SIRT1-mediated deacetylation of FOXO3a transcription factor supports pro-angiogenic activity of interferon-deficient tumor-associated neutrophils

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Abstract
Angiogenesis plays an important role during tumor growth and metastasis. We could previously show that Type I interferon (IFN)-deficient tumor-associated neutrophils (TANs) show strong pro-angiogenic activity, and stimulate tumor angiogenesis and growth. However, the exact mechanism responsible for their pro-angiogenic shift is not clear. Here, we set out to delineate the molecular mechanism and factors regulating pro-angiogenic properties of neutrophils in the context of Type I IFN availability. We demonstrate that neutrophils from IFN-deficient (Ifnar1−/−) mice efficiently release pro-angiogenic factors, such as VEGF, MMP9 or BV8, and thus significantly support the vascular normalization of tumors by increasing the maturation of perivascular cells. Mechanistically, we could show here that the expression of pro-angiogenic factors in neutrophils is controlled by the transcription factor forkhead box protein O3a (FOXO3a), which activity depends on its post-translational modifications, such as deacetylation or phosphorylation. In TANs isolated from Ifnar1−/− mice, we observe significantly elevated SIRT1, resulting in SIRT1-mediated deacetylation of FOXO3a, its nuclear retention and activation. Activated FOXO3a supports in turn the transcription of pro-angiogenic genes in TANs. In the absence of SIRT1, or after its inhibition in neutrophils, elevated kinase MEK/ERK and PI3K/AKT activity is observed, leading to FOXO3a phosphorylation, cytoplasmic transfer and inactivation. In summary, we have found that FOXO3a is a key transcription factor controlling the angiogenic switch of neutrophils. Post-translational FOXO3a modifications regulate its transcriptional activity and, as a result, the expression of pro-angiogenic factors supporting development of vascular network in growing tumors.

Abbreviations: αSMA, α-smooth muscle actin; AKT, protein kinase B; BV8, prokineticin 2 (PROK2); EX527, selective SIRT1 inhibitor (Selisistat); FOXO3a, forkhead box protein O3a; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase; MMP9, matrix metalloproteinase 9; RAS, rat sarcoma viral oncogene homolog; SIRT1, NAD-dependent deacetylase sirtuin-1 (silent mating type information regulation 2 homolog).

Iris Helfrich and Jadwiga Jablonska contributed equally to this study.

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Therefore, targeting FOXO3a activity could provide a novel strategy of anti-angiogenic targeted therapy for cancer.

**KEYWORDS**
deacetylation, FOXO3a, melanoma, pro-angiogenic neutrophils, SIRT1, tumor angiogenesis, tumor-associated neutrophils, Type I IFN, vascular normalization

**What’s new?**
Angiogenesis plays an important role in tumor growth and metastasis. Tumor-associated neutrophils (TANs) are known to affect tumor angiogenesis, but how? In this study, the authors found that a lack of interferon-1 (IFN-1) activates a molecular cascade within TANs that involves Sirtuin 1 (SIRT1) and the transcription factor FOXO3a. This cascade, in turn, can stimulate the release of pro-angiogenic factors into the tumor microenvironment. These insights into the molecular mechanisms of TAN regulation suggest new therapeutic targets for anti-angiogenic cancer therapies.

**1 | INTRODUCTION**

All solid tumors, like normal organs, need a proper vasculature to grow beyond a limited size. The initiation of tumor neovascularization involves the sprouting of pre-existent vascular endothelial cells and the recruitment of pericytes. Lately, tumor-associated neutrophils (TANs) gain increasing attention as cells influencing tumor angiogenesis. In fact, these cells orchestrate a broad spectrum of anti- or pro-tumor activities, depending on tumor environment. In this context, we could previously demonstrate that Type I interferons (IFNs) impair pro-angiogenic activity of neutrophils. In agreement, IFN-deficient mice show elevated intratumoral accumulation of pro-angiogenic neutrophils that support tumor vascularization and growth. Pro-angiogenic TANs show deregulation of multiple signaling pathways, including elevated Granulocyte colony-stimulating factor (G-CSF) signaling. Many factors involved in this pathway demonstrate regulatory activity, such as Sirtuin 1 (SIRT1), which is a potent protein deacetylase, and was shown to regulate multiple cellular functions, such as survival, differentiation and glucose metabolism, and to be overexpressed in solid tumors and hematopoietic malignancies. Nevertheless, the exact mechanism responsible for pro-angiogenic bias of IFN-deficient neutrophils is not clear.

Considering the emerging importance of TANs in the regulation of tumor angiogenesis, we seek to delineate the underlying mechanism and factors supporting the angiogenic potential of these cells. We could demonstrate here that dynamics of vessel development and pericyte maturation in melanoma depends strongly on IFN availability, and that tumor vasculature is established significantly earlier in Ifnar1−/− animals. Moreover, we observe that pro-angiogenic Ifnar1−/− TANs strongly support these processes via the release of angiogenic factors, and that their angiogenic potential is supported by the SIRT1-mediated activation of forkhead box protein O3a (FOXO3a) transcription factor. These results offer unexplored insights in the regulation of pro-angiogenic activity of TANs and offer alternative therapeutic targets to dampen tumor angiogenesis and progression.

**2 | MATERIALS AND METHODS**

**2.1 | Tumor cell lines and animals**

Melanoma B16F10 and CM tumor cells were used in all experiments. B16F10 (Research Resource Identifier [RRID]: CVCL_0159) was purchased from ATCC. The murine melanoma cell line CM was established from a spontaneously developed primary cutaneous melanoma of the MT/ret model expressing the human ret proto-oncogene under the control of the mouse metallothionein I promoter-enhancer. After a short latency of almost three weeks, ret-transgenic mice start to develop spontaneously multiple cutaneous melanomas and metastases in distant organs.

