Supplementary Materials for

Neutrophils activated by membrane attack complexes increase the permeability of melanoma blood vessels

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Supplemental Material and Methods
Fig. S1. Complement activation in human melanoma. C5a levels in plasma samples obtained from stage IV melanoma patients were analyzed by ELISA. Tumor ulceration had no significant impact on C5a plasma levels (A). Expression levels of C3, C5 and C9 measured in renal cell carcinoma (RCC), breast cancer (BC) and melanoma tissues of primary tumors. Data were obtained from the public transcriptome database cBioportal (B). Gene expression levels of C3 and C5 were determined using threshold cycle (Ct) values normalized to GAPDH ($\Delta$Ct) and expressed as $2^{-\Delta\text{Ct}}$. Gene expression of C3 and C5 in human melanoma cell lines WM457, IGR37, MV3, SBCL-2 and BLM suggest negligible expression of complement factors by melanoma cells (C). Representative images of hematoxylin and eosin stained human stage IV primary melanoma tissues (D). The table shows the breslow thickness of the analyzed tissues (D). Immunofluorescence microscopic analysis of C3d/C3b/iC3b in cryosections (scale bars=20µm) of human melanoma tissues (stage II-III-IV, n=5) and healthy skins. Representative images of stage II and stage III melanoma tissues are shown. Complement activation was detected in stage IV and III melanoma patients.
but not in tissues obtained from stage II patients (E). Human basal cell carcinoma (BCC, n = 6), kerotacanthoma (KA, n = 5), and nevocytic nevi (NCN, n = 5) tissue samples were stained for von Willebrand factor (VWF) (green) and C3 cleavage products (C3b/iC3b/C3d, red) (F) or CD15 (red) and MAC (green) (G). VWF was used as a blood vessel marker. Quantification showed a very limited number of vessels with C3b/iC3b deposition (<5%) in BCC, KA, and NCN tissues (F). In comparison to melanoma, fewer MAC-positive neutrophils were detected in BCC (16%) and KA (12%). MAC-positive neutrophils were absent in NCN (G). Data are presented as mean ± SD, *P< 0.05, **P< 0.005, ***P< 0.0005, ND = not detectable.
Fig. S2. Complement activation in murine tissues. Cryosections of early staged primary tumor (1 Week), lung, liver and kidney tissues were stained for VWF (green) and C3 cleavage fragments (C3b). Nuclei were stained with DAPI (blue). C3b deposited was neither found in early staged melanomas (A, n=4, Scale bar=20µm) nor in lung, liver and kidney tissues of melanoma bearing mice (4 weeks, B, n=4 each). The table shows the quantitative summary of our results. Factor B antibody was validated in sections of liver tissues (C). Bar diagrams derived from contour plot quantifications to determine the endothelial specific content of sialic acid. Sialic acid content in 4 week old tumors was strongly decreased, sialic acid content was less affected in 1 week old melanomas (D). Bar diagrams derived from contour plot quantifications to determine the endothelial specific content of fucose, galactose, mannose and N-acetyl-glucosamine (E). Correlation between melanoma patients’ survival and the expression of NEU3 is presented as Kaplan-Meier diagram (F). Expression levels of NEU3 measured in melanoma tissues of metastatic foci or primary tumors. Data were obtained from the public transcriptome database cBioportal (G). Data are presented as mean ± SD, *P< 0.05, **P< 0.005, ***P< 0.0005. NS = not significant.
Fig. S3. Tissue recruitment of neutrophils and MAC deposition. In cryosections of murine melanomas, C5a colocalize with C3b fragments (A, Scale bar=50µm). Neutrophils are recruited to C5a-rich areas (B, Scale bar=50µm). C5-deficiency did not affect primary tumor growth (C). MAC was neither formed on tumor associated macrophages (F4/80 staining, red), T cells (CD3e staining, red) nor on dendritic cells (CD11c staining, red) (D). Neutrophils in lung, liver and kidney tissues of melanoma bearing animals (n=4 each) were MAC negative (E). Flow cytometry was used to identify the deposition of MAC on neutrophils...
in melanoma, bone marrow, lung, and spleen (F). The location of MAC positive neutrophils in tumoral blood vessels was analyzed (G). Neutrophils in the lumen of blood vessels were MAC negative (G, white arrow), whereas neutrophils were MAC positive upon contact with the endothelium (G, yellow arrows, dotted line reflecting endothelial-luminal interface). Scale bars=20µm. Tables show the quantitative summary of our results. Human melanoma cell culture supernatant stimulation promoted the adhesion of neutrophils on endothelial cell surface (H). FACS analysis of MAC formation showed that MAC formed not on the endothelium or floating neutrophils (Neutrophil control), but exclusively on attached neutrophils (Neutrophil attached) (I). Factor P is expressed by tumor-associated neutrophils (J, Scale bar=20µm). Data are presented as mean ± SD, *P< 0.05, **P< 0.005, ***P< 0.0005.
