Rocks et al. – thoraxjnl-2018-211990.R1

Supplementary material and methods

Cell culture

Luciferase-expressing murine mammary 4T1 (clone 1A4) and human mammary MDA-MB-231 (clone D3H2LN) tumor cells were purchased from Caliper Life Sciences. Murine melanoma B16K1 cells were used as previously described. Cells were grown in DMEM supplemented with 10% heat-inactivated FBS, L-glutamine (2 mmol/l, Gibco, 25030-0124), penicillin-streptomycin (100 U/ml–100 µg/ml, Lonza, DE170602E). All cells used in this study were authenticated by Leibnitz-Institut DSMZ GmbH and used within 10 passages after authentication.

Animal experiments and ethical study approval

Eight week-old male Balb/cJRj or C57BL/6JRj mice (± 20grams) were purchased from Janvier Labs (France). NOD-Scid mice were obtained from Charles Rivers (Belgium). After arrival, mice were allowed to recover with cage enrichment for one week and maintained in cages (20x35 cm) containing maximum six-eight animals under pathogen-free conditions within the accredited Mouse Facility and Transgenics GIGA platform of the University of Liège (Belgium). Food and water were supplied *ad libitum*. The light/dark cycle in the room consisted of 12/12 h with artificial light (approximately 40 Lux in the cage). The temperature was 21 ± 1°C, with a relative humidity of 45 ± 10%. During experimental procedures, animals were monitored twice daily for health status. No adverse effect was noted due to ozone exposure or tumor cell injection. Animals were excluded from the study due to premature death due to technical experimental failure. Animal procedures were conducted in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) and all experiments had previously been approved by the Animal Ethical Committee of the University of Liege (protocol references: # 1933, #1667). Total sample size was determined with the ‘G Power’ software using the "Wilcoxon-Mann-Whitney" type *t* test. A completed ‘ARRIVE Guidelines Checklist’ has been provided.

Generation of MRP8cre-Pad4lox/lox mice

Hemizygous MRP8-Cre-ires/GFP mice[1] and homozygous *Pad4*$^{fl/fl}$ floxed mice[2] were purchased from The Jackson Laboratory (021614, 026708, respectively). Hemizygous MRP8-Cre-ires/GFP mice and homozygous *Pad4*$^{fl/fl}$ floxed mice were crossed to obtain noncarrier MRP8-Cre-ires/GFP hemizygous *Pad4*$^{fl/+}$ mice and hemizygous MRP8-Cre-ires/GFP hemizygous *Pad4*$^{fl/+}$ mice. Noncarrier MRP8-Cre-ires/GFP hemizygous *Pad4*$^{fl/+}$ mice and hemizygous MRP8-Cre-ires/GFP hemizygous *Pad4*$^{fl/+}$ were then crossed to obtain hemizygous MRP8-Cre-ires/GFP homozygous *Pad4*$^{fl/fl}$ mice and littermate control noncarrier MRP8-Cre-ires/GFP homozygous *Pad4*$^{fl/fl}$ mice. These last two strains were bred together to maintain the colony of hemizygous MRP8-Cre-ires/GFP homozygous *Pad4*$^{fl/fl}$ mice and littermate controls.

Ozone exposure

Mice were randomly divided into control air- or ozone-exposed groups. For the experimental ozone exposure model, mice were placed in whole-body inhalation exposure chambers, coupled to airflow and connected with the Ozonosan System (Germany). An oxygen-tank provided oxygen to the Ozonosan system, which converted it into ozone using UV lights. The ozone flow was monitored with a detector, to keep a mean of 2 ppm in the inhalation chamber. Mice were exposed to ozone (2ppm, 3 hours/day) or air (control 'air' group) for three consecutive days. On day 4, mice were either sacrificed to investigate pulmonary inflammation or injected with tumor cells to evaluate the impact of ozone on tumor cell behavior. In this latter case, ozone exposure was sustained for three days per week, every second weekday until sacrifice.