B16F10 cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM), CM cells in RPMI 1640 Medium (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS) (Biochrom, Merck, Darmstadt, Germany) and 1% (vol/vol) penicillin/streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA). Cells were grown in monolayer at 37°C in a humidified CO2 incubator. The cell line was regularly tested for mycoplasma contamination using the Venor GeM Kit (Minerva Biolabs, Berlin, Germany) and all experiments were performed with mycoplasma-free cells. Exponentially growing cells were harvested with 0.05% trypsin, washed and suspended in phosphate buffered saline (PBS). The number of cells was counted using a CASY cell counter (OMNI life science, Bremen, Germany).

Littermates (8-12 weeks old) of C57BL/6J wild-type (WT) and Ifnar1−/− strains were used in all experiments. Mice were bred and kept under specific pathogen free conditions in the animal facility of the University Hospital Essen (Essen, Germany). Experiments were done in accordance with government and institute guidelines and regulations.
2.2 | Primers

RT-qPCRs (real-time quantitative polymerase chain reactions) were performed with the following primers:

Rps9-f: 5'-TTGACCTGAGCAAGGAT-3', Rps9-r: 5'-AATC CAGCTTCTGTCGCTTT-3', Bv8-f: 5’-GCCAGCGAGGATCATCAT TT-3’, Bv8-r: 5’-AAATGCGGATATCAGGAA-3’. S100a8-f: 5’-TGGCATGTTGATAAAGTTG-3’, S100a8-r: 5’- GGCAGAAGCTGCTGCTAC-3’.

2.3 | Tumor model

Murine melanoma cells were injected subcutaneously (5 × 10^5 s.c.) into C57BL/6J WT and Ifnar1<sup>−/−</sup> mice and tumor growth was measured by caliper every 2 to 3 days. Tumor volume was calculated:

\[ V = \frac{4}{3} \pi (h \times w^2)/8 \]

2.4 | Immunohistochemistry and vascular network analysis

Tumors were dissected and snap frozen in liquid nitrogen. Consecutive cryosections (5 μm thickness) of murine tumors were fixed and processed for immunostaining as described before. In brief, to observe neutrophil infiltration in tumor tissue, cryosections were stained with rat anti-mouse/human-CD11b (clone: M1/70 BioLegend, San Diego, CA), rat anti-mouse-Ly6G (Clone 1A8, BioLegend, San Diego, CA) and rabbit anti-mouse-laminin (Clone AP1001.1, Immunodiagnostic AG, Bensheim, Germany). For the visualization of endothelial-pericyte association, double-stainings were performed by using rabbit anti-mouse laminin combined with rabbit anti-mouse-desmin (Abcam, Cambridge, UK), rat anti-mouse-CD140b (PDGFRβ), Affymetrix, California or anti-mouse-α-smooth muscle actin (αSMA, clone 1A4, Sigma-Aldrich/MERCK, Darmstadt, Germany), DAPI (4’,6-diamidino-2-phenylindole) (BioLegend, San Diego, CA) staining was used for the detection of nuclei. Hypoxic tumor areas were detected by the formation of pimonidazole adducts after injection of pimonidazole hydrochloride compound into tumor-bearing animals 20 minutes prior killing. Tumor sections were immunostained by using the Hypoxyprobe™-1 Plus Kit following manufacturer’s protocol (Natural Pharmacia International, Burlington, MA). The hypoxic area index was quantified as pimonidazole-positive tumor area (μm^2) by using the average of three tumor sections per tumor (top, middle, base) of concordant distance. For the detection of vessel perfusion, tumor-bearing C57BL/6J WT and Ifnar1<sup>−/−</sup> mice were intravenously injected (i.v.) using 150 μg FITC (fluorescein isothiocyanate) conjugated lectin/150 μL 0.9% NaCl from Bandeiraea simplicifolia (Sigma-Aldrich/MERCK, Darmstadt, Germany) 20 minutes before cervical dislocation. A perfusion index was quantified as the percentage of lectin<sup>+/CD31</sup> tumor vessels. For assessment of vascular stability, mice received 1 mg/100 μL Alexa 488-conjugated dextran (10,000 MW, Invitrogen, Oregon) via i.v. injection. After 15 minutes of incubation, mice were killed and histological analyses were performed by measuring the dextran-positive area around intratumoral blood vessels in WT and Ifnar1<sup>−/−</sup> tumors at indicated day post-transplantation (p.t.). Immunofluorescence was examined by Olympus X51 or Zeiss AxioObserver.Z1 inverted microscope with ApoTome optical sectioning equipped with filters for DAPI, FITC, Alexa Fluor 488, GFP, DsRed and Cy3. Images from 10 fields of view each equal to 0.5 mm^2 were processed with Zen Blue 2012 (Carl Zeiss AG, Jena, Germany) and analyzed with FIJI (ImageJ)<sup>11</sup> and AngioTool softwares.<sup>12</sup>

2.5 | Flow cytometry

Single-cell suspensions from tumors were prepared: erythrocytes were removed using erythrocyte lysis buffer. Single-cell suspensions were prepared for flow cytometry staining (gating strategy Figure S1A,B).

