of WT mice to *P. aeruginosa* infection.

WT Lung Neutrophils Release Elevated Levels of Reactive Oxygen Species and Neutrophil Extracellular Traps in Response to *Pseudomonas* Infection

As neutrophils are the major antibacterial effector cells during *P. aeruginosa* infection and their activation seem to be altered

in IFN-deficient mice, we aimed to characterize antibacterial functions of neutrophils isolated from both mouse strains. For this purpose, we challenged isolated mouse lung neutrophils (>95% purity, Figure S1) with *P. aeruginosa* MOI 10. Notably, significantly impaired capacity to release NETs was observed in IFN-deficient neutrophils, as compared to WT (Figures 2A,B). Moreover, the ability to produce long NETs was also significantly reduced in such neutrophils (Figures 2A–C).

Reactive oxygen species (ROS) are involved in direct cytotoxicity of neutrophils, but are also suggested to trigger the NET release by these cells (36). Therefore, we measured the production of ROS by lung-associated neutrophils in response to *P. aeruginosa* infection and compared WT with IFN-deficient mice. We could observe that IFN-deficient neutrophils have lower capability to produce ROS in these conditions (Figure 2D).

Subsequently, we estimated migration, phagocytosis, endocytosis and phagolysosomal degradation in neutrophils infected with *P. aeruginosa*. Moreover, we assessed the expression of key antibacterial genes in such neutrophils, and compared WT and IFN-deficient animals. IFN-deficient neutrophils showed significantly higher migratory capacity toward bacterial lipopolysaccharide (LPS) (Figure S2A). Other functions of neutrophils, such as phagocytosis (Figure S2B), endocytosis and phagolysosomal processing (Figure S2C) were not significantly different between WT and IFN-deficient mice. Furthermore, the expression of molecules responsible for antibacterial functions of neutrophils, such as defensins, lysozyme, myeloperoxidase, NADPH-oxidase, was not significantly altered between WT and IFN-deficient neutrophils (Figure S2D).

Altogether, we observed that WT neutrophils release more ROS and NETs in response to *Pseudomonas* infection, while other antibacterial functions of neutrophils are not significantly altered. Elevated ROS and NETs are probably responsible for prominent tissue damage that is observed in the lungs of infected WT mice.

WT Neutrophils Induce Strong Biofilm Formation by *P. aeruginosa*

WT mice show higher tissue damage after *Pseudomonas* infection, but also elevated bacteria count in lung. Since WT neutrophils release higher amounts of ROS and NETs, we were interested if this influences also the control of bacteria by these cells. Therefore, we co-incubated *P. aeruginosa* with isolated WT and IFN-deficient lung neutrophils, and assessed survival of bacteria. In agreement with our previous findings, neutrophils released high amount of NETs (histone 1⁺) in response to *Pseudomonas*. This was significantly higher in WT neutrophils, compared to IFN-deficient neutrophils (Figures 3A,B). Of note, co-incubation of *P. aeruginosa* with neutrophils resulted in significantly elevated survival of bacteria, compared to bacteria alone (control). Moreover, *Pseudomonas* were mainly found in NETs (Figures 3A,C).

To validate the role of NETs in this experimental setting, we disrupted them using DNase and estimated *P. aeruginosa* counts. Interestingly, treatment with DNase reduced survival of bacteria (Figures 3A–C), suggesting an importance of NETs for *Pseudomonas* persistence.

It is known that *Pseudomonas* survival in tissues depends strongly on the ability of this bacterium to form biofilms.

