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TITLE:

Transfer of Manipulated Tumor-Associated Neutrophils into Tumor-Bearing Mice to Study their Angiogenic Potential In vivo

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KEYWORDS:

Tumor-associated neutrophils, neutrophil polarization, angiogenesis, neutrophil transfer, tumor growth, Nicotinamide Phosphoribosyltransferase (NAMPT) inhibitor

SUMMARY:

Here, we show therapeutic potential of anti-angiogenic tumor-associated neutrophils after their transfer into tumor-bearing mice. This protocol can be used to manipulate neutrophil activity ex vivo and to subsequently evaluate their functionality in vivo in developing tumors. It is an appropriate model for studying potential neutrophil-based immunotherapies.

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

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 growth[1]. 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 signaling[2]. 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 proliferation[5]. NAMPT is overexpressed in multiple cancer types, including colorectal, ovarian, breast, gastric, prostate cancer and gliomas[6]. 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 differentiation[4], inhibits apoptosis and stimulate expression of multiple cytokines or matrix-degrading enzymes in macrophages[7].

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[1,2,8-10]. 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 effects[11]. Treatment with small molecules, such as NAMPT inhibitor FK866, that modulate neutrophil tumoriogenicity, 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[12-14]. 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

1.1. Prepare mycoplasma-negative cells grown to a 90% confluent monolayer (approximately 10 x 10^6 cells/T75 flask) in complete Iscove's Modified Dulbecco's Medium (IMDMc: IMDM + 10% Fetal Bovine Serum (FBS) + 1% penicillin-streptomycin).

1.2. 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.

1.3. 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.

1.4. 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).

1.5. Remove the supernatant and resuspend the pellet in 1 mL of PBS.

1.6. Count the cells, and resuspend them to the concentration of 3 x 10^6/mL PBS (for the step 2) or 6 x 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

2.1. 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.

2.2. Shave the skin of the mouse on the flank with an electrical shaver, and disinfect the skin with tissue wet with 70% ethanol.

2.3. Collect the prepared B16F10 melanoma cells at a concentration of 3 x 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.

2.3.1. Mix the cells well before every injection. Use needles not less than 0.4 mm in diameter as to not disturb tumor cells.

2.4. 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.

2.5. At day 14, sacrifice the mice in the CO_2 chamber.

2.6. 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

3.1. Place tumors into sterile 6-well plates, 5 tumors per well. Cut tumors into 2-3 mm pieces with sterile scissors.

3.1.1. 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% CO_2 in a humid incubator, and mix with a 10 mL syringe without a needle every 15 min 3 times.

3.2. 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.

3.3. Lyse erythrocytes with a lysis buffer (NH_4Cl 150 mM, $KHCO_3$ 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.

3.4. 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.

3.5. Resuspend the pellet in 1 mL of PBS. Add 3 µL of Fc-block antibodies (CD16/CD32, stock 0.5 mg/mL), and incubate on ice for 15 min.

3.6. Add antibodies: 10 µL of Ly6G-PE (stock 0.2 mg/mL) and 10 µL of CD11b-APC (stock 0.2 mg/mL). Add 20 µL 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.

3.7. Add PBS up to 15 mL, centrifuge at 460 x *g*, 4 °C for 5 min, and remove the supernatant.

3.8. Resuspend the pellet in DMEMc to the concentration approximately 10 x 10^6/mL, and keep on ice.

3.9. 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 µm nozzle, a threshold rate of maximal 22,000 events/second and a flow rate of 1-3.

3.10. Check the purity of the sorted neutrophils using a cytometer for a recommended purity of >95%.

3.11. 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 x 10^6/mL.

NOTE: The expected number of neutrophils in one 14-day B16F10 tumor (10 mm diameter) is approximately 3 x 10^4 cells.

4. NAMPT inhibition in TANs in vitro

4.1. Prepare FK866 (NAMPT inhibitor) stock in dimethylsulfoxide (DMSO) at a final concentration of 100 mM.

4.2. Seed sorted neutrophils (step 3.11) into 2 wells of a 96-well U-bottom plate (1.5 x 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% CO_2 in a humid incubator.

4.3. 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.

4.4. 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 x 10^6 cells/mL (in 0.75 mL) (for step 5) or in PBS to the final concentration of 0.6 x 10^6 cells/mL (in 0.25 mL) (for step 6).

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

5.1. 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% CO_2 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 response[15].

5.2. 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% CO_2 humid incubator to allow the matrix to polymerize. Prepare at least 3 wells per condition.

5.3. 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.

5.3.1. Place the plate in a 37 °C, 5% CO_2 humid incubator for another 30 min to allow the polymerization of the second matrix layer.

5.4. Add 150 µL/well of supplemented endothelial cell growth medium and $2x10^4$ *Ifnar1*$^{-/-}$ TANs (control and FK866-treated) (step 4.4).

5.5. Incubate the plate for 14 days at 37 °C, 5% CO_2 in a humid incubator.

5.6. 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 allogenic tumor model

6.1. Prepare B16F10 melanoma cells (step 1.6) in PBS at a concentration of 6×10^6 cells/mL.

6.2. 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.

6.3. 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.

6.4. 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.

6.5. At day 2, prepare 2 types of neutrophils: FK866-treated neutrophils and control untreated

neutrophils (step 4.4) in PBS at a concentration 6 x 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

7.1 Monitor tumor growth every other day. Evaluate the tumor size with calipers and calculate the tumor volume with the formula $V=4/3*\pi*(h*w^2)/8$ (h=height, w=width, depth= width).

7.2 Sacrifice mice at the day 14 in the CO_2 chamber. Remove tumors and measure the tumor weights.

7.3 Freeze tumors in optimum cutting temperature compound in liquid nitrogen, and store at -80 °C.

7.4 Thaw the samples to -20 °C and prepare 5 µm 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.

7.5 Block with Fc-block antibodies (CD16/CD32, stock 0.5 mg/mL 1:500) in PBS for 1 h at 20 °C.

7.6 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.

7.7 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.

7.8 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.

7.9 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, *Ifnar1*$^{-/-}$ neutrophils were isolated from tumors and treated with NAMPT inhibitor FK866 for 2 h. Untreated *Ifnar1*$^{-/-}$ 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 *Ifnar1*$^{-/-}$ 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 *Ifnar1*$^{-/-}$ neutrophils (**Figure 5A,B**).

FIGURE AND TABLE LEGENDS:

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.

Figure 2. Gating strategy for TANs sorting. CD11b^{+} Ly6G^{hi} alive neutrophils are sorted from tumors with the purity ≥95%.

Figure 3. Suppression of angiogenic properties of TANs after FK866 treatment. Angiogenic properties of sorted *Ifnar1*$^{-/-}$ 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.

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.

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 μm. (**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.

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 arrival[16]. 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 apoptosis[16]. In sum, the protocol demonstrates the potential of ex vitro manipulated anti-angiogenic neutrophils to functionally suppress tumor growth in mouse melanoma tumor model.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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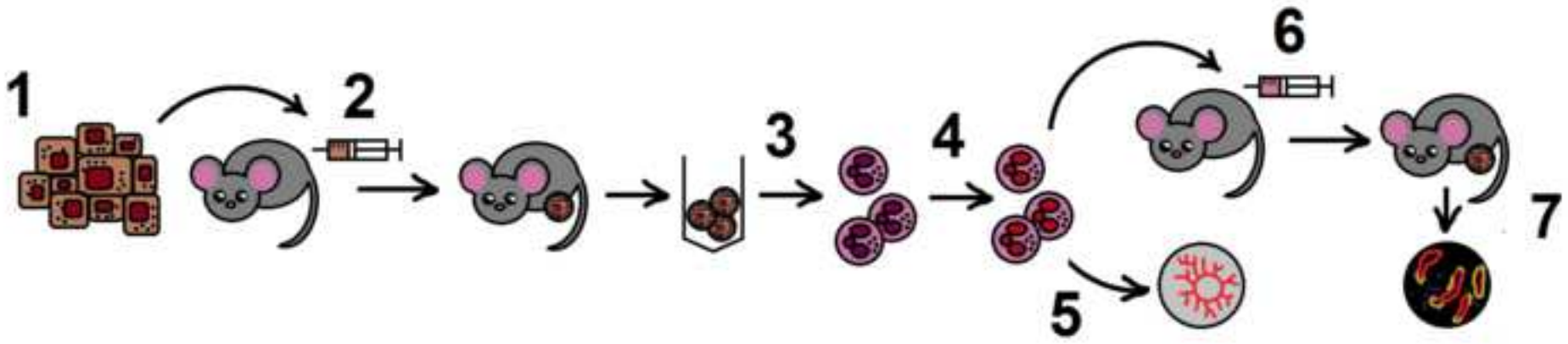