Single-cell suspensions were stained in PBS with conjugated antibodies listed below, and flow cytometry was performed using BD FACSCanto and data was analyzed using BD FACSDiva software (BD Biosciences, Franklin Lakes, NJ). Rat anti-mouse-CD16/32 (Clone 2.4G2, BD Pharmingen, BD, NJ), rat anti-mouse-human/CD11b (clone: M1/70 BioLegend, San Diego, CA), rat anti-mouse-Ly6G (Clone 1A8, BioLegend, San Diego, CA), rabbit anti-FOXO3a (Clone 75D8, Cell Signaling, Danvers, MA), anti-pFOXO3a (ab47285, Abcam, Cambridge, UK) and to determine viable cells viability dyes eFluor 780 (eBioscience, Affymetrix, CA) and DAPI (BioLegend, San Diego, CA) were used. For intracellular staining, Cytofix-Cytoperm buffer was used (BD Biosciences, Franklin Lakes, NJ).

2.6 | TAN isolation

Heparinized blood was collected via heart puncture and tumors were harvested and digested using dispase II 0.2 μg/mL, collagenase D 0.2 μg/mL and DNase I 100 μg/mL (all from Sigma-Aldrich, Merck KGaA, St. Louis, MO) solution in Dulbecco’s modified Eagle medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% (vol/vol) heat-inactivated FCS and 1% (vol/vol) penicillin/streptomycin. Cells were meshed through 50-μm filters (Cell Trics, Sysmex, Goerlitz, Germany) and erythrocytes lysed in ACK buffer. Single-cell suspensions were stained with Ly6G and CD11b. Viable CD11b<sup>+</sup>Ly6G<sup>+</sup> TANs were sorted using a FACS Aria cell sorter (BD Biosciences, Franklin Lakes, NJ) and the purity of cells was assessed (≥90%) (Figure S1).

2.7 | Immunofluorescence and subcellular localization

Isolated WT and Ifnar1<sup>−/−</sup> tumor associated neutrophils, 15 000/well were incubated with or without treatment with 1 μM EX-527/SIRT1
inhibitor), in glass-bottom 96-well plate (MatTek Corporation, MA) pre-coated with poly-β-lysine 1 mg/mL (Sigma-Aldrich/Merck, Darmstadt, Germany) for 4 hours at +37°C, 5% CO2. Samples were fixed with paraformaldehyde (Thermo Fisher Scientific, Waltham, MA) to the final concentration 4%, permeabilized with 0.2% Triton X-100 (Sigma-Aldrich/Merck, Darmstadt, Germany) containing buffer and then blocked in 5% BSA (bovine serum albumin). Rabbit Anti-FOXO3a (Clone 75D8, Cell Signaling, Danvers, MA) and anti-pFOXO3a (ab47285, Abcam, Cambridge, UK) were used to define the level of subcellular localization and phallolidin (Flash Phallolidin Green 488, BioLegend, San Diego, CA) was used to determine cytoskeleton. Stainings were mounted with ProLong Gold Antifade Mountant with DAPI (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Immunofluorescence imaging was performed by Zeiss ELYRA PS.1 Super-resolution and TIRF (total internal reflection fluorescence) microscope combined with a LSM 710 confocal and fluorescence intensity of FOXO3a and pFOXO3a proteins in nuclear and cytosolic compartments of neutrophils were analyzed using FIJI (ImageJ) software.

2.8 | Tumor lysis

Regarding the strong pigmentation of B16F10 biopsies, which does not allow to receive reliable photometric-based protein measurements, tumor samples of the same weight were used and lysed in 2x Cell Lysis Buffer (cat. 9803, Cell Signaling) complemented with Protease Inhibitor Cocktail (cat. 11836153001, Roche, Basel, Switzerland), 1% PMSF (phenylmethylsulfonyl fluoride) and 0.1% SDS (sodium dodecylsulfate), using the tissue protein extraction program of a Gentle MACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.9 | Western blot

Tumor lysates were separated by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis). Proteins were transferred on 0.45 μM nitrocellulose membranes and probed over night at 4°C with the primary antibodies. Following antibodies were used: Erk (Clone 137F5), pErk (Clone 197G2), Akt (Clone C67E7), pAkt (Clone 193H12), STAT3 (Clone 124H6), pSTAT3 (Clone 124H6) and FoxO3a (Clone 75D8cat. 2497) (all Cell Signaling, Danvers, MA), pFOXO3a (ab47285, Abcam, Cambridge, UK) and actin (cat. 691001, MP Biomedicals, Valiant, China). Membranes were incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibodies, allowing signal visualization with Pierce ECL Western Blotting Substrate (cat. 32106, Thermo Fischer Scientific, Waltham, MA). Signals were quantified using ImageJ software. All protein signals were normalized to actin. Afterward phospho-protein signals were normalized to the total protein expression and the fold change was calculated for each individual protein. Stripping of nitrocellulose membranes was performed by using Restore Western Blot Stripping Buffer (cat. 21095, Thermo Fischer Scientific, Waltham, MA) according to the manufacturer's instructions. After reblocking of the membrane, immunodetection was performed as described before.

2.10 | Proteome profiler antibody array

Cytokine profiles were analyzed by using a Proteome Profiler Antibody Array (Mouse XL Cytokine Array Kit, cat. ARY028, R&D Systems, Bio-Technne, Minneapolis, MN) according to manufacturer's instructions. In brief, antibody-coated membranes were incubated with tumor lysates at 4°C over night, washed three times and followed by incubation with a biotin-conjugated antibody cocktail for 1 hour at room temperature. Membranes were washed and HRP-conjugated antibodies added, allowing the detection of cytokines by enhanced chemiluminescence reaction. Quantification of signal intensity was done using FIJI (ImageJ) software. Signal intensities were normalized to the control spots intensities on the corresponding membranes.