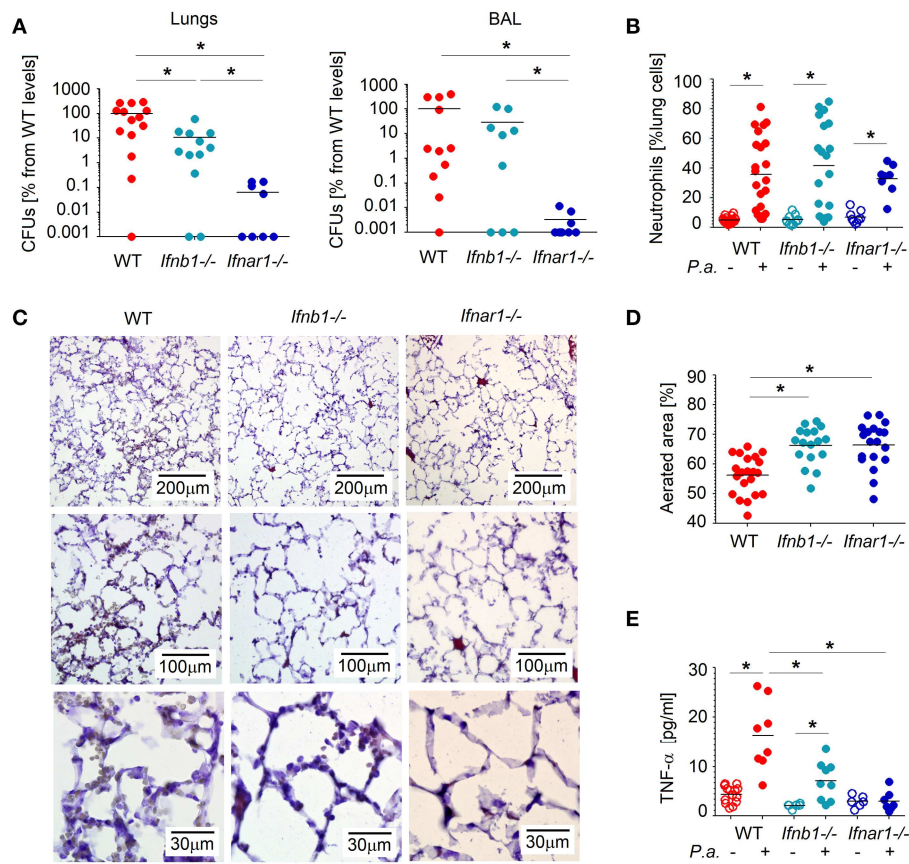


FIGURE 1 | Type I IFNs contribute to tissue damage and *P. aeruginosa* survival in lungs. **(A)** Elevated bacteria load in lungs and in broncho-alveolar lavage (BAL) of WT mice. WT, *Ifnb1*^{-/-} and *Ifnar1*^{-/-} mice were infected with *P. aeruginosa* and sacrificed after 20 h. Bacterial load in organs was determined by serial dilutions and plating on LB agar, CFUs were determined. At least 4 animals per group in 2 independent experiments were used. **(B)** Elevated infiltration of neutrophils into lungs of WT mice. Neutrophils in lungs were determined by flow cytometry. **(C,D)** Better aerated lungs in IFN-deficient mice after *Pseudomonas* infection. Five micrometer lung cryosections were stained with hematoxylin-eosin, scale bars: 200, 100, and 30 μm. At least 5 fields of view in 4 lungs per group were analyzed. **(E)** Systemic TNF-α levels before and after infection were measured in plasma with ELISA. *P.a.*, *Pseudomonas aeruginosa*. Data are shown as individual values and mean, **p* < 0.05.

Therefore, we assessed biofilm development after co-incubation of bacteria with lung neutrophils and observed significantly elevated production of biofilms in the presence of neutrophils, as compared to bacteria alone. Moreover, WT neutrophils seemed to be more efficient stimulators of biofilm formation than IFN-deficient neutrophils. Since biofilm formation by *Pseudomonas* correlates with higher NETs release from neutrophils, we tested biofilm production by bacteria in the presence of neutrophils treated with DNase. Staining with crystal violet proved diminished biofilm formation in this experimental setting (Figures 3D,E).

We hypothesized that NETs released from neutrophils trigger *Pseudomonas* biofilm formation. This in turn should support the persistence of bacteria in lungs of infected mice. To test this *in vivo*, we infected mice with *P. aeruginosa* and stained their lungs to visualize NETs components. In agreement with our *in vitro* data, we observed higher amounts of histone1 positive NETs in lungs of infected WT mice, as compared to IFN-deficient *Ifnb1*^{-/-} or *Ifnar1*^{-/-} animals (Figures 3F,G). Higher NETs

presence in lungs of WT mice correlated positively with elevated bacteria counts.

Thus, upon infection with *P. aeruginosa*, NETs that are released from lung-associated neutrophils support formation of biofilms by the bacterium. Biofilms support *Pseudomonas* persistence in the lung, by protecting it from the immune system. This phenomenon is responsible for the observed elevated counts of bacteria in lungs of WT mice, as compared with IFN-deficient animals.

rmIFN-β Treatment Supports NETs Formation by Neutrophils

To proof that enhanced NETs release is due to IFN availability, we treated mice with 1,000 U of rmIFN-β for 3 days, isolated lung neutrophils and estimated NETs formation in response to *Pseudomonas* infection. Indeed, we could observe significantly elevated NETs release upon rmIFN-β treatment (Figures 4A,B). Interestingly, also the structure of produced NETs was altered (Figure 4A). In agreement with our hypothesis that NETs stimulate biofilm production by *P. aeruginosa*, elevated biofilm