Figure 2

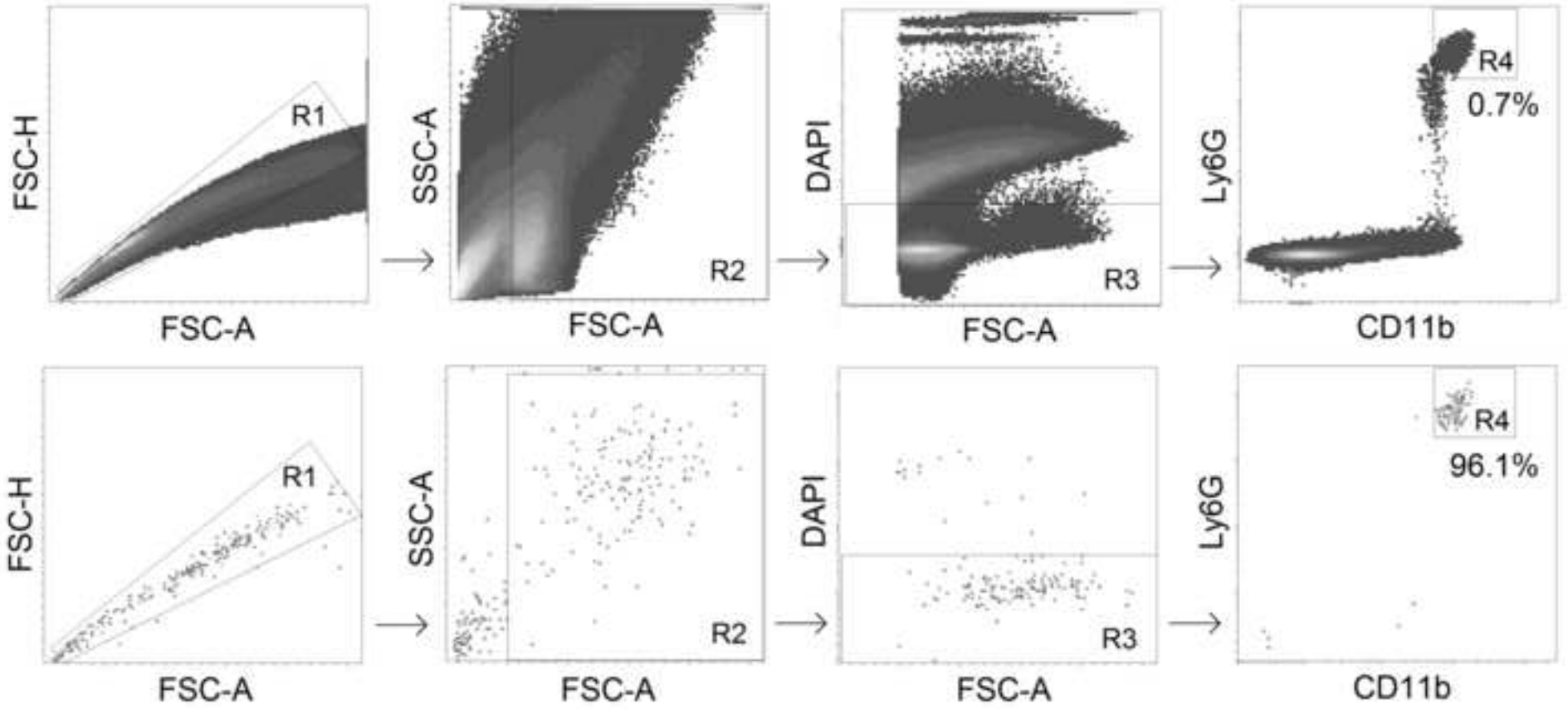

Figure 3

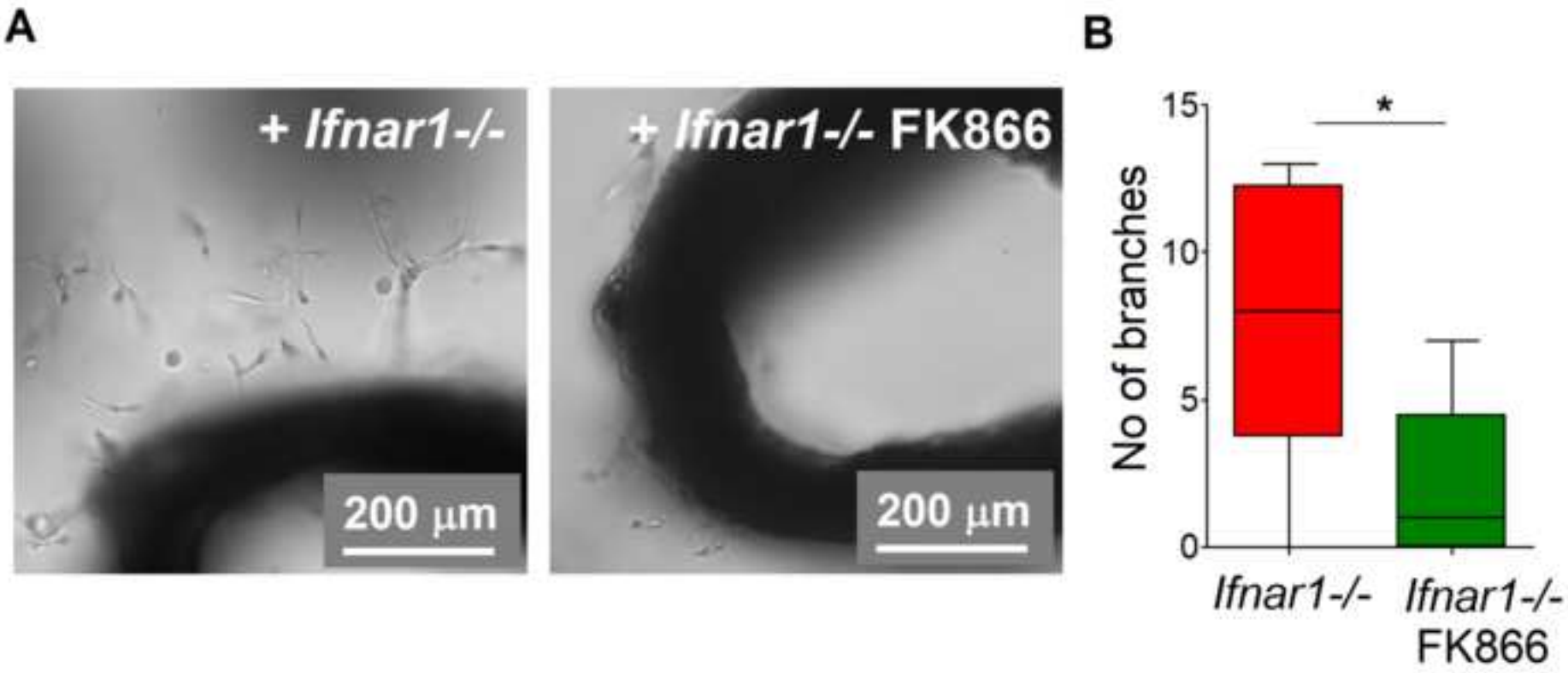

Figure 4

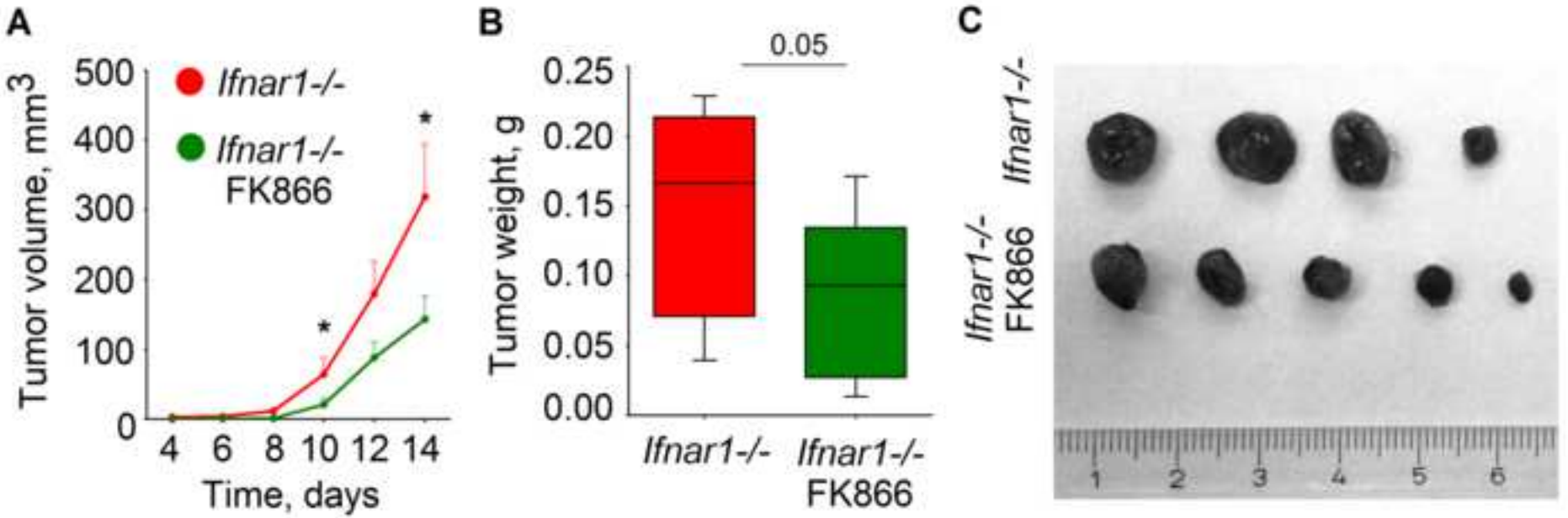

Figure 5

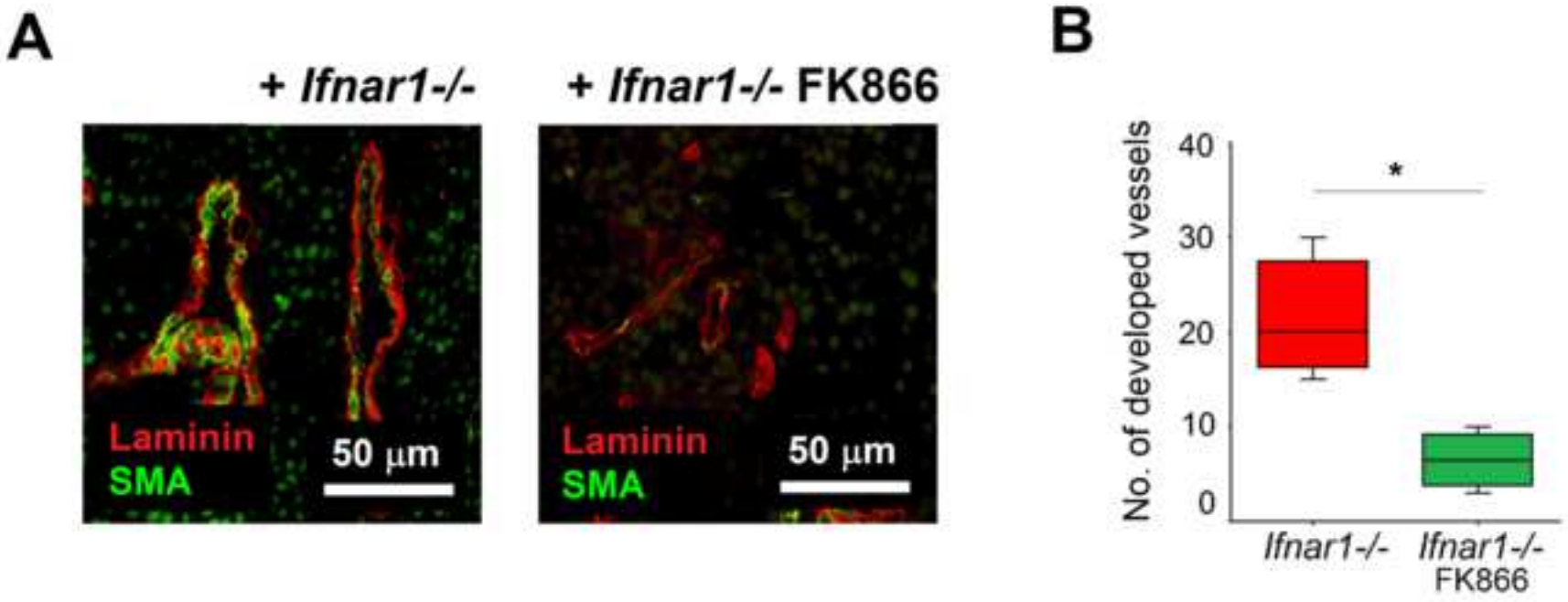

Table of Materials

Click here to access/download;Table of Materials;Kopie von JoVE_Materials_final_sorted.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description	
15 ml tubes	Sarstedt AG & Co., Nümbrecht, Germany	62,554,502		
50 ml tubes	Cellstar, Greiner Bio One International GmbH, Frickenhausen, Germany	227261		
5ml / 10ml / 25ml sterile tipps for the automatic pipette	Cellstar, Greiner Bio One International GmbH, Frickenhausen, Germany	6006180 / 607180 / 760180		
6 well flat-bottom cell culture plates	Sarstedt AG & Co., Nümbrecht, Germany	833,920		
96 well flat-bottom cell culture plates	Cellstar, Greiner Bio One International GmbH, Frickenhausen, Germany	655180		
96 well U-bottom cell culture plates	Cellstar, Greiner Bio One International GmbH, Frickenhausen, Germany	65018		
AMG EVOS fl digital inverted microscope	AMG, Bothel, U.S.			
anti-mouse CD11b	BD Pharmigen, Becton Dickinson, Franklin Lakes, U.S.	553312	clone M1/70, APC-conjugated, 0.2mg/mL	
anti-mouse Ly6G	BioLegend, California, U.S.	127608	clone 1A8, PE-conjugated, 0.2mg/mL	
BD FACS AriaII	BD Biosciences, Becton Dickinson, Franklin Lakes, U.S.		cell sorter	
Caliper	Vogel Germany, Kevelaer, Germany			
Casy cell counter	Innovatis, Roche Innovatis AG, Bielefeld, Germany			
Cell Trics 50µm / 100 µm sterile filters	Sysmex Partec GmbH, Goerlitz, Germany	04-004-2327 / 04-004-2328		
Centrifuge Rotina 420 R	Andreas Hettich, Tuttlingen, Germany	4706		
Collagenase D	Sigma-Aldrich/Merck, Darmstadt, Germany	11088858001		
DAPI (4',6-Diamidino-2-Phenylindole, Dilactate)	BioLegend, California, U.S.	422801	Stock: 5mg/ml	
Dispase I	Sigma-Aldrich/Merck, Darmstadt, Germany	D4818-2MG		
DMEM	Gibco, Life Technologies/Thermo Fisher Scientific, Massachusetts, U.S.	41966-029	DMEM complete: DMEM + 10% FBS + 1% penicillin-streptomycin	
DMSO (Dimethylsufoxide)	WAK-Chemie Medical GmbH, Steinbach, Germany	WAK-DMSO-10	CryoSure-DMSO	
DNase I	Sigma-Aldrich/Merck, Darmstadt, Germany	DN25-100MG		
DPBS	Gibco, Life Technologies/Thermo Fisher Scientific, Massachusetts, U.S.	14190-094		
Endothelial cell growth medium	PromoCell, Heidelberg, Germany	c-22010		
FBS (Fetal Bovine Serum)	Biochrom, Berlin, Germany	S0115		
Fc-block (Anti-mouse CD16/32)	BD Pharmingen, Becton Dickinson,Becton Dickinson, Franklin Lakes, U.S.	553142	clone 2.4G2, Stock: 0.5mg/mL	
FK 866 hydrochloride	Axon Medchem, Groningen, Netherlands	Axon 1546	Stock: 100 mM	
Goat Anti-Rabbit IgG H&L	Abcam, Cambridge, U.K.	ab97075	Cy3-conjugated, Stock: 0.5 mg/mL	
Heracell 240i CO2 Incubator	Thermo Fisher Scientific, Waltham, U.S.	51026334		
IMDM	Gibco, Life Technologies/Thermo Fisher Scientific, Massachusetts, U.S.	12440-053	IMDM complete: IMDM + 10% FBS + 1% penicillin-streptomycin	
Isis GT420 shaver	B. Braun Asculap, Suhl, Germany	90200714		
Matrigel Matrix basement membrane	Corning Life Sciences, Amsterdam, Netherlands	7205011		
Microtome Cryostat Microm HM 505 N	Microm International GmbH, Walldorf, Germany			
Monoclonal Anti-Actin, α-Smooth Muscle	Sigma-Aldrich/Merck, Darmstadt, Germany	F3777	FITC-conjugated, no information about stock concentration	
Needles 0.4 mm x 16 mm	BD Microlance, Becton Dicson, Becton Dickinson, Franklin Lakes, U.S.	302200		
Neomount	Merck, Darmstadt, Germany	HX67590916		
Normal goat serum	Jackson ImmunoResearch Laboratories, West Grove, U.S.	005-000-121		