Lung neutrophil depletion

To deplete neutrophils, a purified anti-mouse Ly6G antibody (clone 1A8, 200µg/200µl, BioLegend, 127602) was administered intraperitoneally on days 1, 4, 8 and 10 of the experimental setup. Rat IgG2b, κ monoclonal antibody was used as an isotype control immunoglobulin (200µg/200µl).

Intravenous injection of tumor cells

After exposure to ozone for three consecutive days (2ppm, 3 hours/day), 4T1, MDA-MB-231 or B16K1 cells ($1x10^5$ cells/100µl in serum-free medium) were injected intravenously in the lateral tail vein of Balb/cJRj (for 4T1, MDA-MB-231 cells) or C57BL/6JRj (for B16K1 cells) mice.

Subcutaneous injection of tumor cells

After ozone exposure for three consecutive days (2ppm, 3 hours/day), 4T1 cells were subcutaneously injected into both left and right flanks of mice ($2x10^5$ cells/200µl in serum-free medium). Primary tumors were measured using a caliper on a weekly basis to evaluate tumor volumes. Primary tumor volumes were calculated by measuring length and width of tumors and using the following formula: (length) × (width) 2×0.4^3. The mean volume of both left and right tumors *per* mouse was considered for statistical evaluation.

Biophotonic imaging of tumor cells

In vivo imaging of luciferase-expressing 4T1 or MDA-MB-231 tumor cell dissemination to lung tissues was performed using the Xenogen IVIS 200 ® imaging system (Caliper Life Sciences) as described previously[4]. To evaluate the impact of ozone-induced inflammation on early metastatic steps or on tumor development, luciferase-activity of 4T1 or MDA-MB-231 tumor cells was monitored 30 minutes, 2, 24, 48, 72 hours or 7 days after tail vein injection. Briefly, twelve minutes before acquiring *in vivo* images, mice were intraperitoneally injected with Beetle luciferin (150 mg/kg in PBS, Promega, E1605) and anesthetized with 2.5% isoflurane-oxygen mixture. Mice were then placed in dorsal position in the IVIS® 200 Imaging System and a digital grayscale image followed by a pseudocolour image representing the detected photons emerging from luciferase-expressing tumor cells were acquired.

The extent of tumor cell implantation in lung tissues was quantitatively assessed by determining "regions of interest" (ROI) around lungs and by measuring bioluminescence intensity using Living Image software version 4.5 (Caliper Life Sciences). Data displayed are in 'Photon mode' and units are expressed as photons/s/cm2/sr.

DNase I treatment

To denature NET meshes, mice were treated on a daily basis with an intraperitoneal injection of desoxyribonuclease I (DNase I; 1000U/100µl, Sigma Aldrich, D5025).

Isolation of mouse lung neutrophils

Mice were subjected to ozone or air during 3 consecutive days. On day 4, mice were sacrificed; lungs were collected and digested in culture medium containing type IV collagenase (1mg/ml, Life Technologies, 17018029). After lysis of red blood cells (Red Blood Cell Lysis Buffer, Sigma-Aldrich, 11814389001), cells were filtered through a 41 µm-cell

strainer. Neutrophils were isolated with the Neutrophil Isolation Kit (Miltenyi Biotec, 130-097-658), an MS column and an OctoMACS Separator (Miltenyi Biotec), and prepared for further intratracheal instillations (5.10^5 neutrophils in serum-free medium) or for *in vitro* experiments (*vide infra*). To collect neutrophil-conditioned supernatants, ozone- or air-challenged neutrophils were cultured in serum-free medium (5.10^5 cells per well (24-well plates)) during 24 hours. Supernatants were then centrifuged and immediately stored at -80°C for further assays.