Fig. S4. MAC formation and impact of C3a and C5a signaling on ROS release and NET formation.
Antibody sensitized neutrophils were treated with 10% NHS or HIS for 30 min and then washed with PBS. MAC specific antibodies were used for flow cytometry and formation of MAC on NHS-treated neutrophils is shown (A). A C9 specific antibody was used to probe neutrophil lysates by Western blot. The amount of C9 deposition was increased on NHS-treated neutrophils compared with HIS treated neutrophils (B). Sensitized neutrophils were treated with 10% NHS for 30 min to induce MAC formation on neutrophils, 10% HIS was used as a control. Intracellular ROS was quantified by microscopic analysis of DCFDA fluorescence intensities (20 μM, 30 min, scale bar=20µm)(C). After priming with PMA (50nM), luminol-based chemiluminescence was used to measure ROS production by control and MAC-positive neutrophils (C). FACS showed that stimulation of neutrophils with recombinant C5a (100ng/ml) induced cell swelling (D) but failed to induce ROS generation (E). C3aR antagonist and C5aR antagonist treatment was insufficient to block ROS formation (F). ROS production by neutrophils was decreased by Eculizumab and C5- and C6-
depleted serum (G). C5a stimulation did not to induce release of DNA-histone fragments in the HIS or NHS treatment group. CD15 antibody primed neutrophils released significantly increased levels of DNA-Histones fragments in the presence of NHS (H). C3aR antagonist and C5aR antagonist treatment did not block the NETosis (I). NETosis was not reduced in tumor-bearing mice upon treatment with the C5aR antagonist PMX-53 (1mg/kg) (J, scale bars=20µm). Data are presented as mean ± SD, *P< 0.05, **P< 0.005, NS, not significant.
Fig. S5. LMWH treatment blocked NET formation. Tinzaparin and NAH reduced the release of DNA-histone fragments (Tinzaparin, 8 µM; NAH, 8 µM) (A) and citrullinated histone 3 (B) from NHS-treated neutrophils. Colocalisation of CitH3 (green) and Ly6G+ neutrophils (red) was analyzed in cryosections of primary tumors (n=4) by immunofluorescence microscopy (CitH3, green) and neutrophils (C). Quantification revealed a significant reduction of CitH3 positive neutrophils after tinzaparin treatment. Scale bars = 20µm. Data are presented as mean ± SD, *P < 0.05, **P < 0.005.
Fig. S6. Vascular margination and transmigration of neutrophils in human and murine tumors. Cryosections were stained with anti-CD15 (red) and anti-VWF (green) antibodies in human melanoma tumor. Anti-Ly6g (Red) and anti-VWF (green) antibody were used for the staining of murine tumors. Nuclei were stained with DAPI. Neutrophils accumulated in close proximity to the tumor blood vessels. Scale bars = 50µm.
Fig. S7. Vascular recruitment of neutrophils and blood vessel leakage. Cryosections of primary tumors of C6-def mice were stained for Ly6G (red) and MAC (green). C6 deficiency abolished MAC formation (A), while recruitment of neutrophils to the tumor tissue was not affected (B). Immunofluorescence microscopic images of Ly6g (white) and IgG (red) in cryosections of Cl-amidine (10mg/kg, n=5) treated mice (C) and C5-def mice (D). Green dotted lines mark the endothelial-luminal interface. Quantitative analysis revealed no significant difference of IgG leakage between vessels with or without neutrophils. Scale bars = 20µm. Data are presented as mean ± SD, NS, not significant.
**Fig. S8. Neutrophils in vivo depletion.** Immunofluorescence microscopic images of neutrophils (Green, clone REAL-176) in cryosections of tumor tissues of isotype control (clone 2A3, 100µg/mouse) or ly6g antibody (clone 1A8, 100µg/mouse) treated mice (A). Scale bars = 20µm. Flow cytometry was used to confirm in vivo depletion of neutrophils in blood of anti-Ly6g-treated (clone 1A8) mice. Control animals were treated with an isotype control (clone 2A3). Representative dot plots of neutrophils gated on CD45+ cells (FITC). CD11b+ (Pacific blue) and ly6g+ (APC, clone RB6-8C5) cells were identified as neutrophils (B). Data are presented as mean ± SD, *P< 0.05, **P< 0.005.
Supplemental Materials and Methods