Penicillin Streptomycin	Gibco, Life Technologies/Thermo Fisher Scientific, Massachusetts, U.S.	15140-122		
Pipetus automatic pipette	Hirschmann Laborgeräte, Eberstadt, Germany	9907200		
ProLong Gold Antifade Mountant with DAPI	Invitrogen, Thermo Fisher Scientific, Massachusetts, U.S.	P36935		
rabbit anti mouse Laminin gamma 1 chain	Immundiagnostik, Bensheim, Germany	AP1001.1	No information about stock concentration	
StemPro Accutase	Gibco, Life Technologies/Thermo Fisher Scientific, Massachusetts, U.S.	A1105-01		
Sterile disposal scalpel (no. 15)	MedWare, Naples, U.S.	120920		
Syringes 1 ml	BD Plastipak, Becton Dickinson, Franklin Lakes, U.S.	303172		
Syringes 10 ml	BD Discardit II, Becton Dickinson, Franklin Lakes, U.S.	309110		
T75 sterile cell culture flasks	Sarstedt AG & Co., Nümbrecht, Germany	833,911,302		
Tissue-Tek O.C.T. Compound	Sakura Finetek, Torrance, U.S.	4583		
Zeiss AxioObserver.Z1 Inverted Microscope with ApoTome Optical Sectioning	Carl Zeiss, Oberkochen, Germany			

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Author(s): E. Pylaeva, I. Spyra, S. Bordbari, S. Lang, J. Jablonska

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Essen, March 25th, 2019

Dear Dr. Nam Nguyen, dear Reviewers

First, we would like to thank the Editor and Reviewers for taking their time and for a thorough revision of our manuscript JoVE59807 entitled: "Transfer of manipulated tumor-associated neutrophils into tumor-bearing mice to study their angiogenic potential *in vivo* ".

We carefully addressed all the reviewer concerns and questions. Please find enclosed the revised version of our manuscript and below a point-by-point response to all comments. We feel that the revised version of our manuscript is significantly improved and should be now suitable for the publication in the JoVE.

With best regards,
Yours,

Jadwiga Jablonska

Point to point response:

Question	Answer
Editorial board	
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.	Thank you for the comment, we corrected this accordingly
2. Please revise lines 58-59, 197-199, 201-203, 207-209 to avoid previously published text.	done
3. Are any figures reprinted from a previous publication?	There are no figure reprinted from the previous publication
4. Keywords: Please provide at least 6 keywords or phrases.	The 6th keyword is added
5. Introduction: Please expand to include the advantages of the presented method over alternative techniques with applicable references to previous studies, description of the context of the technique in the wider body of literature and information that can help readers to determine if the method is appropriate for their application.	done
6. Please define all abbreviations before use.	done
7. Please abbreviate liters to L (L, mL, µL) to avoid confusion.	done
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9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.	done
10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.	done
11. Line 84: How to disinfect the mouse? Please describe.	done
12. Line 88: Please describe how to control tumor growth, sacrifice the mouse and remove tumors.	done
13. Line 89: Please provide the composition of the DMEM complete. What container is used?	done

14. Lines 92-94: Are the tumors from one mouse or multiple mice? What container is used for digestion? In 1 ml of what? It is unclear. What volume of digestion solution is used?	done
15. Line 95: Is the residue left on the filter discarded? Please clarify. How many filters/tubes are used per mouse?	done
16. Line 96: Add PBS to what? The 15 ml tube? What volume of PBS is used?	done