Collection of bronchoalveolar lavage (BAL)

After sacrifice, a bronchoalveolar lavage was performed by injecting 4x1ml of PBS-EDTA 0.05mM into lungs. Collected bronchoalveolar lavage fluid (BALF) was centrifuged and supernatants were stored at −80°C for further analyses. Cell pellets were suspended in PBS-EDTA and total cell counts were evaluated using a Particle Coulter and Size Analyzer (Z2, Beckman Coulter). For differential cell counts, cytocentrifuged cells were stained with Diff-Quick and their identity was evaluated by a blinded observer based on morphological criteria. Three hundred cells per slide were reckoned.

Lung tissue histology

Bronchial wall inflammation scoring

A hematoxylin-eosin (HE)-staining was performed on histological slides allowing the assignment of a bronchial wall inflammation scoring. Hence, a value from 0 to 2 was adjudged to each bronchus, depending on the extension of inflammation. A score of 0 was attributed to bronchi without inflammation, a score 1 corresponded to occasional mononuclear cells observed around bronchi, while a score of 2 represented bronchi surrounded by one to five layers of inflammatory cells. Eight bronchi of the left lung per mouse were analyzed and the average value was used as individual score for each mouse.

Quantification of pulmonary metastatic burden

To assess the extent of metastatic dissemination, five HE-stained slides per mouse lung were prepared and entirely scanned using the NanoZoomer 2.0-HT system equipped with a TRI-CCD Camera (0.46 µm/pixel (20X) scanning resolution), each quantified lung tissue sample separated from the next one by 50 µm. Metastatic dissemination to lungs was evaluated by measuring the ratio between tumor area and total area of lung tissue using the Cytomine software (University of Liège, Belgium), which follows a hybrid human-computer approach

based on machine learning and human proofreading[5]. The average value of metastatic dissemination measured on all five lung tissue slides was used as individual score for each mouse.

Lung extravasation assay

To measure short-term extravasation of 4T1 tumor cells to lung parenchyma of mice exposed to air or ozone, 4T1 cells were previously incubated in serum-free DMEM with a red-fluorescent CMTPX Cell Tracker (2.5 µM, Thermofisher Scientific, C34552), rinsed and re-incubated in serum-free DMEM medium before being intravenously injected into Balb/cJRj mice. After 48 hours, mice were anesthetized by an intraperitoneal injection (200 µl) of ketamine (10 mg/ml; Merial, 1534-601) and xylazine (1 mg/ml; VMD, 11J19C). Pulmonary vasculature was fixed by a perfusion of PFA 4%-PBS (PFA, 10 ml/minute) through the right ventricle during 2 minutes whereas the blood/fixative mix was allowed to escape out of the lung vascular network through a small incision in the left atrium. Lungs were harvested, fixed in 4% PFA at 4°C for 4 hours, incubated in PBS - sucrose (30%) - azide (0.1%) at 4°C overnight, and frozen in Tissue-Tek (VWR, 25608-930). 5 µm-thick-frozen sections were rinsed in PBS, dried at room temperature and mounted with DAPI Fluoromount-G (SouthernBiotech, 0100-20). Red CMTPX-labeled tumor cells remaining in the lungs after the perfusion were considered as extravasated tumor cells and imaged using NanoZoomer 2.0-HT system with a LX2000 source (0.23 µm/pixel (40X) scanning resolution). Whole lungs were considered for cell counting and results are expressed as ratio between number of red 4T1 cells and total lung area analyzed.

***In vivo* lung vascular permeability assay**

Mice were subjected to ozone or air during 3 consecutive days. On day 4, mice were intravenously injected with tetramethylrhodamin-conjugated dextran (50mg/kg; Thermofisher Scientific, D3307). Ten minutes later, mice were anesthetized with a xylazine-ketamine mixture and perfused with PFA 4%-PBS as described in the 'Lung extravasation assay' section. Lungs were collected, fixed in 4% PFA-PBS overnight and embedded in paraffin. 5µm-thick sections (two sections prepared from two different sites of each mouse lung) were deparaffinized, rehydrated and mounted with DAPI Fluoromount-G. Sections were imaged using NanoZoomer 2.0-HT system and LX2000 source (0.23 µm/pixel (40X) scanning resolution). Released rhodamine-dextran from blood vessels was evaluated in whole lung tissues using computer-assisted quantification. Image processing and measurements were

performed using the image analysis toolbox of Matlab R2016a (9.0.341360; Matworks,Inc.,Natick. MA, USA). Results are expressed as median dextran density defined as the ratio between the area occupied by the dextran-stained regions and the whole lung area analyzed. The average value of dextran density measured on both lung tissue slides was used as individual score for each mouse.

