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## SUPPLEMENTARY INFORMATION

### **MET is required for the recruitment of anti-tumoural neutrophils**

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## SUPPLEMENTARY METHODS

**Cell lines:** Murine Lewis lung carcinoma cells (LLC), melanoma B16F10, and human non-small cell lung carcinoma A549 cells were obtained from American Type Culture Collection (ATCC); the murine pancreatic tumour cell line Panc02 and the murine fibrosarcoma cell line T241 were respectively a gift from Dr. Cavallaro (IEO, Milano, IT) and Dr. Claesson-Welsh (Rudbeck Laboratory, Uppsala, SE). LLC, B16F10, A549, and T241 cells were cultured in DMEM (Gibco) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and containing 10% FBS (DMEM 10% FBS). Panc02 cells were cultured in RPMI (Gibco) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and containing 10% FBS. All these murine tumour cell lines are syngeneic in a C57BL/6 background, allowing implantation in *Met* conditional knockout mice or chimeras. Human Umbilical Vein Endothelial Cells (HUVEC) were isolated from human umbilical cords and maintained in M199 (Invitrogen) supplemented with 20% FBS, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.15% Heparin, 20 µg/ml ECGS (M199 complete). 0.1% pork gelatin was used to favour the adhesion of HUVEC to the flask bottom. All cells were maintained in a humidified incubator in 5% CO<sub>2</sub> and 95% air at 37°C. Three or four different short hairpin RNA lentiviral vectors (Sigma) were used to silence *Met* in LLC, B16F10 or T241 (LLC sh*Met*, B16F10 sh*Met* or T241 sh*Met*; see below), or to silence *TNFA* in HUVEC (HUVEC sh*TNFA*). Scramble lentiviral vectors were used as control. Transduced cells were selected with 8 µg/ml puromycin. All cancer cell lines and primary HUVEC underwent mycoplasma testing before their use. Negative mycoplasma contamination status was verified using LookOut Mycoplasma PCR Kit (Sigma) and MycoAlert Mycoplasma Detection Kit plus Assay Control (Lonza). Panc02 and T241 cells were both authenticated by Idexx Bioresearch. All cells were passaged in the laboratory for no longer than 6 months after receipt. Supplementary Table 2 lists the sequences of all the shRNA constructs used in this study.

**Tumour-conditioned medium (TCM) and LLC (or A549)-conditioned medium (CCM)**

**preparation:** Two grams of endstage LLC tumour explanted from WT mice were chopped and incubated at 37°C in 7 ml of DMEM (supplemented with 2 mM glutamine, 100 units/ml penicillin/100 µg/ml streptomycin) FBS-free (DMEM 0% FBS).  $5 \times 10^4$  LLC (or A549) were seeded in a 6-multiwell plate in DMEM 10% FBS and incubated at 37°C. Medium alone (DMEM 0% FBS or DMEM 10% FBS, respectively) was used to prepare mock controls. After 72 hours, the medium was filtered, supplemented with 2 mM glutamine and 20 mM HEPES and stored at -20° C. TCM and mock medium (DMEM 0% FBS) were diluted 1:5 in DMEM 10% FBS; CCM and mock medium (DMEM 10% FBS ) were diluted 4:5 in DMEM FBS-free.

**Lung colonisation assay:** In the experimental metastasis assays,  $0.5 \times 10^6$  B16F10 cells were injected in the tail vein and lungs were collected after 12 days. To quantify pulmonary seeding, lungs were homogenized in Trizol (Ambion) and RNA was purified with the RNeasy Mini kit (Ambion) according to manufacturer's instructions. The expression of the melanocyte specific gene S100B was measured as readout of lung colonization by qRT-PCR following reverse transcription to cDNA with the QuantiTect Reverse Transcription kit (Qiagen).

**Endothelial cell isolation:** Lungs were collected and a single cell suspension was obtained as described in Methods. Endothelial cells were obtained by performing a negative selection for CD45 (30F-11) and F4/80 (CI:A3-1) followed by a positive selection for CD31 (MEC 13.3) by using magnetic beads (Dynabeads, Invitrogen) according to the manufacturer's protocol.

**Mouse white blood cell (WBC) isolation:** Blood was collected from the retro-orbital vein in 10% heparin. For the isolation of WBC, the blood was diluted in 1.25% dextran in saline to allow the sedimentation of red blood cells (RBC). After 30', the supernatant was collected and washed in PBS 0.1% BSA. The remaining RBC were lysed in a hypotonic solution of 0.2% NaCl for 30 seconds and brought in isotonic condition with 1.6% NaCl. WBC were washed in PBS 0.1% BSA, counted and resuspended accordingly with the experimental setting.

**Peritoneal macrophages:** 5 ml of sterile PBS were injected in the peritoneum of anesthetized mice and collected after 3 minutes. Cells were centrifuged, washed and culture overnight.