17. Line 104: 5 □l of DAPI or Ly6G-PE? It is unclear.	done
18. Line 106: What volume of PBS is added?	done
19. Line 107: What volume of DMEM complete is used? What does it mean by "sort into cold DMEM complete"?	done
20. Line 108: Is the sample analyzed by FACS?	done
21. Line 110: How to check the purity of the sorted neutrophils?	done
22. Line 115: Please list an approximate volume to prepare.	done
23. Line 125: Please provide the composition of the endothelial cell growth medium.	done
24. Line 129: Please describe how these are actually performed and specify all surgical tools used.	done
25. Line 141: Please describe how to prepare melanoma cells. What does mln mean?	done
26. Line 142: What does p.4.6 mean? Also p.6.2, p.1.8?	done
27. Line 160: At what temperature are the sections fixed?	done
28. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.	done
29. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.	done
30. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.	We have highlighted essential steps
31. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.	done
32. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.	done
33. Figure 3: Please include a space between the numbers and their corresponding units of the scale bar. Please describe panel A and B in the figure legend.	done

34. Figure 4: In panel A, please make the number "3" in "mm3" a superscript. Please describe the right panel in the figure legend and probably label it as panel C.	done
35. Figure 5: Please include a space between the numbers and their corresponding units of the scale bar.	done
36. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.	done
37. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below: Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).	done
Reviewer #1	
-It seems Fig 4A and Fig 4B were already used in their recently published papers (Fig4b in References 14).	Yes, the data were published in our previous manuscript, since the story is based on this manuscript. We have modified the figure so that it does not resemble IJC figure.
-Numbering is wrong. My guess it happens after removal of part 1. It looks like it was not updated.	done
-Step 5.7 is not a repeat of step 5.1-5.4 as described by the authors.	Thank you for the comment, we corrected this
-On page 2 / 6, line 88: it is better to give a tumor size at which the mice need to be sacrificed, rather than a day.	The day reflects the stage of the tumor development, tumors should not exceed 15 mm in diameter according to the animal care regulations. We sacrifice mice for this experiment at day 14. Tumor size at this time depends on mice and treatment
-On page 2 / 6, line 104: although the figure 2 shows CD11b staining, this antibody is not included here. Please add it.	done
-On page 2 / 6, line 116: In which volume do you seed these TAN ? Also specify how many TAN per well and not merely "into 2 equal parts" as you might obtain different amounts of TAN depending on the tumor. Your inhibitor might be titrated differently if you don't use the exact same number of TAN per well every time.	The reviewer is right, we corrected this
-On page 2 / 6, line 118: although the quantity of DMSO is low after the dilution of the inhibitor, still some DMSO should be added to the control well not just DMEM.	The reviewer is right, we corrected this mistake
-On page 2 / 6, line 125: do TAN survive in this endothelial cell growth medium, without serum neither cytokines?	TANs due to their activation in the tumor milieu survive in this medium up to 3 days.