Flow Cytometry

After sacrifice, lung tissues were harvested and digested in type IV collagen (1mg/ml) at 37°C. After digestion, red blood cells were lysed using Red Blood Cell Lysis Buffer. Cells were then filtered through a cell strainer. CD45-positive cells were isolated using CD45 microbeads (Miltenyi Biotec, 130-052-301) and then stained using following antibodies: PE-conjugated anti-Ly6B.2 (AbD Serotec, MCA771PE), V450 Horizon-conjugated anti-CD45 (BD Biosciences 560501), BV510 Horizon-conjugated CD3 (BD Biosciences, 563024), PerCP-CY5.5-conjugated anti-GR1 (BD Biosciences, 552093). Neutrophils ($CD45^+$, $CD3^-$, $GR1^+$, $Ly6B.2^+$) were identified using FACSCanto^{TM} II and their relative numbers in lung tissues were quantified with BD FACSDiva software (BD Biosciences).

Total protein and albumin dosage

Total proteins were extracted from crushed lung tissues using a 2M-urea solution. Protein concentrations in lung tissues or BALF were determined using DC protein Assay kit (Bio-Rad Laboratories, 5000116). Albumin leakage in BALF was measured with the quantitative Mouse Albumin ELISA kit (Alpha Diagnostic International, 6300) following manufacturer's instructions.

ELISA assay

ELISA measuring CXCL1, CXCL2 or G-CSF protein levels in supernatants of neutrophils isolated from ozone- or air-exposed lungs were performed following protocols of Duoset ELISA development kits (R&D Systems-Biotechne, DY453, DY452 and DY414, respectively).

Quantification of dsDNA levels in BALF or neutrophil supernatant

DsDNA levels in neutrophil supernatants or BALF were measured using the Quant-iT PicoGreen dsDNA kit according to the manufacturer's instructions (Thermofisher Scientific, P7589).

Western blotting

Lung tissues were homogenized in RIPA buffer supplemented with protease-inhibitor cocktail (Complete protease inhibitor cocktail, Sigma-Aldrich, 04693116001). Primary antibodies used for immunostaining were anti-citrullinated histone 3 (rabbit polyclonal anti-CIT H3, Abcam, ab5103) and anti-HSC70 (mouse monoclonal anti-HSC70, Santa Cruz Biotechnology, sc-7298). After incubation with HRP-conjugated secondary antibodies, immunoreactions were revealed using the enhanced chemoluminescence kit (Thermofisher Scientific, NEL103001EA). Images were acquired by a LAS4000 digital camera (FujiFilm).

Identification of neutrophil extracellular DNA Traps (NETs)

Hoechst staining: Isolated air- or ozone-treated lung neutrophils ($1x10^5$) were seeded into Lab-tek® chamber slide systems (8 chambers/system; Sigma-Aldrich, C7182) in serum-free DMEM and treated or not with Phorbol 12-myristate 13-acetate (PMA - 80 nM, eBioscience, P8139), a *Pad4* inhibitor (*Pad4*i, Cl-amidine, 200 µM, Calbiochem, 506282) or desoxyribonuclease I (DNase I, 2U/500µl, Sigma Aldrich, D5025). After 24 hours, neutrophils were fixed in 4% PFA and DNA was stained with Hoechst 33342 (Thermofisher Scientific, H3570). Computer-assisted quantification of changes in neutrophil morphology was performed by measuring the factor shape of each cell, defined as the length of a cell divided by its area. The used algorithm was implemented in the Matlab software environment (Matlab R2016a (9.0.341360; Matworks,Inc.,Natick. MA, USA). Results are expressed as length cell density, which is the median value of the factor shape on at least 2000 cells per experimental condition.