Mouse procedures
All animal experiments were approved by the government animal care authorities of Hamburg or the Animal Care and Use Committee of Shanghai Medical College of Fudan University. C57BL/6J (RRID: IMSR_JAX:000664) wild type and C5-def mice (RRID:IMSR_JAX:000461) were purchased from the Jackson Laboratory, backcrossed as previously reported (1) and maintained under specific pathogen free conditions. C6-def mice were kindly provided by Anita Ignatius (Universitätsklinikum Ulm) and John D. Lambris (University of Pennsylvania). Animals were assigned to experimental groups using simple randomization. 7.5×10^5 ret transgenic melanoma cells or B16F10-Luc2 melanoma cells in 100 µl phosphate-buffered saline (PBS) per mouse were i.d. injected into 8-12 week old mice. After 4 weeks, mice were sacrificed and tumors were embedded in Tissue Tek for cryosectioning. Heparin treatments took place the first day before the injection of melanoma cells, and mice were treated daily with tinzaparin (0.6IU/g, Innohep; Leo pharma) or NaCl subcutaneously. For anti-NET therapies, Cl-amidine (10mg per kg body weight; Millipore) or PBS was injected intraperitoneally on days 25, 26, 27 and 28. For pharmacological inhibition of C5aR in vivo, PMX-53 (1mg per kg body weight; Sigma) or PBS was injected daily on the fourth week intraperitoneally. Tetramethylrhodamine-labeled dextran (5mg/mouse) was injected intravenously in WT and C6-def mice on day 28 and mice were sacrificed after 2 hours. Antibody-mediated depletion of neutrophil was performed by daily intraperitoneal injections of 100 µg/mouse anti-Ly6G (Clone 1A8) or control-IgG2a (Clone 2A3, both from BioXcell) in WT and C6-def mice from day 21 to 28.

Samples of human melanoma patients’
The full study protocol involving the analysis of human materials such as blood and tumor tissue was conducted according to the guidelines of the Declaration of Helsinki and was approved by the responsible ethics committees of the University Medical Center Mannheim (2010-318 N-MA) and of the Medical Association of Hamburg (PV4954). Written informed consent was obtained from all patients involved in the study before recruitment to the study protocol. EDTA-plasma samples and melanoma tumor tissues were obtained from stage II, III and IV melanoma patients. Skin tumors tissue samples were obtained from the department of human pathology, University Medical Center Hamburg-Eppendorf, Germany.