	During this time, TANs release cytokines and growth factors that stimulate endothelial cells and initiate angiogenic processes. Our experience shows that their longer presence is not needed.
-On page 3 / 6, line 151: with this 2nd injection i.v. this time, most neutrophils will go to the lungs, liver and some in the spleen, but very few in the tumor (which is not even palpable at 1-4 days). So I'm not really sure what could be achieved here.	Growing tumors secret neutrophil-specific chemotactic factors, as the accumulation of these cells is observed in the place where tumor cells were injected already at day 1. It happens long before tumors are palpable. Probably, such released chemokines attracts iv injected neutrophils, since our previous experiments demonstrate their homing to the site of injected tumors.
-On page 3 / 6, line 155: This formula is accurate for ultrasonography measurements but not for calipers. For calipers, it is probably better to use V = (l2 x L)/2, where l and L are the shortest and longest diameters (in mm) of the tumors, respectively.	Our observations show that the shortest diameter = depth. Therefore, we use this formula in our publications. However, since we mainly compare growth of tumors between different conditions or mouse strains the formula that we use is not critical.
-On page 4 / 6, lines 178-179: Here, neutrophils were injected twice in the flank while earlier (page 3 / 6, line 151), the second injection was i.v. What is accurate?	The second injection was i.v. – we corrected this now
Reviewer #2	
While all the steps are listed, it would be appropriate to add clarifications and rationale to the different steps at various points so the researchers understand why specific steps are performed the way they are and understand the importance of these steps.	Thank you for the comment. The reviewer is right, we corrected this accordingly
It would be important to change the wording on the animal use (see comments below) as they are determined by country/organization/animal care committee	done
It would be important to provide a table or graph that outlines the yield per tumor or tumor size so that researchers can plan their experiments accordingly. It is also likely that combining several tumors would result in relatively less loss/higher yields than individual tumors.	Thank you for the comment, we agree with that. We corrected this accordingly
The figure with the timeline is not very clear. There are 2 groups of donor mice that provide neutrophils for the recipient on 2 different days. The aortic ring assay is likely to be run in parallel, but the abstract suggests sequential order (not likely that the neutrophils will be alive after the 12 days sitting in a well)	Thank you for the comment. The reviewer is right. These two experiments are run in parallel. First, controls the angiogenic capacity of neutrophils *in vitro*, second one *in vivo*. We corrected the

	figure and hope that it is clear now
Line 83: is there a specific reason why female mouse are used. It would be good if the authors indicate whether this would work in male mice as well	It is an interesting question the reviewer raises here. Despite of the fact that male and female innate and adaptive immune responses are comparable, we observe in our experiments that female mice grow bigger tumors. Moreover, males due to their aggressiveness are prone to infractions of the tumor, which increases inflammation and influences tumor growth. Therefore, to obtain comparable results, we always stay with the same gender.
Line 85 presumably the B16F10 is injected s.c.	The reviewer is right, corrected
Line 88: letting tumors grow by days rather than size might be in violation of many animal care standards and protocols. Please indicate a maximum tumor size that you work with and describe the consistency of the tumor. At this time point it is likely that the tumor has become necrotic in the middle and might fall apart/rupture upon harvest.	The reviewer is right. We stick to animal care standards and we indicated reviewer concerns in the text.
Line 89 please clarify DMEM complete	done
TAN isolation, Please indicate at the top that except for the 1st step the cells should be kept cold to improve viability and yield (so the reader will know that it is not an option)	done
Please indicate at the beginning that this will be flow sorting (if the authors have ever tried another method (such as magnetic beads it would be good to indicate, even if it is to warn people against it).	The reviewer is right, we included this into the manuscript
Indicate what machine you are using as there can be large differences in "fragile cell" viability using jet-in-air vs cuvette systems.	The information about sorter is included into M&M table
Line 108 : For the sorting, please provide a note indicating that the neutrophils will have high FSC and SSC and that the gating strategy involves these large gates.	The reviewer is right, it is shown on the Figure 2, which displays our sorting strategy (R2).
Line 109 : Please indicate why the "tail" of the Ly6C CD11b cells is not included in the gating strategy	We think the reviewer means Ly6G "tail". Such gating excludes monocytes that are present in the tail.
Line 110 : please add that the checking for purity is done by re-running it through the flow cytometer.	done
Please indicate something about expected yield/tumor, viability, the effect of combining tumors or multiple tumors on the yield. Please indicate something about your preferred manner of counting since the numbers are so low and it is	done

likely that flow sorting and the manipulations will affect the numbers.	
NAMPT inhibition in vitro Line 116: At point 2 indicate how many cells/well (please provide a not if you have seen any effects on viability with different seeding densities	done
Line 125 : At point 6, this step is for the angiogenic assay and not for the transfer into vivo	done
Line 129 : Please explain why the male aorta is used	done
Line 134 please proved more information on the embedding (pushed down? More Matrigel added? The originally cast Matrigel should be relatively sturdy by now	done
For the figure 3, it would be good provide the scoring system for the branching or provide the reference with the scoring system	This is an interesting idea. We will try to include it into our next studies. For the need of this protocol counting of branches was sufficient
Adoptive transfer Line 141 : please indicate the fluid used (PBS?)	The reviewer is correct, done
Line 143 : please indicate the fluid used (PBS?)	done
Line 149 : housing will be dependent on the specific animal facilities and can vary widely between facilities/countries). If there is a need to do small group housing, please elaborate.	There is no need for special housing, females can be kept according to our animal care regulations
Line 150 : presumably step 1 is not repeated. Please indicate why there is a repeat step and what would happen if this is done on a different day (this will tell the researchers how much flexibility they have in the planning)	Neutrophils have the maximal effect during the initiation of angiogenic processes in the early stages of the tumor development (0-3 days). Depletion or transfer of these cells later have no effect on the tumor vascularization
Tumor growth Line 156: please indicate that harvest would be determined by tumor size/ restrictions of the animal welfare guidelines	done
Line 168 please provide some information on the quantitation of the images (or provide a reference)	done
Reviewer #3	
-Although the protocol concerned is one that was used in the IJC paper, effort should be made to make the commentary, etc., more in keeping with this protocol and not reiterate the findings of the IJC paper too much. Critical assessment of this protocol and how it compares with others could be expanded and this would help to make it distinct from the IJC paper.	Thank you for this comment. We tried to address this in the manuscript
It is not always clear whether vehicle controls are to be used and their formulation.	Thank you for this comment, we addressed it in the manuscript
Although reasonably clear, the paper does need considerable copy editing to make it suitable for publication.	We went through the manuscript and edited it accordingly. We hope the reviewer is satisfied

Final or stock concentrations of all Ab should be listed not just their dilution factor.	We included this information into M&M table, when provided by the manufacturer

TITLE:

Transfer of ~~*ex vivo*~~ manipulated tumor-associated neutrophils into tumor-bearing mice to study their ~~influence on tumor angiogenesis and growth~~ angiogenic potential *in vivo*.

AUTHORS AND AFFILIATIONS:

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KEYWORDS:

Tumor-associated neutrophils, neutrophil polarization, angiogenesis, neutrophil transfer, tumor growth, Nicotinamide Phosphoribosyltransferase (NAMPT) inhibitor

SUMMARY:

Here we show therapeutic potential of anti-angiogenic tumor-associated neutrophils after their transfer into tumor-bearing mice. This protocol can be used to manipulate neutrophil activity *ex vivo* and to subsequently ~~to~~ evaluate their functionality *in* ~~*in*~~ *vivo* ~~system~~in developing tumors. It is an appropriate model for studying potential neutrophil-based immunotherapies.