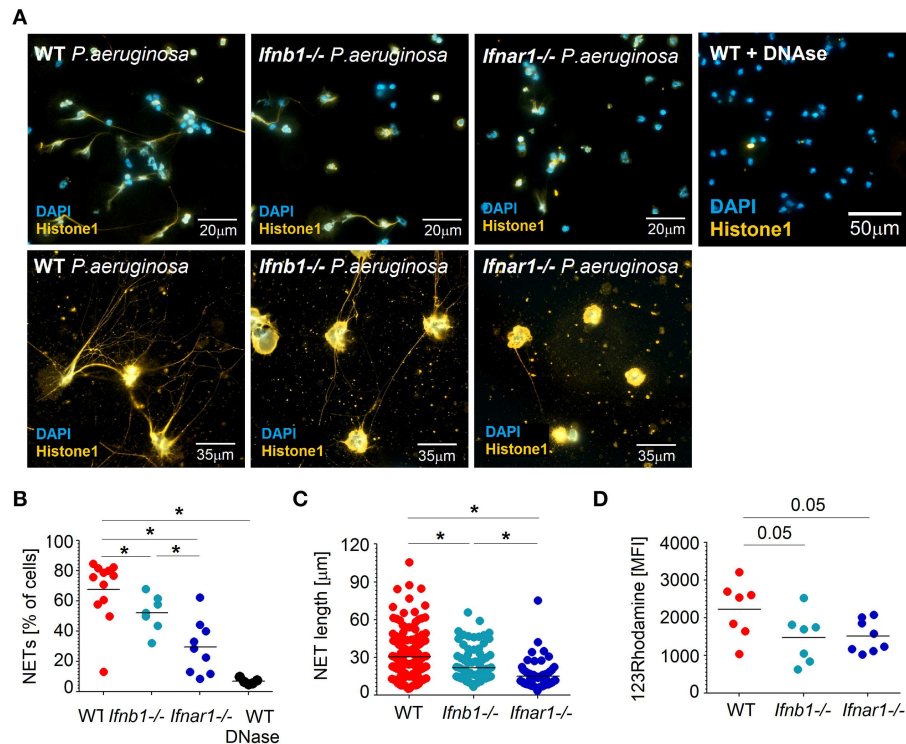


FIGURE 2 | Lower ROS production and NET formation in IFN-deficient neutrophils after infection with *P. aeruginosa*. **(A–C)** Reduced NETosis in IFN-deficient mice after infection with *P. aeruginosa*. Lung neutrophils isolated from WT, *Ifnb1*^{-/-} and *Ifnar1*^{-/-} mice were challenged with *P. aeruginosa* MOI 10 for 4 h, NETs were stained with anti-histone 1 (orange) and DAPI (blue). Exemplified staining of NETs **(A)**, scale bars: 20 and 13 μ m. Quantification of NET-positive cells **(B)** and NET length **(C)**. At least 9 fields of view per group were analyzed. Treatment with DNase proves the major role of DNA as a component of NETs. **(D)** ROS production in presence of *P. aeruginosa* is higher in WT mice. Single cell lung suspension from WT, *Ifnb1*^{-/-} and *Ifnar1*^{-/-} mice was challenged with *P. aeruginosa* MOI 10 for 1 h, stained with 123Rhodamine and released ROS evaluated in neutrophils using flow cytometry. At least 7 animals per group were included. Data are shown as individual values and mean, * $p < 0.05$.

formation was observed after co-incubation of bacteria with IFN- β pretreated neutrophils (**Figures 4C,D**). High numbers of bacteria associated with histone-rich areas after IFN- β treatment were proven (**Figures 4E,F**).

Thus, IFNs stimulate lung-associated neutrophils to release NETs during *P. aeruginosa* infection. This in turn triggers the formation of bacterial biofilms.

NET-Derived DNA Is Essential for *P. aeruginosa* Biofilm Formation

To prove the involvement of NETs in biofilm formation, *P. aeruginosa pelA* mutant strain PA24480 was used. Pel is cationic exopolysaccharide that cross-links extracellular DNA to the biofilm matrix (37), therefore PA24480 mutant (lacking Pel exopolysaccharide) is unable to bind to the extracellular NETs DNA. We isolated lung neutrophils and co-cultured them with *Pseudomonas* PA24480. NETs release and biofilm formation was evaluated. We could observe that biofilm formation by *Pseudomonas* PA24480 was significantly inhibited for both WT and IFN-deficient neutrophils (**Figures 5A,B**). Importantly, the capacity to induce NETs was comparable between PA24480 and wild type PA14 strain (**Figure 5C**). Therefore, we assume that the inhibition of biofilm formation by PA24480 strain is solely due to its inability to bind NETs DNA.

To corroborate this *in vivo*, we infected WT and IFN-deficient mice with *Pseudomonas* PA24480, and compared the bacterial load in lungs after 20 h. As expected, we could not observe any significant differences in bacterial load between WT and IFN-deficient mice (**Figure 5D**). Devoid of biofilm protection, *P. aeruginosa* in WT mice had no advantage over those in IFN-deficient mice.

Taken together, binding of bacteria to DNA in NETs is an essential step facilitating the production of biofilms by *P. aeruginosa* and supporting its persistence in infected lungs.

N-Acetylcysteine Prevents NET-Dependent Biofilm Formation by *P. aeruginosa* and Leads to Effective Bacteria Elimination From the Lung

We could demonstrate that NET-dependent biofilm formation supports *P. aeruginosa*-persistence in infected lungs. Since production of ROS by neutrophils is one of the triggers for NETs formation, the reduction of ROS may provide therapeutic solution for persistent *Pseudomonas* lung infections. One of the factors that are known to efficiently reduce ROS production in neutrophils is N-acetylcysteine (NAC) (38). Here, we set