Immunocytochemistry: Isolated lung neutrophils were permeabilized and incubated overnight at 4°C with rabbit anti-citrullinated histone H3 (CIT H3) antibody (Abcam, ab5103). Secondary donkey anti-rabbit IgG AlexaFluor 555-conjugated antibody (Thermofisher Scientific, A-31572) was added with Hoechst 33342 to identify NETs. NETs were imaged using digital slide-scanner NanoZoomer 2.0-HT and LX2000 source (0.23 µm/pixel (40X) scanning resolution) (Hamamatsu Photonics).

Immunohistochemistry for NET identification

To identify NETs in lung tissues, histological lung tissue sections were deparaffinized and boiled in 10 mM sodium carbonate buffer for antigen retrieval. Lung sections were permeabilized, blocked and incubated in blocking buffer with rabbit anti-citrullinated histone

H3 (CIT H3; Abcam, ab5103) and goat anti-MPO (R&D Systems-Biotechne, AF3667) antibodies. Secondary donkey anti-rabbit and anti-goat IgG antibodies conjugated with AlexaFluor 555 (Thermofisher Scientific, A-31572) and AlexaFluor 488 (Thermofisher Scientific, A-11055), respectively, were added in blocking buffer containing Hoechst 33342. Samples were scanned using NanoZoomer scanner and LX2000 source (0.23 µm/pixel (40X) scanning resolution).

Proliferation assay

Cell proliferation was evaluated 24, 48 or 72 hours after 4T1 cell seeding ($2x10^3$/well) in 96-well plates using the CyQUANT cell proliferation assay kit (Thermofisher Scientific, C7026), according to the manufacturer's instructions. Briefly, 4T1 cells were plated in sixplicates in either DMEM containing 10% FBS (positive control), in DMEM +1% FBS (vehicle: negative control) or in media conditioned by ozone- or air-stimulated neutrophils supplemented with 1% FBS. Plates were read with a SpectraMax i3 multi-mode microplate reader (Molecular Devices) with an excitation maximum at 480 nm and an emission maximum at 520 nm. All readings were normalized to the vehicle (Negative Control) considered as baseline proliferation.

Boyden chamber assay

The ability of supernatants derived from *ex vivo*-neutrophils previously isolated from ozone- or air-exposed lungs to induce tumor cell migration *in vitro* was studied using Boyden chambers. Cell migration was assessed by using 24-well transwell chambers with filters of 8 µm pore size (Costar). Lower wells contained media conditioned by neutrophils isolated from ozone- or air-exposed lungs supplemented with 1% FBS and 1% BSA. 4T1 cells ($1x10^5$) were added to each upper well in serum-free media supplemented with 0.1% BSA and incubated at 37°C for 16 hours. At the end of the experiment, cells were fixed in methanol at -20°C during 30 minutes and stained with Giemsa. Membranes were removed from the inserts using a scalpel, placed on microscope slides and digitalized with a NanoZoomer 2.0-HT system equipped with a TRI-CCD Camera (0.23 µm/pixel (40X) scanning resolution). Migrating cells were counted on eight random fields and the average value was used as an individual score for each membrane. Each experimental condition was performed in quadruplicates.

Adhesion assay

4T1 cells were previously stained with a red-fluorescent CMTPX Cell Tracker (Invitrogen) (see 'Lung extravasation assay' section for 4T1 cell staining), then 5×10^4 4T1 cells were added on a confluent SVEC4.10 endothelial cell monolayer cultivated in Lab-tek® chamber slide systems (4 chambers/system; Sigma-Aldrich). After 45 minutes incubation in presence of media conditioned by air- or ozone-exposed neutrophils, non-adherent 4T1 cells were washed away and slides were mounted with DAPI Fluoromount G (Southern Biotech). Membranes were digitalized with a NanoZoomer 2.0-HT system and LX2000 source (0.23 µm/pixel (40X) scanning resolution). Per each sample, adherent red 4T1 cells were counted on four randomly selected microscopic fields and the average value was used as individual score for each sample. Each experimental condition was performed in quadruplicates.