ABSTRACT:

The contribution of neutrophils to the regulation of tumorigenesis is recently getting growing 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 tumor context are type I ~~IFNs~~interferons. In presence of ~~IFNs~~interferons neutrophils gain anti-tumor properties, including cytotoxicity or stimulation of the immune system. On the opposite, the absence of ~~IFN~~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~~nicotinamide phosphoribosyltransferase (NAMPT) signaling pathway in these cells. Inhibition of this pathway in ~~TANs~~ tumor-associated neutrophils leads to their potent anti-angiogenic phenotype. Here, we demonstrate ~~the potential of~~ our ~~neutrophils transfer~~newly established model ~~to~~allowing *in vivo* ~~study~~evaluation of tumorigenic ~~functionality of these cells.~~potential of manipulated tumor-associated neutrophils (TANs). Shortly, pro-

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angiogenic ~~TANs are~~ tumor-associated neutrophils can be isolated from tumor-bearing ~~IFN~~interferon-deficient mice and repolarized into anti-angiogenic ~~cells using~~ phenotype by blocking of NAMPT ~~inhibitor FK866. Their~~signaling. The angiogenic activity ~~is confirmed~~of these cells can be subsequently evaluated using aortic ring assay. ~~Such anti~~Anti-angiogenic TANs ~~are subsequently~~ can be transferred into tumor-bearing ~~WT~~wild type recipients and ~~in the following 14 days~~ tumor growth ~~is~~should be monitored for 14 days. At day 14 mice are sacrificed, tumors removed, cut and their vascularization assessed. Overall, ~~the results indicated that transfer of modified neutrophils into tumor bearing mice could be useful for evaluating their in vivo functionality in tumor context~~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 cellline models.

INTRODUCTION:

Type I Interferons (IFNs) ~~are essential for~~ play an important role in the stimulation of host responses to neoplasias~~. Lack~~, as the lack of type I IFN signaling results in significantly elevated tumor ~~angiogenesis and~~ growth [1]. ~~The molecular~~One of the mechanisms ~~of these effects include activation of~~ 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 ~~cascade in the absence of type I~~ signaling [2]. ~~G-CSF~~Colony-stimulating factor 3 (CSF3~~) is a potent regulator of tumor growth and~~), or granulocyte colony-stimulating factor, was shown to activate signaling ~~pathway~~ involving nicotinamide phosphoribosyltransferase (NAMPT) [3,4]. NAMPT is a rate-limiting enzyme for ~~NAD~~nicotinamide adenine dinucleotide synthesis, which enhances glycolysis and regulates DNA repair, gene expression, and stress response promoting cancer cells survival and proliferation [5]. NAMPT is overexpressed in multiple cancer types, including colorectal, ovarian, breast, gastric, prostate cancer and gliomas [6].

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~~. NAMPT regulates~~ – it drives their differentiation ~~of hematopoietic precursors into myeloid-lineage cells~~ [4], inhibits apoptosis ~~of cells~~ and ~~was also shown to~~ stimulate expression of multiple cytokines~~,~~ or matrix-degrading enzymes ~~and chemokines~~ in macrophages [7]. ~~Pharmacological inhibition of NAMPT results in suppression of tumor growth, but severe side effects of the systemic introduction of the drugs - gastrointestinal toxicitiy and thrombocytopenia - limit the usage of such a treatment [11-13].~~

Tumor-associated neutrophils ~~(TANs)~~ represent important modulators of tumor growth. TANs 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 [1,2,8-10]. Importantly, such pro-angiogenic TANs show elevated activity of NAMPT, suggesting its essential role in pro-tumor polarization of neutrophils.

~~Therefore, we suggest here the protocol of the selective inhibition of NAMPT in TANs as an efficient approach to suppress their pro-angiogenic capacity and as a result to limit tumor growth.~~ 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

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monoclonal antibodies are immunogenic, and their administration is associated with a range of life-threatening side effects [11]. Treatment with small molecules, such as NAMPT inhibitor FK866, that modulate neutrophil tumoriogenicity, 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 [12-14]. 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 to avoid systemic toxic side-effects of the compounds, while its effect on the target cells will be sustained.

PROTOCOL:

Note: All the ~~procedure~~procedures including animal subjects have been approved by the regulatory authorities: LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz NRW) ~~Germany~~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).

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

~~1~~1. Preparation of B16F10 melanoma cell line.

1.1. Prepare mycoplasma-negative cells grown to 90% confluent monolayer (approximately 10 x 10^6 cells/T75 flask) in complete Iscove's Modified Dulbecco's Medium (IMDMc: IMDM + 10% Fetal Bovine Serum (FBS) + 1% penicillin-streptomycin)

1.2. Remove the medium, rinse the cells with Phosphate Buffered Saline (PBS) and apply 6 ml of a cell detachment solution containing proteolytic and collagenolytic enzymes (see "Table of materials"), incubate at +37 °C for 2 min

1.3. Knock the flask gently to mobilize remaining adherent cells from the bottom, collect suspension of cells in 15 mL tubes and centrifugate at 300g for 7 min, +20 °C.

1.5. Remove the supernatant, resuspend the pellet well in 1 mL PBS, add 14 mL PBS and centrifugate (300g, 7 min, +20 °C).

1.6. Remove the supernatant and resuspend the pellet in 1 mL PBS.

1.7. Count cells, resuspend them to the concentration of 3 x 10^6/mL PBS (for the step 2) or 6 x 10^6/mL PBS (for the step 6) for injection. Keep cells on ice for maximum 30 min.

2. Allogenic tumor model in mice.

~~1.~~ ~~Female~~ 2.1. Take 10 female *Ifnar1*$^{-/-}$ mice 8-12 weeks old kept under Specific-Pathogen-Free (SPF) conditions.

~~Catch the mouse, shave~~Note: Female mice are more 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.~~ 2.2. Shave the skin of the mouse on the flank ~~on both sides~~ with electrical shaver, disinfect

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the skin with tissue wet with 70% ethanol.

~~3.~~ ~~Prepare~~2.3. Collect the prepared B16F10 melanoma cells ~~3x10^6 / ml~~in concentration 3 x 10^6/mL PBS (see the step 1) in 1 mL syringe and 0,4 x 19 mm needle, inject 100 ~~µl~~ µL of suspension ~~on the flank of mouse with insulin syringe (0,4 x 19 mm needle).~~subcutaneously.

Note: mix the cells well before ~~the injection.~~ every injection. Use the needles not less than 0,4 mm in diameter not to disturb tumor cells.

~~Control tumor growth~~2.4. Place up to 5 mice in one cage, control tumor size (length, width and depth) with caliper for 14 days~~,~~

2.5. At day 14 sacrifice the mice ~~at~~in the ~~day 14,~~CO_2 chamber

~~4.~~ 2.6 Disinfect the skin with 70% ethanol and remove tumors~~, keep in~~ with scissors and forceps in a sterile Petri dish. Keep tumors in 50 mL tube in complete Dulbecco's Modified Eagle Medium (DMEMc: DMEM ~~complete~~+ 10% FBS + 1% penicillin-streptomycin) on ice.

~~2~~Note: According to the animal regulations, tumor size should not exceed 15 mm in diameter, mice with bigger or necrotic/open tumors should be sacrificed beforehand.

3. TAN isolation.

~~1.~~ 3.1. Place tumors into sterile 6-well plates, 5 tumors per well. Cut tumors into 2-3 mm pieces with sterile scissors, digest with 1 mL dispase/~~collagenaseA/~~ collagenase D/ DNase I solution (0.~~2mg/~~2 mg/ 0.~~2mg/ 100mg~~2 mg/ 100 mg in 1 ~~ml),~~mL DMEMc) per 1 tumor, incubate ~~45 min~~ at +37 °C, 5% CO_2 in humid incubator, mix with the 10 mL syringe without needle every 15 min 3 times.