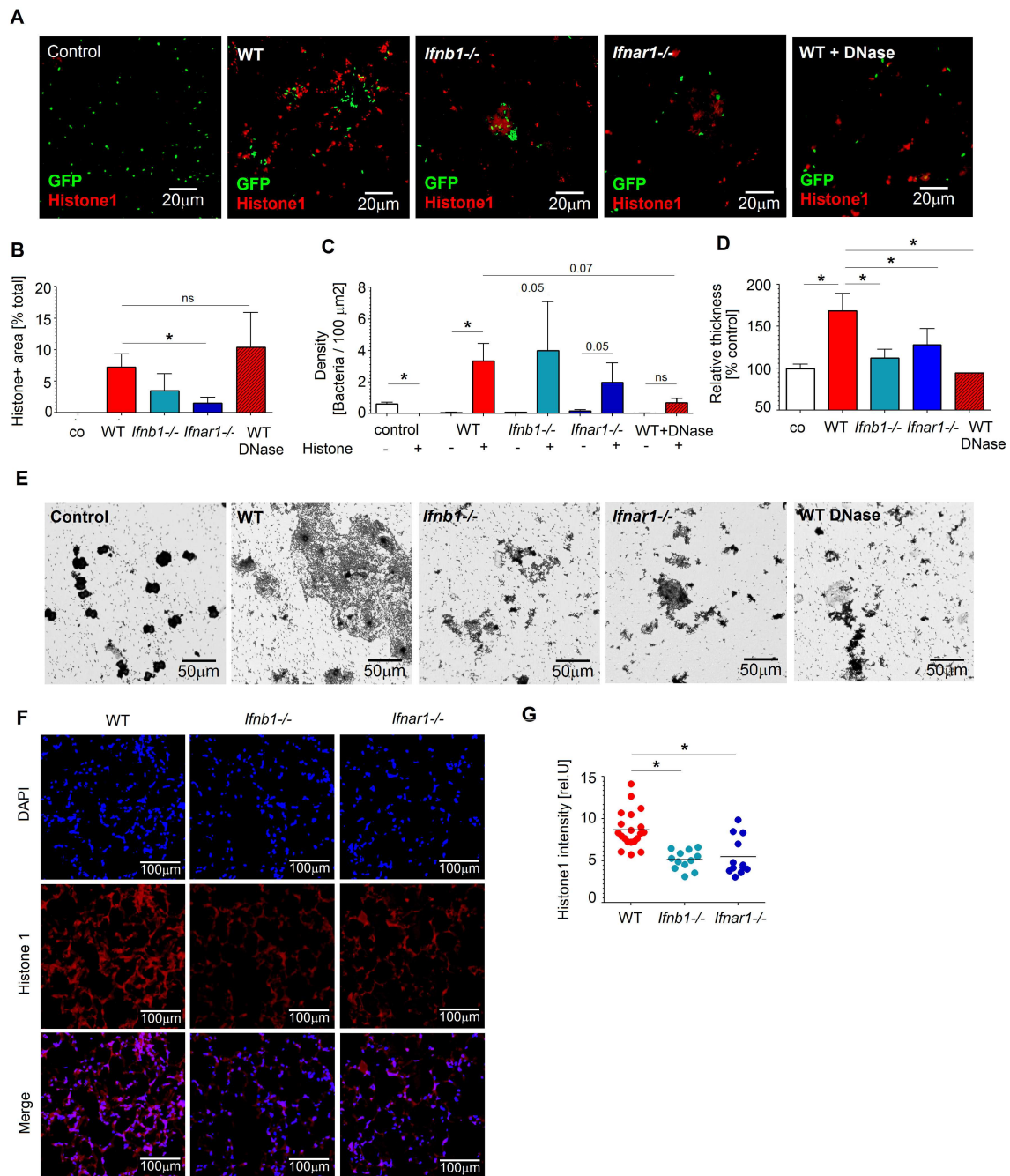


FIGURE 3 | IFN-sufficient neutrophils support biofilm formation *in vitro* and *in vivo* after *Pseudomonas* infection. **(A–C)** Higher numbers of bacteria associated with histone-rich areas after *in vitro* infection of WT lung neutrophils. Lung neutrophils isolated from WT, *lfnb1*^{-/-} and *lfnar1*^{-/-} mice were challenged with *P. aeruginosa* GFP MOI 10 for 3 days, wells were stained for histone 1 (red). Control—bacteria without neutrophils; Exemplified staining, scale bars: 20 μm **(A)**. Quantification of histone1-positive areas **(B)** and *P. aeruginosa* accumulation in histone 1-rich vs. histone 1-negative areas **(C)**. Quantification was done in triplicate with at least 5 fields of view per well. **(D,E)** Elevated biofilm formation after incubation of WT neutrophils with *P. aeruginosa*. Isolated lung neutrophils were challenged with bacteria MOI 10 for 3 days, biofilms were stained with crystal violet, control—bacteria alone without neutrophils. Exemplified biofilm staining, scale bars: 50 μm **(D)**. Quantification of biofilms was performed using acetic acid and OD measurements **(E)**. Data are shown as mean ± SEM, **p* < 0.05. **(F,G)** Elevated accumulation of neutrophil-derived NETs (histone 1⁺) in lungs of WT mice after *P. aeruginosa* infection. WT, *lfnb1*^{-/-} and *lfnar1*^{-/-} mice were *i.t.* infected, sacrificed after 20 h, 5 μm cryosections of lungs were stained with anti-histone 1 (red) and DAPI (blue). Exemplified lung staining, scale bars: 100 μm **(F)**, Quantification of the histone 1 intensity in lungs was analyzed in 4–5 fields of view in 4 lungs per group. Data are shown as individual values and mean, **p* < 0.05.