References

1. Passegue E, Wagner EF, Weissman IL. JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. Cell 2004;**119**(3):431-43.
2. Hemmers S, Teijaro JR, Arandjelovic S, et al. PAD4-mediated neutrophil extracellular trap formation is not required for immunity against influenza infection. PloS one 2011;**6**(7):e22043.
3. Chabottaux V, Sounni NE, Pennington CJ, et al. Membrane-type 4 matrix metalloproteinase promotes breast cancer growth and metastases. Cancer research 2006;**66**(10):5165-72.
4. Rocks N, Bekaert S, Coia I, et al. Curcumin-cyclodextrin complexes potentiate gemcitabine effects in an orthotopic mouse model of lung cancer. British journal of cancer 2012;**107**(7):1083-92.
5. Maree R, Rollus L, Stevens B, et al. Collaborative analysis of multi-gigapixel imaging data using Cytomine. Bioinformatics 2016;**32**(9):1395-401.

Supplementary figure legends

Supplemental figure 1: Ozone impacts early steps of MDA-MB-231 mammary tumor cell metastasis. A. Timeline of a 3-day ozone exposure followed by an intravenous MDA-MB-231 cell injection (black arrow). The presence of tumor cells in lungs was monitored 24 hours after MDA-MB-231 injection (white arrow). B. Representative images of MDA-MB-231-related luciferase imaging (Photon mode) in lungs of mice (red circles) exposed to air or ozone. C. Luciferase activity was quantified in ROI determined around lungs in mice treated with air or ozone, (***$p<0.001$; Mann Whitney test). Error bars represent the interquartile range; n=10 mice/experimental group.

Supplemental figure 2: Media conditioned by air- (CM PNN Air) or ozone-challenged neutrophils (CM PNN Ozone) do not affect 4T1 cell proliferation, migration and adhesion rates. A. CyQUANT analysis of 4T1 cell proliferation 24 hours, 48 hours and 72 hours after treatment of 4T1 cells with media conditioned by air- or ozone-challenged neutrophils. All readings were normalized to the vehicle (Negative Control) considered as baseline proliferation and expressed as fold increase from baseline. Bars represent the median value per experimental group and the interquartile range is shown by horizontal lines; n=6 wells/experimental group (**$p<0.01$ *versus* Positive Control; Kruskal-Wallis test). B. The influence of media conditioned by air- or ozone-challenged neutrophils on *in vitro* 4T1 tumor cell migration was assessed in an *in vitro* Boyden Chamber Assay. Representative images of Boyden Chamber filters bearing migrating 4T1 cells are shown here (scale bar: 250 µm). C. Quantification of migrating 4T1 cells per microscopic field (8 random microscopic fields analyzed per sample). The middle horizontal line represents the median value of migrating cells while upper and lower lines represent the interquartile range (n=4 wells/experimental group; Mann-Whitney test). D. Representative pictures of each experimental condition of the adhesion test (scale bar: 250 µm). Upper panels show the SVEC4.10 cell layer as well as adhering 4T1 cells stained by DAPI while lower panels only show adhering CMTPX-red stained 4T1 cells. E. Quantification of 4T1 cell numbers adhering to a confluent murine endothelial SVEC4.10 cell monolayer per microscopic field. Cells were treated either with media conditioned by air- (CM PNN Air) or ozone-challenged neutrophils (CM PNN Ozone). The middle horizontal line represents the median value of adhering cells while upper and lower lines represent the interquartile range (n=4 wells/ experimental group; Mann-Whitney test).

Results are representative of two experiments performed individually.