~~2. Mesh~~3.2. To remove undigested fibers, mesh cells through 100 µm filters into 15 ~~ml~~mL tubes~~.~~

~~3.~~ (one well – one filter – one tube). Add PBS to 15 mL, centrifugate tubes at ~~460 g~~460g, +4 °C for 5 min, remove the supernatant.

~~4.~~ 3.3. Lyse erythrocytes with ~~ACK~~a lysis buffer (NH_4Cl 150 mM, ~~$KHCO_3$10~~$KHCO_3$ 10 mM, ~~Na_2EDTA~~EDTA 0.1 mM, ~~room temperature~~pH 7.3, +20 °C) by adding 1 ~~ml~~mL into each tube, mix well, ~~block~~ combine the solution from all tubes into one, stop the reaction after 2 minutes with ~~9 ml~~ 11 mL of ice-cold ~~DMEM complete~~(+4 °C) DMEMc.

~~5.~~ 3.4. Centrifugate at 460g, +4 °C for 5 min, remove the supernatant, resuspend the pellet with ~~5 ml~~15 mL cold PBS, centrifugate at ~~460 g~~460g, +4 °C for 5 min, remove the supernatant.

~~6.~~ 3.5. Resuspend the pellet in ~~1000 µl~~1 mL PBS ~~+~~. Add 3 µl ~~FC~~Fc-block antibodies (CD16/CD32, stock 0.5 mg/mL), incubate on ice for 15 min.

~~7.~~ 3.6. Add ~~DAPI (5 mg/ml Stock) 1:100 and~~antibodies: Ly6G-PE ~~1:200 (5 µl),~~(stock 0.2 mg/mL) 10 µL and CD11b-APC (stock 0.2 mg/mL) 10 µL'. Add 6-Diamidin-2-phenylindol viability dye (DAPI, stock 5 mg/mL) 20 µL and incubate on ice in darkness for 30 min.

Note: Another combination of viability dyes and fluorescent conjugates of antibodies can be used.

~~8.~~ 3.7. Add PBS up to 15 mL, centrifugate at ~~460 g~~460g, +4 °C for 5 min, remove the supernatant.

~~9.~~ 3.8. Resuspend the pellet in ~~DMEM complete~~DMEMc to the concentration approximately 10 x 10^6/mL, keep on ice~~, sort into cold DMEM complete~~.

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~~10.~~ 3.9. Sort ~~single~~CD11b^{+} Ly6G^{hi} alive (DAPI-negative) ~~Ly6G-positive~~ neutrophils ~~according to the~~ with a fluorescence-activated cell sorter (gating strategy ~~(~~see Figure 2).

Note: The tube with cell suspension and the tube with DMEMc for sorted cells should be kept at +4 °C, the optimal sorting settings are at 70 µm nozzle, threshold rate maximal 22.000 events/second and flow rate 1-3.

~~11.~~ 3.10. Check the purity of the sorted neutrophils using cytometer, recommended purity ~~is not less than~~ > 95%.

~~12.~~ 3.11. Centrifugate ~~the~~ sorted neutrophils at 460g, +4 °C for 5 min, remove the supernatant, resuspend the sorted cells in ~~DMEM complete~~DMEMc to the concentration of 1 ~~x10^{6}/ml~~x 10^{6}/mL

~~3~~Note: The expected number of neutrophils in one 14-day B16F10 tumor (10 mm diameter) is approximately 3 x 10^{4} cells.

4. NAMPT inhibition in TANs in vitro.

~~1.~~ 4.1. Prepare FK866 (NAMPT inhibitor) stock in Dimethylsulfoxide (DMSO~~, with the~~), final concentration ~~of~~ 100 mM.

~~2. Divide the~~4.2. Seed sorted neutrophils (~~p.~~step 3.12) into 2 ~~equal parts, seed into 2~~ wells of 96-well U-bottom plate~~,~~ (1.5 x 10^{5} neutrophils/well), add FK866 ~~stock~~ into the intervention well ~~to the~~ (final concentration 100 nM~~,~~), add the equal amount of ~~DMEM complete~~DMEMc with DMSO into the control well.

~~3.~~ Incubate for 2 hours at ~~+~~37 °C, 5% CO_2 humid incubator.

~~4.~~ 4.3. Centrifugate at 460g ~~+4°C for 5 min, remove the supernatant, resuspend in 200 µl PBS each well~~

~~5. Centrifugate at 460g +4,~~ +4 °C for 5 min, remove the supernatant, resuspend in 200 µ~~l~~L PBS each well. Repeat 2 times.

~~6.~~ 4.4. Centrifugate at 460g, +4 °C for 5 min, remove the supernatant, resuspend in ~~serum-free Endothelial cell growth medium to the final concentration 0.2 mln/ml (for the step 5) or in PBS~~commercial endothelial cell growth medium (supplemented with endothelial cell growth supplement 4 µL/mL, recombinant human epidermal growth factor 0.1 ng/mL, recombinant human basic fibroblast growth factor 1 ng/mL, heparin 90 µg/mL and hydrocortisone 1 µg/ml) to the final concentration 0.~~6 mln/ml~~2 x 10^{6} cells/mL (in 0.75 mL) (for the step 5) or in PBS to the final concentration 0.6 x 10^{6} cells/mL (in 0.25 mL) (for the step 6).

45. Estimation of angiogenic properties of TANs ~~in~~using the aortic ring assay.

~~1.~~ 5.1. Dissect thoracic aorta from a male C57BL/6J (WT) mouse, clean and cut into 0.5 mm width rings. Place all rings in ~~serum-free Endothelial~~a well of a 24-well plate with 1 mL supplemented endothelial cell growth medium, incubate overnight at +37 °C, 5% CO_2 in 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 response [15].

~~2.~~ 5.2. Fill the wells of the 96-well flat-bottom plate with 50 ~~µl Matrigel~~µL of solubilized

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basement membrane matrix, let the gel stay for 30 min in 37~~c~~ °C 5% CO_2 humid incubator~~.~~ to allow the matrix to polymerize. Prepare at least 3 wells per condition.

5.3. Embed the rings in ~~Matrigel~~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~~. Add another 50 µL of solubilized basement membrane matrix to cover each ring, place the plate in 37 °C 5% CO_2 humid incubator for another 30 min to allow the polymerization of the second matrix layer.

~~3.~~ 5.4. Add 150~~µl~~ µL/well of supplemented endothelial cell growth medium and ~~20000~~ ~~Ifnar~~2x10^4 *Ifnar1*$^{-/-}$ TANs (control and FK866-treated) (~~p.~~step 4.6).

~~4.~~ 5.5. Incubate the plate for ~~12~~14 days at +37 °C, 5% CO_2 in humid incubator, image using standard phase-contrast microscope in dynamics, estimate 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**.

~~5~~6. Adoptive transfer of treated neutrophils in the allogenic tumor model.

~~1.~~ 6.1. Prepare B16F10 melanoma cells (~~p.~~step 1.8) in PBS in concentration ~~6 mln/ml~~6x10^6 cells/mL.

~~2.~~ 6.2. Prepare 2 types of neutrophils~~–~~: FK866-treated neutrophils~~,~~ and control untreated neutrophils (~~p.~~step 4.6) in PBS in concentration ~~0.~~6 ~~mln/ml~~x 10^5 cells/mL, mix ~~in the proportion 1:1~~neutrophils with B16F10 melanoma cells (the final neutrophil to tumor cells ratio 1:10) to have 2 types of cell mixtures.

~~3.~~ 6.3. Take 10 Female WT mice 8-12 weeks old, 5 in each group.

~~4. Catch the mouse, shave~~ Shave the skin on the flank with electrical shaver, disinfect with 70% ethanol.

~~5.~~ 6.4. Inject ~~100µl~~100 µL of cell suspension (~~p.~~step 6.2) ~~s.c.~~subcutaneously with insulin syringe and needle with 0.4 mm diameter, to both groups

~~6.~~ of mice. Place mice ~~3~~1-5 from the same group in one cage.