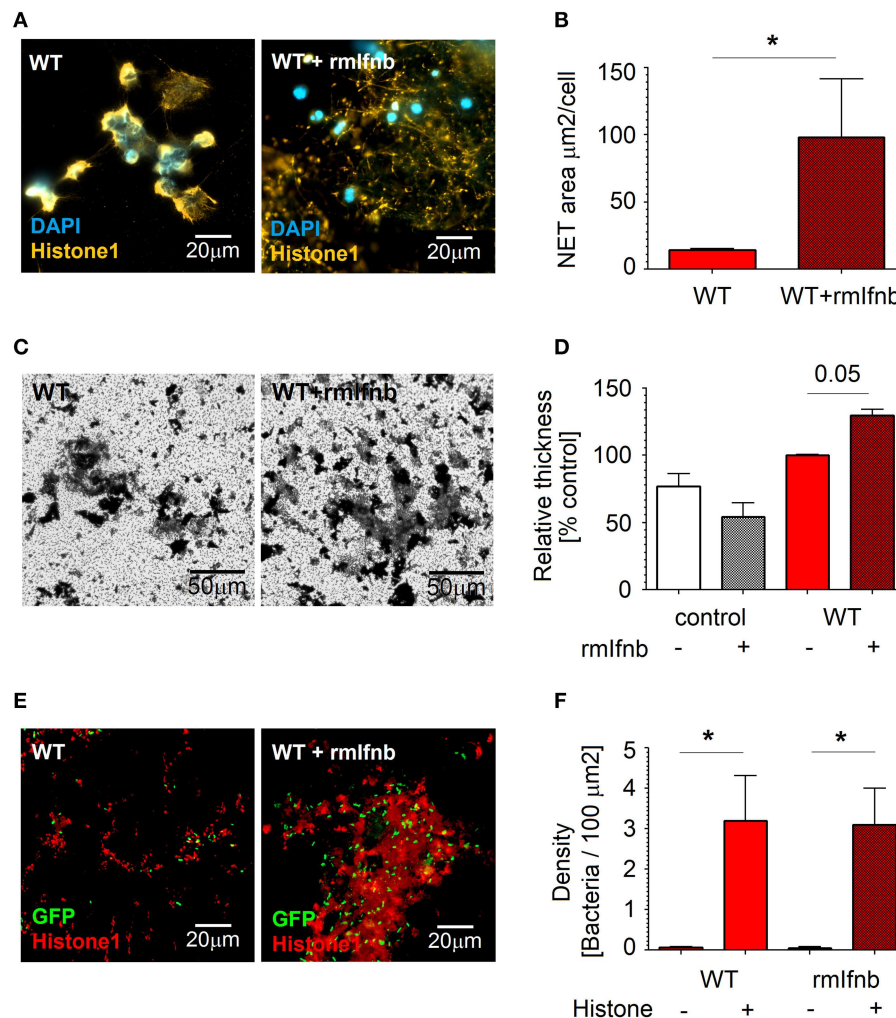


FIGURE 4 | rmlfnb stimulates NETs release by neutrophils and supports biofilm formation. **(A,B)** NETosis is enhanced by rmlFNβ. Lung neutrophils were isolated from untreated and rmlfnb-treated WT mice. Cells were challenged with *P. aeruginosa* MOI 10 for 4 h, NETs were stained with anti-histone 1 (orange) and DAPI (blue). Exemplified staining, scale bars: 20 μm. **(A)** Quantification of the area covered with NETs. **(B)** At least 5 fields of view per group were analyzed. **(C,D)** Elevated biofilm production after IFN treatment. Lung neutrophils isolated from untreated and rmlFNβ-treated WT mice were challenged with *P. aeruginosa* MOI 10 for 3 days, biofilms were stained with crystal violet. Exemplified staining, scale bars: 50 μm. **(C)** Quantification of biofilms was performed after dilution with acetic acid and OD measurement. **(D)** Data are shown as mean ± SEM, **p* < 0.05. **(E,F)** Higher numbers of bacteria associated with histone-rich areas after *in vitro* infection of WT lung neutrophils. Lung neutrophils isolated from WT naive and IFN-β-treated animals were challenged with *P. aeruginosa* GFP MOI 10 for 3 days, wells were stained for histone 1 (red). Exemplified staining, scale bars: 20 μm **(E)**. Quantification of *P. aeruginosa* accumulation in histone 1-rich vs. histone 1-negative areas **(F)**.

out to determine the effect of NAC on NETs formation and consequential biofilm development by *P. aeruginosa*.

First, we isolated WT lung neutrophils, co-incubated them with bacteria and quantified NETs release and biofilm production in presence vs. absence of NAC. Importantly, treatment of neutrophils with NAC led to significant suppression of NETs release (**Figures 6A,B**). In agreement, biofilm formation by *P. aeruginosa* was also diminished in this experimental setting (**Figures 6C,D**).

Next, we have evaluated therapeutic effectiveness of NAC in preventing *Pseudomonas* persistence in lungs of infected mice. We treated WT mice with NAC and infected them with *Pseudomonas*. Notably, we could observe reduced counts of bacteria in the lungs and BAL of mice treated with NAC,

as compared to untreated animals (**Figure 6E**). NAC-treated animals showed also diminished lung tissue damage (preserved aeration areas) (**Figures 6F,G**) and lower amounts of NET components (histone 1) (**Figures 6H,I**).

Thus, prevention of NET release by NAC leads to reduced survival of bacteria in lungs and at the same time to lower lung tissue damage. Therefore, NAC treatment may provide a potent tool in prophylaxis and treatment of persistent *P. aeruginosa* infections.

In sum, in this manuscript we could demonstrate that during *Pseudomonas aeruginosa* infection type I IFNs excessively activate neutrophils to release NETs. Binding to such NETs triggers formation of biofilms by *P. aeruginosa*. Accumulating biofilms provide in turn a protective niche that supports bacteria