2.11 | Aortic ring assay

Aortic ring assay was performed as described previously.13 Shortly, thoracic aortae from WT mice were dissected, fat and connective tissue were removed and 0.5 mm aorta rings were placed in 96-well plates coated with type I collagen (Enzo life sciences, Farmingdale, NY). Once embedded, rings were covered with the endothelial cell growth medium (Promocell GmbH, Heidelberg, Germany). Subsequently, 2 × 10^6 TANs were added (WT, Ifnar1−/− or EX-527-treated Ifnar1−/−). The wells imaged 12 days after incubation in 37°C using AMG EVOS digital phase contrast inverted microscope and analyzed by FIJI (ImageJ) software.

2.12 | ELISA (Enzyme-linked Immuno Sorbent Assay)

TANs were isolated from WT and Ifnar1−/− melanoma B16F10-bearing mice (as described above), and coincubated with B16F10 tumor cells in ratio 10 000:10 000 in 100 μL DMEMc for 24 hours. 10 000 cells B16F10 alone in 100 μL DMEMc were used as a control. Supernatants were collected and the concentration of Vegf and Mmp9 were estimated according to the manufacturer’s protocol (R&D, Minneapolis, MN).

2.13 | Statistics

Statistical analyses were performed using Kruskal-Wallis ANOVA (english analysis of variance) method for multiple comparisons with the Bonferroni correction, Mann-Whitney U test was performed for two independent samples. P < 0.05 was considered significant.

3 | RESULTS

3.1 | Type I IFN-deficiency drives vessel normalization in growing tumors

Type I IFN-deficient (Ifnar1−/−) mice show enhanced tumor growth after transplantation of B16F10 melanoma cells, when compared to
their WT counterparts (Figure 1A,B). This phenomenon is associated with an elevated infiltration of CD11b+ Ly6G+ neutrophils into the tumor site of Ifnar1−/− mice (Figure 1C,D), which accumulate preferentially in close vicinity to the tumor vascular network (Figure 1E). Importantly, Ifnar1−/− neutrophils express significantly more pro-angiogenic factors, such as Vegf, Mmp9, Bv8 and S100a8, than WT counterparts (Figure 1F,G).

As tumor growth depends on the efficient vasculature, and this seems to be supported by pro-angiogenic action of neutrophils, we have assessed the vascular tumor network in Ifnar1−/− vs WT mice. Interestingly,

FIGURE 1 Ifnar1−/− mice show elevated B16F10 tumor growth and increased pro-angiogenic neutrophil counts in tumors. (A, B) B16F10 tumor size and weight. (C) Quantification of neutrophil infiltration in tumor cryo-sections, 10 fields of view (1 mm²) were analyzed.

(D) Exemplified staining showing tumor-infiltrating neutrophils, CD11b (red), Ly6G (blue), laminin (green). (E) Exemplified staining showing neutrophil localization in tumors—close proximity to vessels mainly in Ifnar1−/− (arrows: neutrophils). (F) Pro-angiogenic factors expressed in isolated TANs. TANs were sorted and relative expression of proangiogenic gene expression was assessed by qRT-PCR in Ifnar1−/− mice compared to WT (expression set to 1) mice.

(G) Pro-angiogenic factors released by isolated tumor-associated neutrophils in response to tumor B16F10 cells in vitro. Protein concentration of Vegf and Mmp9 was assessed with Enzyme-Linked Immunosorbent Assay (ELISA) in 24 hours cell culture supernatants. Tumor cells were injected s.c. into the flank of mice and tumor growth and neutrophil infiltration in tumor cryosections were assessed 14 days post-tumor induction. Scale bars: 200, 50 μm. Neutrophils were sorted from tumor tissue, RNA isolated and real-time quantitative PCR (RT qPCR) performed. A representative result from two replicate experiments is shown with n = 5 for all cohorts. For comparison of two independent groups, Mann-Whitney U test was used. Data are shown as median, interquartile range, minimal and maximal values (B, C); median and interquartile range (A, F); each replicate with the line on mean (G), *P < .05; ***P < .001; ****P < .0001. TANs, tumor-associated neutrophils; WT, wild-type.
FIGURE 2  Lack of Type I IFN receptor signaling supports accelerated tumor angiogenesis and vascular normalization (pericyte maturation) in growing tumors. (A-F) Quantification of vascularization of tumors (number of vessels (A), total area of vessels (B), vessel perimeter (C) and maturation stages of pericytes: PDGFRβ (D), desmin (E) and SMA coverage (F) area), calculated in 10 fields of view. (G-I) Representative images of pericyte staining in tumor sections at Day 14 post-tumor induction. Laminin (red) and PDGFRβ, Desmin, SMA (green). Scale bars: 200 and 50 μm. (J-L) Kinetics of tumor vascularization in Ifnar1−/− and WT mice. Number of vessels (J), area of vessels (K), total vessel perimeter (L). (M-O) Quantification of pericyte maturation: immature pericyte staining: PDGFRβ (M), intermediate stage of maturation: Desmin (N) and fully matured vessels: SMA (O). Tumor cells were injected s.c. into the flank of mice. Mice were sacrificed in different time points (d1, d4, d9 and d14), cryosections prepared and stained, pericyte maturation markers were analyzed. Vascularization and pericyte coverage was calculated in 10 fields of view (each equal to 0.5 mm²). Two independent experiments were performed, with at least five mice per group (n = 5). For comparison of two independent groups, Mann-Whitney U test was used. Data are shown as mean ± SEM (A-F); median and interquartile range (J-O). *P < .05; **P < .01; ***P < .001; ****P < .0001. IFN, interferon; SMA, smooth muscle actin; WT, wild-type
whereas the number of vessels does not differ between WT and Ifnar1<sup>−/−</sup> tumors (Figure 2A), the area covered by vascular network is significantly higher in tumors growing in Ifnar1<sup>−/−</sup> mice (Figure 2B). This was due to the increase in vessel perimeter in such animals (Figure 2C). Thus, we next addressed the vascular function in both groups.