Immunofluorescence staining
Cells and tissue cryosections (10 µm) were fixed in 4% paraformaldehyde (PFA) for 15 min, and then washed and blocked with 10% goat serum. The following primary antibodies were used: mouse anti-human C3d (Abcam, RRID: AB_443879), rabbit anti-human VWF (Dakocytomation, RRID: AB_2315602), mouse anti-human CD15 (Abcam, RRID: AB_2105355), rabbit anti-human TCC-FITC (AE11, Hycult, RRID: AB_10989636), rat anti-mouse F4/80 (BD Bioscience, RRID: AB_2739222), hamster anti-mouse CD11c-APC (biolegend, RRID: AB_313779), rat anti-mouse CD3e (BD Bioscience, RRID:AB_395697), rat anti-mouse C1q (Hycult, RRID: AB_533002), rabbit anti-mouse Factor B (Lifespan, Cat. #114998), rat anti-mouse C4 (Hycult, RRID: AB_533012), rat anti-mouse MBL-C (Hycult, RRID: AB_533138), rat anti-mouse C3b/iC3b/C3c (Hycult, RRID: AB_533250), rat anti-mouse CD31 (BD Bioscience, RRID: AB_393571), rabbit anti-mouse C5a (Biorbyt, RRID: AB_10752171), rat anti-mouse ly6g (BD bioscience, RRID: AB_394206), rabbit anti mouse C5b-9-FITC (Biobyt, Cat. #Orb102206), rabbit anti-human Histone H3 (Citrulline R2 + R8 + R17, Abcam, RRID: AB_304752). The following secondary antibodies were used: FITC-conjugated goat anti-rat (BD Pharmingen, RRID: AB_395210), FITC-conjugated goat anti-rabbit (BD Pharmingen, RRID: AB_395212), Alexa 555-conjugated goat anti-rat (Invitrogen, RRID: AB_141733), Alexa 555-conjugated goat anti-rabbit (Invitrogen, RRID: AB_141784), Alexa 555-conjugated goat anti-mouse (IgG; Invitrogen, RRID: AB_1500929). FITC-conjugated lectins directed against N-acetyl-glucosamine (wheat germ agglutinin, WGA, Sigma), FITC-conjugated lectins directed against fucose (Aleuria Aurantia Lectin, AAL, vectorlabs), FITC-conjugated lectins directed against galactose (Griffonia Simplicifolia Lectin, GSL, vectorlabs), FITC-conjugated lectins directed against mannose (Lens Culinaris Agglutinin, LCA, vectorlabs) and FITC-conjugated lectins directed against sialic acid (Sambucus Nigra Lectin, SNA, vectorlabs) were used to stain glycans. Nuclei were stained with DAPI and immunofluorescence microscopy was performed using Zeiss Observer z.1 microscope. Images were processed with Zen software and ImageJ (version 1.51j8).

**Lectin staining analysis**

Whole tissue sections, stained with FITC-conjugated lectins (AAL, SNA, LCA, SNA and WGA) and a CD31 directed antibody (primary antibody: BD Bioscience, RRID: AB_393571; secondary antibody: Invitrogen, RRID: AB_141733) to label blood vessels, were scanned by a fluorescence microscope (Observer z.1, Zeiss). Images of superimposed lectin and blood vessel staining were imported to imageJ (version 1.51j8). Imported images were completely divided into regions of interests (ROI) with a dimension of 5x5 µm. Fluorescence intensities measured in the ROIs of the lectin and the CD31 staining were recorded separately. In subsequent calculations, single ROIs are considered as independent event. Using a script programmed in octave (version 5.2.0) frequencies of fluorescence intensities per event were calculated.
and arranged in a 256x256 matrix. For quantitative analyses, frequencies of events with a defined fluorescence intensity were summed (gated) and presented in relation to the total number of events excluding signals of background noise. The applied codes indicating also the applied gating strategies are freely available (https://github.com/gorzelac/tissue-lectin.git).