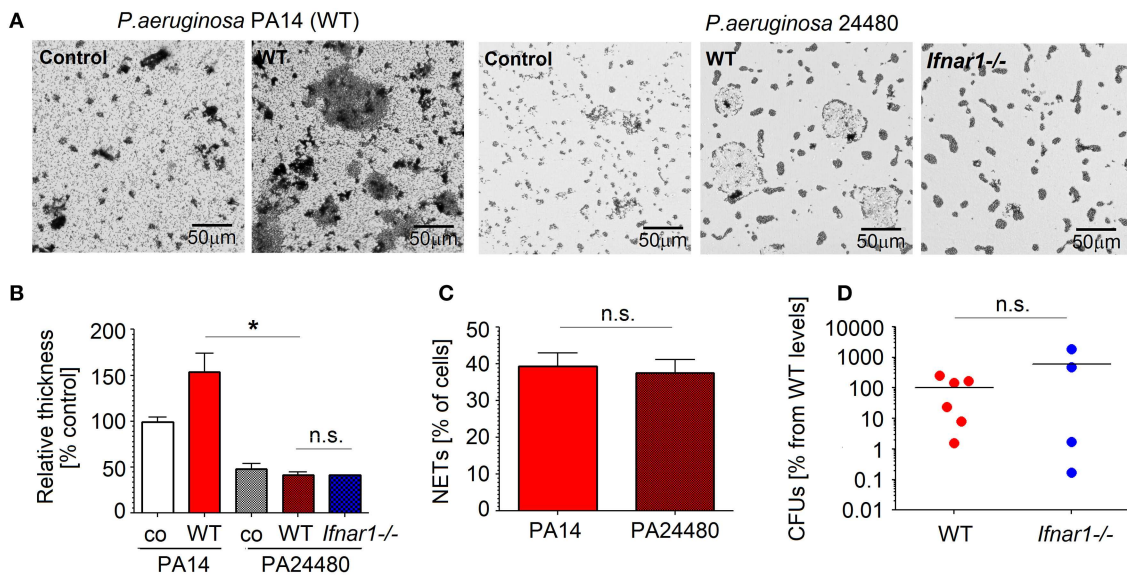


FIGURE 5 | NETs released by neutrophils are important components of *P. aeruginosa* biofilms. **(A,B)** *Pseudomonas pelA* mutant strain 24480 which is unable bind to eDNA shows reduced biofilm formation. Neutrophils isolated from WT mice were challenged with *P. aeruginosa* PA14 and 24480 strain at MOI 10 for 3 days. Biofilms were stained with crystal violet, control—bacteria only. Exemplified biofilm staining, scale bars: 50 μ m. **(A)** Quantification of biofilms was performed using acetic acid and OD measurement **(B).** **(C)** Comparable NET formation in response to different *P. aeruginosa* strains: PA14 and 24480 strain. Neutrophils isolated from WT mice were challenged with *P. aeruginosa* PA14 and 24480 strains at MOI 10 for 4 h, NETs were stained with anti-histone 1 and DAPI, the number of NET-positive cells was quantified. At least 5 fields of view per group were analyzed. Data are shown as mean \pm SEM, * $p < 0.05$. **(D)** Inhibited biofilm production by *pelA* *Pseudomonas* mutant abrogated differences in bacteria clearance between infected WT and *Ifnar1*^{-/-} mice. WT and *Ifnar1*^{-/-} mice were infected with 24480 strain, animals were sacrificed after 20 h, bacterial load in organs was determined by serial dilutions and plating on LB agar, CFUs were calculated. At least 4 animals per group were included. Data are shown as individual values and mean, * $p < 0.05$.

persistence in lungs. Therefore, NETs disrupting agents should be considered as anti-bacterial treatment and prophylaxis in diseases associated with type I IFN up-regulation.

DISCUSSION

Viral infections or cancer are associated with the upregulation of type I IFNs and IFN-dependent immune responses, as well as with the enhanced rate of bacterial complications (1, 2). Moreover, infections with bacteria, such as *P. aeruginosa*, are often associated with the elevated type I IFN signaling in lung epithelial cells (13). The data concerning the impact of high IFN levels on the bactericidal functions of neutrophils are limited. Clinical observations show that neutrophils isolated from patients with elevated type I IFN levels, spontaneously produced NETs and displayed indicators of oxidative and mitochondrial stress (39). A similar phenomenon was observed in neutrophils from healthy controls that were exposed to patient plasma samples or exogenous IFN (39).

Here, we demonstrate that type I IFN-mediated activation of neutrophils in lungs may lead not only to the prominent tissue damage, but it can also support biofilm formation by *Pseudomonas* and its tissue persistence.

The role of neutrophils during *P. aeruginosa* infections is not clear. The high (up to 50%) proportion of the neutrophils in the body exists in so called marginating pool in the microcirculatory vessels of the liver, spleen, bone marrow and lungs, and can

be fast mobilized in response to infection. The consumption of neutrophils at the sites of inflammation is compensated by the elevated emergency myelopoiesis (40). Neutrophils rapidly respond to the infection, the release of NETs by these cells can be observed already during first 10 min after contact with the pathogen (41).

Pseudomonas aeruginosa evolved multiple mechanisms to evade host immune responses. For example, ROS produced by neutrophils triggers mutations in *P. aeruginosa*, including those responsible for exopolysaccharide production and biofilm formation (42). This may promote further adaptation and survival of bacteria. Biofilm formation by *P. aeruginosa* could be supported by eDNA released by neutrophils (5). Bacteria within biofilms are protected from the immune system and eventual antibiotic treatment (4, 6), which leads to their high persistence in patients. In agreement, we observe the accumulation of GFP-expressing (alive) bacteria in histone-rich areas (NETs). Obviously, *Pseudomonas* is not only able to survive the contact with neutrophil NETs, but can also utilize NETs components to produce biofilms.

Persistence of the pathogen leads to elevated immune responses and severe cytotoxicity. Excessive activation of neutrophils, or their prolonged survival in tissues during chronic inflammation, is associated with a high tissue damage due to release of proteases, such as neutrophil elastase (43) or matrix metalloproteinase-9 (44). Resulting cleavage of opsonizing complement molecules or receptors on immune cells could lead