Stabilization and maturation of the vascular network depends on the recruitment of mural cells, so called pericytes, and vascular smooth muscle cells, to nascent blood vessels. Therefore, we performed morphometric analyses by using the well-established pericyte markers, known to be expressed at different stages of vascular maturation: PDGFR<sub>b</sub> (Platelet-derived growth factor receptor beta, early stage), desmin (intermediate stage) and αSMA (smooth muscle actin, late stage). In line with the enhanced vascular lumen in Ifnar1<sup>−/−</sup> mice, we observed elevated vascular normalization in these animals, reflected by a significantly higher coverage by PDGFR<sub>b</sub><sup>+</sup> and Desmin<sup>+</sup> pericytes, and enhanced αSMA<sup>+</sup> perivascular cells (Figure 2D-I).

**FIGURE 3** Elevated levels of FOXO3a are detected in nucleus of Ifnar1<sup>−/−</sup> TANs, while inactive cytosolic pFOXO3a form is downregulated. (A) FOXO3a expression in tumor lysates from WT and Ifnar1<sup>−/−</sup> mice. Lysates were collected from WT and Ifnar1<sup>−/−</sup> mice at Day 14 and western blot performed, FOXO3a expression was normalized to β-Actin. (B, D) Quantification of nuclear FOXO3a intensity in WT vs Ifnar1<sup>−/−</sup> TANs (B) and representative immunofluorescence images (D). (C, E) Quantification (C) and representative immunofluorescence images (E) of cytosolic pFOXO3a intensity in WT vs Ifnar1<sup>−/−</sup> TANs. TANs were sorted at Day 14 post-tumor cell injection and stained for FOXO3 and pFOXO3 protein. Scale bars: 2 μm. (F, G) Flow cytometry analysis of neutrophils from WT and Ifnar1<sup>−/−</sup> mice decreased expression of pFOXO3a (F) and decreased ratio of mean fluorescence intensity (MFI) of pFOXO3a to FOXO3a in tumor-associated neutrophils in Ifnar1<sup>−/−</sup> mice in comparison to WT (G). For comparison of two independent groups, Mann-Whitney U test was used. Data are shown as mean ± SEM, and median, interquartile range, minimal and maximal values, *P < .05; ***P < .001; ****P < .0001. TANs, tumor-associated neutrophils; WT, wild-type.
In order to monitor changes in the vasculature along tumor progression, vessel growth and pericyte maturation were followed at Day 1, 4, 9 and 14 post-tumor cell injection. Of note, we could demonstrate significantly accelerated progress of tumor vascularization in Ifnar1−/− mice, in comparison to WT animals (Figure 2j-o).

Caused by the finding of enhanced intratumoral pericyte coverage in the Ifnar1-deficient background, we next addressed the vascular function. Whereas tumors of both genetic backgrounds showed corresponding vascular perfusion (Figure S2A,B), penetration of Alexa-488 coupled dextran, measured 20 minutes after i.v. application, was significantly reduced in Ifnar1−/− transgenic mice (P = .0008) as a result of enhanced vascular maturation in this tumors (Figure S2C,D). Interestingly, prominent tumor hypoxia was detected as a result of enhanced tumor proliferation kinetics starting from Day 9 p.t. in the tumors of Ifnar1-deficient mice when compared to WT controls (Figure S2E,F).

### 3.2 Elevated pro-angiogenic protein expression in tumors growing in Ifnar1−/− mice

Our data show that tumor angiogenesis is regulated by neutrophils. As these neutrophils efficiently infiltrate tumor tissue, we evaluated the expression of pro-angiogenic proteins in the tumor tissue of WT and Ifnar1−/− mice, at early (Day 10 postinjection) and late (Day 14) tumor development stage, using a Proteome Profiler Assay. In total, we detected differences in the expression of 38 proteins with angiogenic activity in tumor tissue (Figure S3A). At Day 10, we could identify the upregulated expression of CCL22, CCL6, FGF-21 and VEGF in tumors from Ifnar1−/− mice (Figure S3B). These factors facilitate tumor progression, metastasis, and promote angiogenesis. At late stage of tumor development (Day 14), we detected in Ifnar1−/− tumors upregulated Fetuin A, promoting tumor angiogenesis (Figure 3A). FOXO3a

### 3.3 Pro-angiogenic Ifnar1−/− neutrophils show elevated activity of FOXO3a

Comparing of gene and protein expression in tumor lysates of WT and Ifnar1−/− mice revealed significantly elevated expression of transcription factor FOXO3a in samples from Ifnar1−/− animals (Figure 3A). FOXO3a is involved in multiple cellular processes, including angiogenesis, and its activity depends on its cellular localization and posttranslational modification. Phosphorylation of this factor by kinases leads to its inactivation and cytoplasmic retention, while deacetylation, leads to FOXO3a activation and nuclear localization.

As tumors from Ifnar1−/− mice show elevated level of FOXO3a, we assessed its activity and posttranslational modification in Ifnar1−/− TANS, and compared it to WT counterparts. We have isolated TANS from tumors of Ifnar1−/− or WT mice, and stain their FOXO3 and pFOXO3. We could demonstrate significantly higher expression of active nuclear form of FOXO3a in pro-angiogenic Ifnar1−/− neutrophils (Figure 3B,D), while expression of inactive pFOXO3a was higher in the cytosol of WT TANS (Figure 3C,E). In agreement, the expression of pFOXO3a and the ratio of inactive pFOXO3a to active FOXO3a, as assessed by flow cytometry, was significantly elevated in WT TANS (Figure 3F, G).