**Characterization of neutrophils in tissues by flow cytometry**

Immune cells isolation from melanoma, bone marrow, spleen and lung was performed as described (2). Flow cytometry (FACS Canto II, BD bioscience) analysis was used to check MAC formation on neutrophils. Anti-ly6g-APC (Biolegend) and anti-CD11b-pacific blue (Biolegend) were used to gate neutrophils. FITC-C5b-9 antibody (Biobyt) was used to detect MAC. Red blood cell Lysis/Fixation Solution (Biolegend) was used for lysing of red blood cells and fixation of the remaining blood elements (such as leukocytes) following immunofluorescent staining with anti-CD45-FITC (Biolegend), anti-ly6g-APC (Clone RB6-8C5, Biolegend) and anti-CD11b-pacific blue (Biolegend).

**Detection of disseminated melanoma cells**

7.5×10^5 B16F10-luc2 (RRID: CVCL_5J17) melanoma cells in 100 µl PBS per mouse were i.d. injected into 8-12 week old WT and C6-def mice. The mice were sacrificed after 28 days, and the blood and lung were collected. Total DNA from blood/lung was purified by using QIAamp DNA mini Kit (Qiagen). DNA samples were diluted 10 times and qPCR reactions were performed using a real-time PCR system (Light cycler 96 system, Roche) and the GoTaq® qPCR Master Mix (Promega). Luc2 primer (FP: TACCGACGCACATATCGAGG; RP: CTCCGCGCTCGTTGTAGATGT) was used. To determine the number of blood flowing melanoma cells we developed a highly sensitive MC-qPCR. Accordingly, we splitted the amplification curve into signals of the products of interest (POI), which was amplified by the luc2 specific primers and into signals of non-intended products (NIP). We assumed that the sequential amplification of the POI and the NIP was of a comparable magnitude and ratio. This ratio was determined by the integration of the melting temperature (Tm) peak areas of POI and NIP. The Tm was determined at the end of each PCR. The integrated melting curve spectrum was the first derivative of the DNA-related SYBR green fluorescence as a function of temperature. Finally, the amplification curve which represents the PCR cycle-related change of the sybr-green fluorescence (flₜₜ) was fitted to equation 1:

\[ f_{\text{ct}} = a_{\text{ct}}(n_{\text{NIP,ct-1}}l_{\text{NIP}} + n_{\text{POI,ct-1}}l_{\text{POI}})C_{\text{flbp}} \]  

\text{equation 1}

where \( a_{\text{ct}} \) is the cycle specific amplification rate which ranges between 2 and 1. \( a_{\text{ct}} \) was directly derived from the amplification curve. The number of templates at the starting point of each cycle corresponds to \( n_{\text{NIP,ct-1}} \) for the NIP and \( n_{\text{POI,ct-1}} \) for the POI. The number of disseminated melanoma cells was related
to \( n_{POI,ct-1} \) by assuming three genomic integrations sites of the probed Luc2 gene. The constant \( C_{fl, bp} \) defines the average sybrgreen fluorescence per base pair (bp) and was determined by a standard curve. The standard curve was prepared by adding defined amounts of melanoma cells into the blood of non-tumor bearing mice. The length (\( l \)) in bp of the POI and NIP was deduced from the melting curve spectra using equation 2 (3):

\[
\begin{align*}
    l = & \frac{R \ln \left( \frac{[\text{primer}]}{4} \right)}{M_e} \\
    & \left( T_m - 16.6 \log ([\text{Na}^+] + 273.15) \right) - \Delta S
\end{align*}
\]

equation 2

Of note, we assumed average enthalpy (\( \Delta H = -8.34 \text{ kcal/K} \)) and entropy values (\( \Delta S = -0.02104 \text{ kcal/K} \)) as the sequence of the NIP was not known. The concentration of sodium equivalents \([\text{Na}^+]\) was 0.013 M and the concentration of primers \([\text{primer}]\) was \(5 \times 10^{-7} \text{ M} \). \( R \) is the gas constant and was set to 0.00199 kcal/K mol.