**Human neutrophil isolation:** 10 ml of venous blood from healthy volunteers were collected in citrate-coated tubes and isolated by erythrocyte sedimentation with dextran and purification with a discontinuous plasma-Percoll gradient as already described<sup>36</sup>.

**Flow sorting of neutrophils from lung cancer patients:** Lung tumour biopsies or healthy tissues were minced in RPMI medium containing 0.1% collagenase type I, 0.2% dispase type I and DNase I 100 U/ml (60 minutes at 37°C), passed through a 19 G needle and filtered. After RBC lysis, cells were resuspended in FACS buffer (PBS containing 2% FBS and 2 mM EDTA) and counted. Myeloid population, enriched using CD11b-conjugated magnetic beads (MACS, Miltenyi Biotec) and separated through magnetic column (MACS, Miltenyi Biotec), was stained with anti-CD66b (G10F5, BD Pharmingen) for 20 minutes on ice and sorted with FACS Aria I (BD Bioscience). Cells were counted and resuspended in RLT buffer (Qiagen) for RNA extraction.

**Histology and immunostainings:** To obtain serial 7- $\mu$ m-thick sections, tissue samples were immediately frozen in OCT compound or fixed in 2% PFA overnight at 4°C, dehydrated and embedded in paraffin. Paraffin slides were firstly rehydrated to further proceed with antigen retrieval in citrate solution (DAKO). Cryosections were thawed in water and fixed in 100% methanol. If necessary, 0.3% H<sub>2</sub>O<sub>2</sub> was added to methanol to block endogenous peroxidases. The sections were blocked with the appropriate serum (DAKO) and incubated overnight with the following antibodies: rat anti-CD45 (30F-11, BD Pharmingen), rat anti-Ly6G (1A8, BD Pharmingen) 1:100, rat anti-CD31 (MEC 13.3, BD Pharmingen) 1:200, rabbit anti-FITC (Serotec) 1:200, goat anti-phosphohistone H3 (Cell Signaling) 1:100, rat anti-F4/80 (CI:A3-1, Serotec) 1:100, mouse anti-NK1.1-biotin (PK136, BD Pharmingen) 1:200, rat anti-CD45R (RA3-6B2, BD Pharmingen) 1:100, rat anti-CD4 (H129.9, BD Pharmingen) 1:100, rat anti-CD8 (53-6.72, BioXCell) 1:100, hamster anti-CD11c biotin (N418, eBioscience) 1:100, mouse anti-3-nitrotyrosin

1:200 (HM.11 Santa Cruz). Appropriate secondary antibodies were used: Alexa488-or Alexa568-conjugated secondary antibodies (Molecular Probes) 1:200, HRP-labelled antibodies (DAKO) 1:100. When necessary, Tyramide Signaling Amplification (Perkin Elmer, Life Sciences) was performed according to the manufacturer's instructions. Whenever sections were stained in fluorescence, ProLong Gold mounting medium with DAPI (Invitrogen) was used. Otherwise, 3,3'-diaminobenzidine was used as detection method followed by Harris' haematoxin counterstaining, dehydration and mounting with DPX. Apoptotic cells were detected by the TUNEL method, using the AptoTag peroxidase in situ apoptosis detection kit (Millipore) according to the manufacturer's instructions. For the double staining TUNEL and LY6G, TUNEL staining was performed as described above, followed by Ly6G staining by using the Vectastain ABC kit (Vector laboratories) according to the manufacturer's instructions. Tumour necrosis and lung metastasis were evaluated by H&E staining. Necrotic area was defined as the area including necrotic cancer cells, inflammatory cells and stromal cells, compared to the total area of the field. Necrotic cells display a more glassy homogeneous appearance in the cytoplasm with increased eosinophilia, while the nuclear changes are reflected by karyolysis, pyknosis, and karyorrhexis. Alternatively, the necrotic tissue was visualized by autofluorescence as previously described<sup>37</sup>. Microscopic analysis was done with an Olympus BX41 microscope and Cell Sense imaging software or a Zeiss Axioplan microscope with KS300 image analysis software. The morphometric analysis was performed by acquiring 4-6 fields per sections on 5 independent sections (at a distance of 40 µm in depth during sectioning) from the same biological tissue sample. The values in the graphs represent the average of the means of, at least, 5 samples and the standard error indicates the variability among the different samples.

**Hypoxia assessment and tumour perfusion:** Tumour hypoxia was detected by injection of 60 mg/kg pimonidazole hydrochloride into tumour-bearing mice 1 hour before tumours harvesting. To detect the formation of pimonidazole adducts, tumour cryosections were immunostained with

Hypoxyprobe-1-Mab1 (Hypoxyprobe kit, Chemicon) following the manufacturer's instructions. Perfused tumour vessels were counted on tumour cryosections from mice injected intravenously with 0.05 mg FITC-conjugated lectin (*Lycopersicon esculentum*; Vector Laboratories).