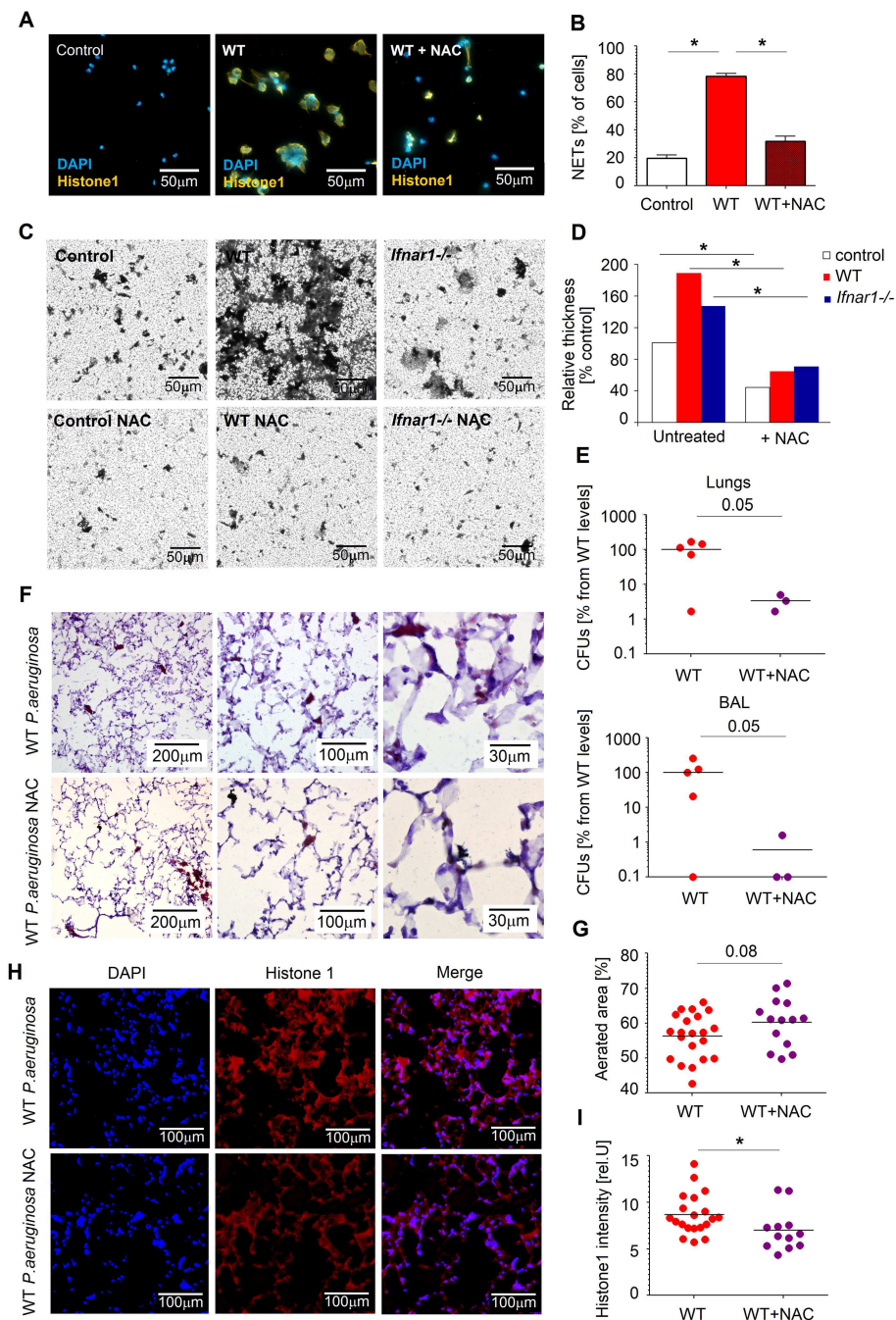


FIGURE 6 | NAC disrupts NETosis and prevents biofilm formation. **(A,B)** NAC prevents NETs formation by WT neutrophils. Neutrophils were challenged with *P. aeruginosa* MOI 10 for 4 h in presence and absence of NAC, NETs were stained with anti-histone 1 (orange) and DAPI (blue). Exemplified staining, scale bars: 50 μm **(A)**. Quantification of NET-positive cells **(B)**. At least 5 fields of view per group were analyzed. **(C,D)** NAC treatment of neutrophils and following inhibition of NETosis prevents biofilm formation by *Pseudomonas*. Neutrophils were challenged with *P. aeruginosa* as described above (+ and -NAC), biofilms were stained with crystal violet, control—bacteria only. Exemplified biofilm staining, scale bars: 50 μm. **(C)** Quantification of biofilms was performed using acetic acid and OD measurement. **(D)** Data are shown as mean ± SEM, * $p < 0.05$. **(E)** Reduced bacteria load in lungs of NAC-treated mice. WT mice were treated with NAC at day -1 and 1 h before *Pseudomonas* infection. Mice were sacrificed after 20 h, bacterial load in lungs and BAL determined by plating of serial dilutions on LB agar. CFUs were estimated. At least 3 animals per group were included. **(F,G)** Less lung injury after NAC treatment of mice. Mice were infected as described above in E, lungs were isolated, 5 μm cryosections prepared and stained with hematoxylin-eosin. Representative staining is shown, scale bars: 200, 100, and 30 μm **(F)**. Quantification of aerated areas in at least 5 fields of view in 4 lungs per group **(G)**. **(H,I)** Reduced NETs content in lungs of mice treated with NAC. WT mice were infected as described above, animals were sacrificed after 20 h, 5 μm cryosections of lungs were stained with anti-histone 1 (red) and DAPI (blue). Representative staining of NETs in lungs is shown, scale bars: 100 μm **(H)**. Quantification of NETs in lungs in 4–5 fields of view in 4 lungs per group **(I)**. Data are shown as individual values and mean, * $p < 0.05$.

to immune mismatch and thus to the escape from the immune control (45, 46).

We could demonstrate that type I IFNs excessively activate neutrophils and trigger their ROS production and NETs release in response to *P. aeruginosa* infection. This supports biofilm formation by the bacteria and their survival in lungs. Interestingly, the effect of type I IFNs varies between different models of bacterial, fungal and parasite infections. In certain studies the protective role of IFNs for the host was proven during the microbial infections (19–24), while others revealed increased tissue damage and bacteria colonization in the presence of IFNs (25–29). These differences might be partly explained by the involvement of the distinct cell subsets in antibacterial immune responses, which is due to the diverse, extra- or intracellular, localization of pathogens. Moreover, bacteria developed various protecting mechanisms, such as biofilm formation, that are influencing the outcome of infection. Numerous bacterial species, including *P. aeruginosa*, utilize extracellular eDNA for the biofilm matrix formation (3). Possibly, the mechanism responsible for it includes surface modifications that protect from DNA-induced membrane destabilization and NET-mediated killing (47). Another host-driven mechanism of *P. aeruginosa* resistance, independent from biofilm formation, could be the aggregation of bacteria that is induced by entropic forces generated by host polymers abundant at chronic infection sites, such as DNA, F-actin or mucin (48).