In line with these findings, MEK/ERK and PI3K/AKT kinases, which are involved in FOXO3a phosphorylation, were upregulated in WT tumor lysates, as compared to Ifnar1−/− (Figure 4A,B). Thus, elevated kinase activity results in phosphorylation of FOXO3a and its repressed activity in WT neutrophils.

### 3.4 Inhibition of FOXO3a deacetylation in pro-angiogenic Ifnar1−/− neutrophils leads to its cytosolic transfer and inactivation

Deacetylation of FOXO3a is mediated by sirtuins, such as SIRT1, and leads to its activation and nucleus retention. In agreement with
Ifnar1<sup>−/−</sup> TANs, which are characterized with higher level of active nuclear FOXO3a, show a significant upregulation of SIRT1 deacetylase, as compared to WT (Figure 5A).

To proof the role of SIRT1 in the activation of FOXO3a in TANs, we inhibited this factor using its molecular inhibitor EX527 and evaluated the expression and activity of FOXO3a. Indeed, we could observe diminished FOXO3a gene expression and its reduced nuclear retention in treated Ifnar1<sup>−/−</sup> cells (Figure 5B-D). Moreover, elevated levels of inactive pFOXO3a were detected in the cytosol of EX527-treated neutrophils, as compared to untreated cells (Figure 5E,F).

**FIGURE 5** Legend on next page.
Inhibition of SIRT1 activity by EX527 leads to elevated phosphorylation of FOXO3a in and Mann-Whitney tumor-bearing mice. Ifnar1 tumor angiogenesis and growth when transferred into tumor-bearing mice (Figure 5).

To evaluate the effect of EX527-treatment on pro-angiogenic functionality of TANs, we performed quantitative aortic ring angiogenesis assay using TANs as angiogenic stimulus. This assay allows ex vivo assessment of all steps of efficient angiogenesis, including endothelial cell proliferation, migration, tube formation, microvessel branching, perivascular recruitment and remodeling. TANs from WT and Ifnar1−/− mice were isolated, treated with EX527 and applied on aortic rings. After 14 days of coculture, number and length of developed microvessels were evaluated. We could observe significantly elevated angiogenic activity of Ifnar1−/− TANs in comparison to WT TANs (Figure S5H). Moreover, treatment of Ifnar1−/− TANs with EX527 significantly reduced the pro-angiogenic capacity of such TANs, which was now comparable to WT levels. As expected, inhibition of SIRT1 in WT TANs has shown no visible effect on angiogenic properties of TANs, possibly due to already low amounts of this molecule in WT neutrophils.

3.5 TANs with inactive FOXO3a fail to support tumor angiogenesis and growth when transferred into tumor-bearing mice

To confirm the role of SIRT1-mediated activation of FOXO3a in pro-angiogenic capacity of neutrophils, a rescue experiment was performed. Neutrophils were isolated from WT or Ifnar1−/− mice, incubated with EX527 as described previously, mixed with B16F10 cells and injected s.c. into WT mice. At Day 3 p.t., tumor-bearing mice received EX527-treated or medium-treated WT or Ifnar1−/− neutrophils (i.v.), respectively. Tumor growth was monitored for 14 days. As expected, inhibition of SIRT1 in WT TANs has shown no visible effect on angiogenic properties of TANs, possibly due to already low amounts of this molecule in WT neutrophils. This effect (Figure 6A,B). As expected, mice injected with WT neutrophils show decreased tumor growth and EX527-treatment of WT TANs did not significantly change their effect on tumor growth. Therefore, we have excluded this group (EX527-treated WT) from further analyses.

In line with our ex vivo data, mice injected with EX527-treated Ifnar1−/− neutrophils showed significantly reduced maturation of tumor vasculature, in comparison to mice injected with untreated Ifnar1−/− neutrophils (Figure 6C-K), indicating once more that SIRT1-mediated deacetylation is required for the activation of FOXO3a and pro-angiogenic function of TANs.

3.6 Regulation of angiogenic properties of TANs by post-translational modification of FOXO3a is a common mechanism in preclinical melanoma

We next asked if our observation in B16F10 melanoma model is cell line- or tumor entity-specific. Therefore, we extended our analyses by using a second syngeneic melanoma model—CM. CM melanoma cells were s.c. injected into Ifnar1−/− and WT control mice, as described for B16F10, and tumor growth was monitored for 14 days (Figure S4A). After 14 days, mice were sacrificed, tumors removed, tumor weight and tumor angiogenesis assessed. CM tumors growing in Ifnar1−/− mice showed significantly accelerated growth in comparison to WT mice, similar to B16F10 cells. Tumors in Ifnar1−/− mice grew faster and reached significantly larger sizes. In addition, higher numbers of TANs infiltrated such tumors (Figure S4B). In all, immunohistological and morphometric analyses showed more advanced tumor angiogenesis in Ifnar1−/− mice (Figure S4C-E), as predicted.