**RNA extraction and qPCR**

All cell lines were tested negative for Mycoplasma using the Venor®GeM Classic kit (Minerva Biolabs GmbH, Germany). Cell line authentication was done through the identification of cell specific single-nucleotide polymorphisms (Eurofins GmbH). RNA was extracted by using RNeasy Plus Mini Kit (Qiagen), and cDNA was synthesized by using Reverse Transcription System (Promega). qPCR reactions were performed using a real-time PCR system (Light cycler 96 system, Roche) and the GoTaq® qPCR Master Mix (Promega). The expression profile of the different cell populations was shown in a heat map. Primers (sequences 5’-3’) are as follows: cd46: FP: AAGTGGTCAAATGTCGATTTCCA, RP: TCGAGGTAAAAACCTTATCGC; cd55: FP: AGAGTTCTGCAATCGTA GCTGC, RP: CACAACAGTACCTGGAAAAAT; cd59: FP: CAGTGCTACAACTGTCC TAACC, RP: TGAGACACGC ATCAAATCAGAT; factor p: FP: TGCTCTGCTTCCACCCAGTATG, RP: CCCTAC GTTTCTGGTAGGCA; gapdh: FP: GGAGCGAGATCCCTCCAAAT, RP: GGCTGTTGTCATACTTCTCATGG.

**ELISA**

ELISA kits for human C3 and C3b were from Abcam; ELISA kits for human C3a and C5a were from Hycult. The tests were performed in accordance with the instructions of the manufacturer. Bioassays were replicated three times.

**Complement assay**

The effect of 8µM tinzaparin (LEO Pharma) and 8µM N-acetylheparin (sigma) on the haemolytic activity
of the classical pathway (CH50) and the alternative pathway (APH50) was assessed according to the described procedures by Wehling (4) et al. Results are presented in reference to a pool of normal human serum as standard (100%).

**Neutrophil isolation**

Blood was drawn from healthy donors into EDTA-coated tube. Neutrophil isolation was performed by using Histopaque-1119 (Sigma) and Percoll Plus (GE Healthcare) gradients as described, which cause minimal neutrophil activation during isolation (5).

**Complement activation on endothelial cells and co-culture with neutrophil**

HUVEC pretreated with melanoma cell conditioned medium for 4 h, were sensitized with CD31 antibodies (Biolegend, RRID: AB_312908, 1:20 dilution, 15 min, 37°C) in HEPES-buffered Ringer’s solution containing 0.1% gelatin. Cells were washed three times and neutrophils were added to HUVECs in the presence of 10% NHS. After 30 min of incubation at 37°C, cells were fixated with 4% PFA in HEPES-buffered Ringer’s solution and subsequently stained with a MAC directed FITC-labeled antibody (AE11, Hycult, RRID: AB_10989636).

HUVEC pretreated with 10 ng/ml tumor necrosis factor alpha for 4 h were incubated with purified C5b,6 (70 nM) and CL555-labeled C7 (1000 nM) in HEPES-buffered Ringer’s solution containing 0.1% gelatin for 10 min at 37°C. Unbound C5b,6 and C7 was removed by three washing steps with HEPES-buffered Ringer’s solution. Neutrophils were added to HUVECs in the presence of purified C8 (700 nM) and C9 (1400 nM) and incubated for 30 min at 37°C. Prior to the staining of MAC with a directly FITC-labeled antibody (AE11, Hycult, RRID: AB_10989636) cell were fixated with 4% PFA in HEPES-buffered Ringer’s solution. Object slides were analyzed by fluorescence microscopy.

**MAC formation on neutrophil**

The induction of MAC on neutrophils was performed as described by Morgan (6). Briefly, antibody anti-human CD15 (Abcam, RRID: AB_2105355, IgM isotype, 1:100 dilution, 15 min) was used to sensitize neutrophils which were then exposed to 10% normal human serum (NHS) to induce MAC formation. Heat inactivated serum (56°C for 30 min; HIS) and C5-depleted serum (Merckmillipore) was used as negative controls.

**MAC detection on neutrophils**

Antibody sensitized neutrophils were exposed to 10% of NHS or HIS for 30 min first and then washed 3
times with PBS. For western blot detection, neutrophils were homogenized and 20µg of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot was performed according to standard protocols using monoclonal antibody to C9 (Santa Cruz, RRID: AB_2894830) for detection. For FACS analysis, cells were incubated with rabbit anti-human TCC-FITC (Hycult, RRID: AB_10989636) antibody for 20 min and acquisition was done by flow cytometry (FACS Canto II, BD bioscience).