The survival of bacteria in biofilms is one of the possible mechanisms associated with the inappropriate regulation of neutrophil functions. NETs released by neutrophils represent the source of various biologically active components, which are shown to induce inflammation or tissue damage through multiple mechanisms. NET-derived histones are cytotoxic themselves and were shown to induce epithelial and endothelial cell death (49). While anti-histone antibodies had protective functions, DNase treatment was not effective (49). Moreover, circulating histones can serve as mediators of lung injury during pathological conditions such as trauma, and their levels correlate with lung tissue damage (50). Histones act as damage-associated molecular pattern proteins, activating the immune system and leading to elevated cytotoxicity (51). Possibly, the elevated lung tissue damage observed in WT mice is induced by the high release of histone-containing NETs.

In this manuscript, we show detrimental effect of the elevated IFN signaling during *P. aeruginosa* infection. We observe that *P. aeruginosa* is able to utilize NET components released from activated neutrophils to form biofilms. In the absence of IFN signaling, the release of ROS and NETs is significantly diminished. This leads to reduced biofilm production and lower bacterial count in lungs of infected IFN-deficient mice, as compared to WT. Different bacterial load between WT and IFN-deficient mice is due to altered activation of neutrophils and different amounts of released NETs. Binding to DNA in NETs is a crucial step facilitating biofilm formation by *Pseudomonas*. This could be proven by the infection with *P. aeruginosa* strain that is unable to bind DNA (*pelA* mutant 24480) (37) and therefore cannot efficiently produce biofilms. Bacteria without the protection of biofilms was efficiently cleared in both mouse strains, IFN-deficient and –sufficient. This was independent

of the various amounts of NETs measured in these mice, as neutrophil ability to release NETs in response to PA24480 strain was comparable to wild type bacterial strain. It proves the essential role of NETs in the stimulation of biofilm formation.

Multiple treatment modalities are insufficient in infectious complications with *P. aeruginosa*. The reasons for that are on the one hand a high intrinsic heterogeneity of the bacteria, and on the other hand the ability of *Pseudomonas* to form protecting biofilms. Therefore, treatment with factors that disturb biofilm formation should be considered as a strategy to prevent or treat *P. aeruginosa* complications. This is particularly important in inflammatory conditions associated with augmented neutrophil activation. We could show here that NAC, which is a reducing agent and a ROS scavenger, decreases biofilm formation by *Pseudomonas*. Besides its capacity to directly disturb bacterial biofilms by reducing disulfide bonds in mucopolysaccharides (52), NAC has an immunotropic activity by suppressing NETs formation by neutrophils (53). Here, we demonstrated promising therapeutic effect of NAC in *P. aeruginosa* infected mice. NAC treatment significantly improved the clearance of bacteria from lungs, diminished lung tissue damage, and decreased amounts of NETs in lungs. *In vitro* experiments corroborated these results, showing a direct inhibitory effect of NAC on NETs release by neutrophils and, later, on the NET-dependent biofilm production by *Pseudomonas*. In addition, we could validate the efficiency of DNase in the prevention of NET-mediated biofilm formation. This is in agreement with data showing improved clearance of *P. aeruginosa* from the lungs of cystic fibrosis patients after the treatment with DNase (54).

In summary, our work describes detrimental role of type I IFNs during lung infection caused by *P. aeruginosa* in mice. IFNs stimulate pro-inflammatory functions of neutrophils that lead to boosted NETs production by these cells. Binding to NETs activates in turn the formation of biofilms by *P. aeruginosa*. Hidden in biofilms, *Pseudomonas* is able to persist in lungs due to the limited sensitivity to antimicrobials, which causes a progressing therapeutic challenge for patients and clinicians. Therefore, we suggest that NETs-disrupting agents should be considered as a treatment or prophylaxis in clinical conditions associated with elevated type I IFNs levels, such as cancer or viral infections.

DATA AVAILABILITY

The material is available upon request to interested researchers.

ETHICS STATEMENT

This study was carried out in accordance with the regulatory authorities LANUV (Das Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen), Germany. Our animal care and used protocols adhere to the regulations of das Deutsche Tierschutzgesetz (TierSchG) and follow FELASA recommendations. The protocol was approved by the regulatory authorities LANUV (Das Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen).

AUTHOR CONTRIBUTIONS

EP: conceptualization, methodology, project administration, formal analysis, writing, and original draft preparation. SB, IS, and AD: formal analysis. VV: formal analysis, software, writing, and original draft preparation. SH: resources, writing, review, and editing. SL: resources, writing, review, and editing. JJ: conceptualization, project administration, supervision, funding acquisition, resources, writing, review, and editing.

FUNDING

The study was supported by Deutsche Forschungsgemeinschaft (DFG/ JA 2461/2-1) and Deutsche Krebshilfe (111647).

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ACKNOWLEDGMENTS

We thank M. Carevic (University Tuebingen, Germany) and C. F. Wu (E-DA Hospital/I-Shou University, Kaohsiung, Taiwan) for the help in the establishment of methods. We also want to thank IMCES, Endocrinology, and Microbiology Department and A. Hasenberg (University hospital Essen, Germany) for equipment support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.02190/full#supplementary-material>

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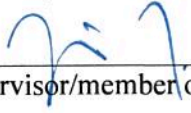
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In accordance with § 6 (para. 2, clause g) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I represent the field to which the topic „Type I IFN-mediated polarization of neutrophils and its impact on cancer progression and bacterial infections“ is assigned in research and teaching and that I support the application of Ekaterina Pylaeva.


Essen, date 01.11.20 Priv.-Doz. Dr. Jadwiga Jablonska-Koch
Name of the scientific supervisor/member of the University of Duisburg-Essen


Signature of the supervisor/member of the University of Duisburg-Essen

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In accordance with § 7 (para. 2, clause d and f) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I have written the herewith submitted dissertation independently using only the materials listed, and have cited all sources taken over verbatim or in content as such.


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