~~7. Note: Repeat steps 1-4 two days later, inject~~6.5 At day 2 prepare 2 types of neutrophils: FK866-treated neutrophils and control ~~neutrophils to the respective mice~~untreated neutrophils (step 4.6) in PBS in concentration 6 x 10^5 cells/mL. Inject 100 µL of cell suspension (step 6.6) i.v. into ~~a~~the tail vein with insulin syringe and needle with 0.4 mm diameter, to both groups of mice. Place mice back to the cage.

~~6~~7. Tumor growth measurement, histological examination.

~~1.~~7.1 Monitor tumor growth every second day, evaluate the tumor size with caliper, calculate tumor volume with the formula V=4/3*π*(h*w^2)/8 (h=height, w=width, depth= width). Sacrifice mice at the day 14, remove tumors, measure tumor weights.

~~2. Sacrifice mice at the day 14, remove tumors, measure tumor weights in groups.~~

~~3.~~7.2 Freeze tumors in ~~O.C.T.~~optimum cutting temperature compound in liquid ~~nitrogen~~nitrogen, store at -80 °C.

~~4.~~ Defreeze the samples to ~~-20C~~20 °C, prepare 5 µm sections ~~with~~using cryotome~~, let them~~.

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Let the cryocuts dry for ~~10 minutes~~30 min at ~~room temperature.~~

~~5.~~7.3 +20 °C. Fix section ~~with ice~~ in -20 °C cold acetone for 2 min~~,~~ and let them dry for 30 ~~minutes~~min at ~~room temperature~~+20 °C.

~~6.~~7.4 Block with ~~3% normal goat serum~~Fc-block antibodies (CD16/CD32, stock 0.5 mg/mL 1:500) in PBS for 1 hour ~~in room temperature, wash with PBS~~at +20 °C.

~~7.~~ Stain with ~~Rabbit~~rabbit anti mouse Laminin ~~Ab~~gamma antibody (1:1500 in PBS, 200µl) for 1 hour ~~in room temperature~~

~~8.~~7.5 at +20°C. Wash ~~in~~with PBS 3x

~~9.~~7.6 Stain with secondary goat anti-~~–~~rabbit ~~Ab (~~antibody (stock 0.5 mg/mL, 1:400 in PBS~~),~~ µL), anti-~~–~~mouse αSMA (1:500 in PBS) and 2 µL DAPI (~~1~~stock 5mg/mL , 1:100 in PBS~~)~~), final volume of antibody solution 200 µl. Incubate for 1 hour ~~in room temperature~~at +20°C in darkness. Wash with PBS 3x

~~10.~~7.7 ~~Wash in PBS, dry~~Dry slides for 20 minutes at +20 °C in darkness, mount with ~~Neomount~~anhydrous mounting medium for microscopy and cover with coverslip. Let it dry 1 hour in 37°C.

~~11.~~7.8 ~~Analyze with Zeiss AxioObserver.Z1 Inverted Microscope~~ 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 1: To perform image analysis, all images must be taken under the same conditions (light, contrast, magnification). In this case, processing parameters are fixed, and image processing becomes completely automatic.

Note 2: representative results are depicted in the **Figures ~~3~~4 and ~~4~~5**.

REPRESENTATIVE RESULTS:

Using the procedure ~~presented~~described here, *Ifnar1*$^{-/-}$ neutrophils were isolated from tumors and treated with NAMPT inhibitor FK866 for 2 hours. Untreated *Ifnar1*$^{-/-}$ 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 3 A and B**).

FK866-treated anti-angiogenic neutrophils were injected subcutaneously into ~~the flank of~~ 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 *Ifnar1*$^{-/-}$ neutrophils (**Figure 4 A and B**).

Histological examination of the extracted tumors proved the significant suppression of angiogenesis ~~of~~in tumors isolated from mice ~~transplanted~~treated with FK866-treated TANs, ~~ascompared~~as compared to those injected with untreated *Ifnar1*$^{-/-}$ neutrophils (**Figure 5 A and B**).

FIGURE AND TABLE LEGENDS:

Figure 1. The scheme of the protocol. Step 1~~–~~. Preparation of ~~cell line~~ B16F10 melanoma cellline; 2~~– allogenic~~. Allogenic tumor model in mice; 3~~– TAN isolation~~. Isolation of TANs from the tumors;

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4 – . Inhibition of NAMPT inhibition in TANs *in vitro*; 5 – estimation. Estimation of angiogenic properties of TANs in the aortic ring assay; 6 –. Adoptive transfer of treated neutrophils in the allogenic tumor model; 7 tumor. Tumor growth measurementmonitoring, histological examination.

Figure 2. Gating strategy for TANs sorting. Single cell suspensions of tumors were prepared, stained using antibodies listed above. $CD11b^{+}$ $Ly6G^{hi}$ alive neutrophils wereare sorted using a FACSAria cell sorter (BD Bioscience), and from tumors with the purity of cells was assessed (≥95%).%.

Figure 3. Suppression of the endothelial branches formation in aortic ring assay by neutrophils angiogenic properties of TANs after FK866 treatment. SortedAngiogenic properties of sorted *Ifnar1*$^{-/-}$ TANs were cultivatedtreated with FK866 (green) or with medium (red), washedand added to aortic rings; branchwere estimated using aorta ring assay. Branch formation was monitored during 14 days. The, representative results at the day 14 are presented (**A**). Treatment with FK866 significantly decreased the number and length of endothelial branches were higher in presence of *Ifnar1*$^{-/-}$ neutrophils, inhibition of Nampt in neutrophils leads to reduced branch formation.(**B**). Data are shown as median, interquartile range and min-max, *p<0.05.

Figure 4. Retardation of tumor growth after adoptive transfer of FK866-treated neutrophils. Sorted *Ifnar1*$^{-/-}$ TANs The influence of TANs on the tumor growth was assessed. TANs were cultivated isolated, treated with FK866 (green) or medium (red), washed, mixed with B16F10 melanoma cells (1:10) and s.c. injected into the flank of WT mice, at d3 tumor-bearing mice received FK866-treated or medium-treated neutrophils (i.v.), respectively. Tumor growth was monitored every second day (**A**), atas 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 was istimated (**B**).and (**C**) size were estimated. Data are shown as median, interquartile range and min-max, *p<0.05.

Figure 5. Suppressed angiogenesis in tumors tumor vascularization after adoptive transfer of FK866-treated neutrophils. ExemplifiedTumors 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 vessels in tumor. Laminin (red),tumors are shown: SMA (green), laminin (red). Scale bars: 10050 µm (**A**). (**B**) Quantification of tumor vascularization of tumors after adoptive transfer of TANs cultivated with FK866 (green) or medium (red) (**B**). Data are shown as median, interquartile range and min-max, *p<0.05.

DISCUSSION:

Despite of the progress in surgical and pharmacological approachescancer treatment, successful treatment of cancer therapy remains a significant challenge. TheSince immune cells are known to play an important role in the regulation of tumor growth, novel immunological methods are introduced to influence the tumor-infiltrating immune cells.inhibiting tumorigenicity of such cells should be established. Here we demonstrate thea novel methodapproach 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.

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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 ~~step~~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 ~~with antibodies~~.

The limiting step of the protocol is the isolation of alive neutrophils from tumors due to relatively low amount of these cells in tumors (not more than 1% of single alive cells in melanoma). This could be only 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 ~~tissue arrival~~[14]. ~~–~~tumor tissue arrival[16]. 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 hours) ~~are~~is suggested, as it has no influence on the viability of TANs, while prolonged treatment induces neutrophil ~~apoptosis~~[14]apoptosis[16].

In sum, the protocol demonstrates the potential of *ex vitro* manipulated anti-angiogenic neutrophils to functionally suppress tumor growth in mouse melanoma tumor model.

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DISCLOSURES:

The authors have nothing to disclose.

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