In agreement with B16F10 data, analysis of FOXO3a activation in TANs showed a significantly enhanced nuclear retention of active FOXO3a in Ifnar1−/− neutrophils (Figure S5A,B), while WT cells exhibit high levels of inactive cytosolic pFOXO3a (Figure S5C,D). Thus, regulation of angiogenic properties of neutrophils by post-translational modification of FOXO3a appears to be a general phenomenon in preclinical melanoma models.
FIGURE 6  Adoptive transfer of EX527-treated neutrophils suppresses maturation of pericytes covering tumor vasculature. (A, B) Comparison of B16F10 tumor growth and weight after adoptive transfer of TANs. Blood and tumor neutrophils were sorted from B16F10 bearing WT and Ifnar1^-/- mice, cultivated with or without EX527, 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 EX527-treated or medium-treated neutrophils (i.v.), respectively. Tumors were removed at Day 14, cryosections were prepared and tumor vascularization was analyzed. (C-E) Quantification of tumor vascularization: number of vessels (C), total area of vessels (D) and vessel perimeter (E). (F-H) Maturation status of pericytes: PDGFRβ (F), Desmin (G) and SMA (H) coverage area. Vascularization and maturation status of pericytes was quantified in 10 fields of view (each equal to 0.5 mm²). (I-K) Representative images used for quantitation showed in (F-H). Two independent experiments were performed, with at least five mice per group. For comparison of multiple independent groups, Kruskal-Wallis test was used. Data are shown as median, interquartile range, minimal and maximal values and mean ± SEM. *P < .05. SMA, smooth muscle actin; TANs, tumor-associated neutrophils; WT, wild-type.
3.7 FOXO3a expression in human melanoma and head-and-neck cancer correlates positively with SIRT1 expression and is negatively correlated with Type I IFNs

In order to assess if the results obtained in our transplantable mouse tumor model translate to the human situation, we decided to analyze expression of Foxo3 in cancer tissue of patients suffering from melanoma. Using the available Gene Expression Omnibus database GSE65904 (reference Figure S6), we could confirm that the expression of Foxo3 in human melanoma tissue positively correlates with Sirt1 expression, suggesting similar regulatory mechanism in human and mice. In addition, a significant negative correlation between Foxo3 and Type I IFNs is observed in melanoma (Figure S6A,B). Interestingly, this phenomenon seems not to be limited to melanoma cancer, as samples obtained from HNC (head-and-neck cancer) patients show comparable correlation between Foxo3 and Sirt1, and Foxo3 and Ifnar1 (Figure S6C,D). Data for HNC were obtained from the available Gene Expression Omnibus database GSE65858 (reference Figure S6).

4 DISCUSSION

Tumor neovascularization is a complex process relying on the proliferation of pre-existing endothelial cells and the formation of new vascular sprouts. Many signaling pathways activated during tumor angiogenesis are identical to those involved during developmental vascular growth, the only difference is the absence of controlling mechanisms. Consequently, tumor-associated vessels represent an abnormal phenotype, are highly fragile and hyperpermeable because of reduced mural cell recruitment and vessel maturation.29

Multiple immune cells are involved in tumor angiogenesis, with neutrophils being one of the most prominent. Previously, we could show that IFN-deficient TANs efficiently support tumor angiogenesis and that removal of such cells suppresses this process.4 However, we could also show that tumor-associated macrophages do not play a significant role in our experimental system, as the depletion of neutrophils aborted angiogenesis and tumor growth, clearly showing no involvement of macrophages.6 Moreover, transfer of pro-angiogenic Ifnar1−/− neutrophils into WT tumor-bearing mice significantly elevates tumor angiogenesis in such mice.5 However, the mechanism controlling angiogenic activity of TANs has not yet been identified. Therefore, we focused here on the morphometric vascular situation in the context of intratumoral neutrophils. The plasticity window for vascular remodeling in tumors is defined by the coverage of pericytes. The extent of such coverage is an important marker of vessel maturation30 and its absence is responsible for the irregular, tortuous and leaky blood vessels in tumors, modulating therapy efficacy.31 Several molecules have been proposed as pericyte markers during maturation of the vascular bed.32 NG-2, Desmin and PDGF-Rβ have been established as markers of early, that is, immature pericytes, whereas αSMA has been reported as a marker of mature mural cells, including pericytes and smooth muscle cells.30,32 In our study, using two different melanoma cell lines, we observed elevated neutrophil infiltration into tumor tissue in Ifnar1−/− mice, compared to WT. Importantly, such neutrophils accumulated in close proximity to vessels and secreted high amounts of pro-angiogenic factors that support proliferation and sprouting of endothelial cells, but also enhance recruitment of pericytes to the vascular wall. In consequence, a highly mature vascular network and normalization of the vascular bed is observed in such Ifnar1−/− tumors.

In highly angiogenic tumors of Ifnar1−/− mice, we have observed elevated hypoxia even though the vasculature was normalized. Possibly, the accelerated tumor growth that is observed from Day 9 p.t. could argue for this phenomenon. We hypothesize that due to the increased tumor expansion during such a short period, vascular sprouting and growth could not keep pace with the rapidly growing tumor volume. Thus, induction of intratumoral hypoxia is the consequence and responsible for the increased detection of pro-angiogenic proteins in TANs in the Ifnar1−/− tumor situation. At later stages of tumor establishment, recruitment of pericytes provides coverage and thus reduced leakage of the vascular network in the absence of Ifnar1.

We could demonstrate a significant upregulation of pro-angiogenic genes encoding VEGF, MMP9, BV8 and S100a8 in Ifnar1−/− TANs. MMP9 is known to degrade extracellular matrix and to secrete resequestered growth factors, such as VEGF, to promote angiogenesis.33,34 Furthermore, we observed upregulation of various chemokines and cytokines that have been previously shown to support tumor growth, tumor angiogenesis and vascular invasion.16–22 Apart from angiogenic regulation, secreted microenvironmental VEGF contribute to the impediment of an efficient reciprocal endothelial-lymphocyte interaction. In addition, receptor molecules, such as the endothelin B receptor on endothelial cells, were shown to impact T-cell adhesion and neutrophil recruitment under inflammatory conditions. Although these adhesion molecules bind leukocyte subpopulations, it is still elusive which compensatory signals modulate in detail the intratumoral presence of monocytes and neutrophils.