**ROS measurement**

Intercellular ROS level was measured with dichlorofluorescin diacetate (DCFDA, Sigma). To block C3aR and C5aR function, neutrophils were treated with C5aR antagonist (DF2593A, 100nM, Sigma) and C3aR antagonist (SB290157, 500nM, Sigma). Neutrophils were stained with 20 µM DCFDA for 30 min. Then neutrophils were washed once, and the dichlorofluorescein (DCF) level was evaluated by fluorescence microscopy (Observer z.1, Zeiss) or flow cytometry (FACS Canto II, BD bioscience). ROS production was monitored by luminol chemiluminescence in a plate reader (Infinite 200 RRO).

**DNA-Histone fragments detection**

Antibody sensitized neutrophils were treated with 10% NHS, HIS, C5 depleted serum (Complementtech) and C6 depleted serum (Complementtech) for 1h. The supernatants were collected and histone-associated DNA fragments were quantified by the ELISA Kit from Roche. Citrullinated histone H3 (H3Cit) in supernatants were evaluated by Western Blot using the antibody from Abcam (Abcam, RRID: AB_304752).

**MAC and NET staining**

Antibody sensitized neutrophils were treated with NHS, HIS or NHS with the addition of tinzaparin (100IU/ml) for 30 min. Anti-human TCC-FITC (Hycult, RRID: AB_10989636) antibody and anti-human citrullinated histone H3 (Abcam, RRID: AB_304752) antibody were used for the MAC and NET immunofluorescence staining. DAPI was used to stain the nuclei and the released DNA.

**Neutrophil stimulation by C5a**

Isolated neutrophils were incubated with human recombinant C5a (100ng/ml, R&D) for 30mins and then FACS was used to check the changes for neutrophil morphology and ROS production. C5a stimulated neutrophils were treated with HIS or NHS for 1 hour. MAC formation was checked by FACS and the
neutrophil supernatants were collected for the NETs analysis.

**Endothelial cell layer integrity staining**

HUVECs were grown to confluence in a 24 wells plate and then co-cultured with MAC positive neutrophils and control neutrophils (5×10^5 per well) for 6 h. After fixation with 4% PFA for 15 min, coverslips were washed with PBS, and blocked with 2% BSA. Mouse anti-human CD31 (DAKO Cytomation) and DAPI were used for following staining.

**ECIS**

The trans-endothelial electrical resistance (TEER) of endothelial cell monolayers was measured by ECIS, using established protocols(7, 8). Briefly, HUVECs (RRID: CVCL_2959) were grown to confluence on gelatin coated gold electrodes, and then co-cultured with MAC positive neutrophils (1×10^5 per well). The real time changes in TEER were measured, and data was presented as change in HUVECs layer resistance normalized to the value at the start time for the co-culture. For inhibition of NETosis, MAC-positive neutrophils were co-cultured with HUVECs in media containing Tinzaparin (100IU/ml) or DNase I (100U/ml, sigma) or C1-amidine (50µM, Millipore).

**Human melanoma cells transmigration assay**

Melanoma cells transmigration assay was performed as described previously(9). MAC formation on neutrophils was performed as mentioned above. MAC positive or control neutrophils (1×10^5 per well) were washed to remove non-cell bound complement factors and then co-cultured with confluent HUVECs for 6 h in cell culture inserts (Greiner Bio-one). After removing neutrophils by washing with PBS human melanoma MV3 cells(1×10^5 per well) were labeled with celltrace calcein green (Invitrogen) and then added to the upper chambers. After 12 h, the microscope was used to detect the migrated labeled cells.

**Statistics**

Statistical analysis was performed with GraphPad Prism 6 software and significance was tested by Student’s t-test. All results are presented as means ± SD as indicated in the legend. P< 0.05 was considered as significant difference.
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