Here, we could observe that pro-angiogenic activity of TANs is controlled by the transcription factor FOXO3a, known to regulate numerous aspects of neutrophil biology. It has been shown that FOXO3a acts as antiapoptotic factor by binding to FasL promoter and thus has an important role in neutrophil survival.35,36 Moreover, FOXO3a may also affect cell-cycle progression and the survival of hematopoietic cells through the regulation of cyclin-dependent kinase inhibitor p27kip1 and Bcl-2 family members.37 This is in agreement with our previous studies showing an upregulation of Bcl-2 and elevated survival of neutrophils from IFN-deficient mice.38 Additionally, it has been reported that neutrophils are mobilized from bone marrow (BM) to infection site by the activation of FOXO1.39 As we observe elevated migration of neutrophils into tumor site in IFN-deficient mice, it is possible that this effect is also mediated by FOXO3a.

The biological function of FOXO3, similarly to other transcription factors, relies on reversible post-translational modifications, such as phosphorylation or acetylation/deacetylation performed by Sir tuins (SIRT).40 The impact of such modifications is diverse, and so kinase-
mediated phosphorylation of FOXO3a results in its inactivation and cytoplasmic retention24 of this factor, similarly acetylation via CBP/p300 attenuates FOXO3a transcriptional activity, while deacetylation activates this factor.26,41 It has been reported that phosphorylation-mediated inactivation of FOXO3a occurs through the phosphatidylinositol-3-kinase/AKT (protein kinase B) and the RAS mitogen-activated protein kinase pathways.32 Cytoplasmic retention and degradation of the phosphorylated FOXO3a inhibits transcription of target genes.43 By contrast, oxidative or nutrient stress signals induce activation of JNK, MST1 and AMPK, which phosphorylate FOXO3a at the conserved residue sites, resulting in nucleus localization of FOXO3a and promoting the transcription of target genes. Moreover, it has been shown that FOXO3a protein is degraded by AKT-triggered ubiquitination-proteasome pathway.42 In agreement, we observed elevated phosphorylation of MEK/ERK and PI3K/AKT kinases in WT TANs. This leads to the phosphorylation of FOXO3a, its cytoplasmic translocation and inactivation. Its inactive form fails to activate pro-angiogenic genes, resulting in impaired angiogenic activity of neutrophils and suppressed tumor angiogenesis.

Our study shows a significant upregulation of SIRT1 in TANs of Ifnar1−/− mice. This is accompanied by the elevated amount of active form of FOXO3a in these cells, and its diminished phosphorylation. SIRT1 is a nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylase, which was shown to efficiently deacetylate FOXO3a,27 resulting in its increased activity.25 In contrast, acetylation of FOXO3a attenuates its transcriptional activity44 and induces its proteasomal degradation.45 It has been reported that SIRT1-mediated deacetylation activates FOXO3a in the nucleus, leading to the expression of several targets genes.25 Moreover, increased SIRT1/SIRT2-FOXO3a interaction was shown to tightly bind FOXO3a to DNA binding domain and so to enhance transcription of target genes.26,41 Our findings are in line with these studies. In addition, we could observe strong positive correlation between SIRT1 and FOXO3 expression in human tumor tissue—both in melanoma, but also in HNC. Type I IFNs seem to regulate this axis also in human specimen, as significant negative regulation between FOXO3 and IFNs is observed.

While comparing angiogenic potential of TANs from WT vs Ifnar1−/− mice, we observed significantly elevated capacity of TANs to stimulate endothelial cell proliferation and sprouting in aortic ring assay. Blocking the activity of SIRT1 in Ifnar1−/− TANs led to the cytoplasmic translocation of FOXO3a, its elevated phosphorylation and deactivation. This was accompanied by the decreased expression of angiogenic mediators and suppressed angiogenic capacity of such cells. Yet, the role of SIRT1-mediated deacetylation of FOXO3a is controversial. And so, Motta et al28 demonstrated recently that SIRT1-mediated deacetylation decreases the activity of FOXO proteins, including FOXO1, FOXO3a and FOXO4. A recent study also demonstrated that SIRT1 could bind to and deacetylate FOXO3a, which in turn increases its ubiquitination and protects cells from apoptosis.46 FOXO3 also can be deacetylated by SIRT2, which increases the DNA-binding activity of FOXO329 and thus increases the expression of its target genes, such as cyclin-dependent kinase inhibitor 1B (p27Kip1), manganese superoxide dismutase and BCL2 like 11 (Bim).47 Possibly, distinct cells may have different mechanisms that regulate the activity of FOXO genes, therefore deacetylation of these factors may affect their activity differently, depending on additional factors present in the microenvironment.

Taking together, our findings demonstrate an essential role of FOXO3a transcription factor in the stimulation of pro-angiogenic functions of TANs. Activity and DNA binding affinity of FOXO3a is controlled by the fine balance between acetylation and phosphorylation.41 Deactivation of FOXO3a could reprogram TANs into anti-angiogenic phenotype and thus lead to the suppression of tumor angiogenesis and growth. This could offer new roads to therapeutically target tumor angiogenesis.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

ETHICS STATEMENT

Animal studies have been approved by the regulatory authorities LANUV (Das Landesamt für Natur, Umwelt und Verbraucherschutz NRW).

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