**Quantitative RT-PCR (qRT-PCR):** For mRNA analysis,  $1 \times 10^5$  or  $3 \times 10^5$  mouse or human blood neutrophils, respectively, were incubated in normoxic (21% oxygen) or hypoxic (1% oxygen) conditions, or stimulated with TCM (plus 50  $\mu\text{g/ml}$  Enbrel or human IgG when indicated), CCM, A549-CM, 100 ng/ml of murine or human TNF- $\alpha$ , 50 ng/ml LPS, or mock medium in 96-multiwell for 4 hours at 37°C. For NF- $\kappa\text{B}$  inhibition,  $0.18 \times 10^6$  neutrophils were pre-treated with 10  $\mu\text{M}$  6-amino-4-(4-phenoxyphenylethylamino) quinazoline (Calbiochem) for 1 hour at 37°C and stimulated with 100 ng/ml of murine TNF- $\alpha$  for 1 hours at 37°C.  $2 \times 10^5$  HUVEC were seeded in 24-multiwell coated with 0.1% gelatin and stimulated with 5 ng/ml IL-1 $\alpha$  in DMEM 10% FBS for 4 hours at 37°C. Cells were washed in PBS, collected in RLT buffer (Qiagen) and kept at -80°C. RNA was extracted with the RNeasy Micro kit (Qiagen) according to manufacturer's instructions. Reverse transcription to cDNA was performed with the SuperScript® III First Strand cDNA Synthesis Kit (Life Technologies) according to manufacturer's protocol. Pre-made assays were purchased from Applied Biosystem, except for *Nos2* that was provided by IDT. cDNA, primer/probe mix and TaqMan Fast Universal PCR Master Mix were prepared in a volume of 10  $\mu\text{l}$  according to manufacturer's instructions (Applied Biosystems). Samples were loaded into an optical 96-well Fast Thermal Cycling plate (Applied Biosystems) and qRT-PCR were performed using an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems)

**ELISA:** To quantify plasma, intra-tumoural, tumour-released (TCM) and neutrophil-released HGF, a murine HGF ELISA kit (R&D) was used according to manufacturer's protocols. Blood was collected from tumour free or tumour bearing mice and plasma was prepared according to manufacturer's instruction. TCM was prepared as above. Tumour proteins were extracted in Extraction Buffer (20M Tris HCl, 150mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA).

$0.2 \times 10^6$  neutrophils were cultured for 20 hours in DMEM complete in presence or absence of 100 ng/ml of murine TNF- $\alpha$ . Medium was collected, spun down and supernatant stored at  $-80^\circ\text{C}$  until use. Mock medium was used as negative control. For phospho-MET quantification,  $6 \times 10^6$  mouse blood neutrophils isolated from tumour bearing mice were cultured in presence or absence of 100 ng/ml of murine TNF- $\alpha$ ; 11 hours later, cells were stimulated or not with 100 ng/ml of mouse HGF in presence of 1 mM orthovanadate for 3 minutes at  $37^\circ\text{C}$ , washed in PBS supplemented with 1 mM orthovanadate and phosStop 1X (Roche) and lysed in Extraction Buffer supplemented with 1 mM orthovanadate, 2X phosStop and Complete Mini protease inhibitor cocktail (Roche) for 20 minutes at  $4^\circ\text{C}$ . After clearance, samples were quantified and the same amount of proteins was used for MET and phospho-MET detection using a sandwich ELISA. Briefly, 96-well microtiter plates (MaxiSorp, Nunc) were coated with 1  $\mu\text{g/ml}$  of anti-mouse MET antibody (AF527, R&D Systems) overnight at  $4^\circ$  and then incubated for 2 hours at room temperature (RT) in blocking buffer (PBS, 0.1% Tween-20, 6% non-fat dry milk). The same amount of proteins for each sample was diluted in blocking buffer and incubated for 2 hours at RT on the ELISA plate. After 6 washes in PBS 0.1% Tween-20, samples were incubated for 2 hours at RT with the mouse anti-MET (3D4, Invitrogen) or the mouse anti-phosphotyrosine (4G10, Merck Millipore) antibodies diluted 1:500 in blocking buffer, then washed 6 times in PBS 0.1% Tween-20, and incubate with goat anti-mouse immunoglobulins conjugated to horseradish peroxidase (sc-2031, Santa Cruz Biotechnology) diluted 1:500 in blocking buffer for 2 hours at RT. Signals was developed by 15 minutes incubation with the 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Promega); after stopping the reaction with  $\text{H}_2\text{SO}_4$ , absorbance was measured at 450 nm and corrected for 630 nm with a spectrophotometer.

**Tumour-derived nitric oxide production:** LLC tumours were collected 8 days after injection, cut in pieces of about 5x5 mm, weighted and incubate at  $37^\circ\text{C}$  in 24-multiwell with 800  $\mu\text{l}$  of DMEM.



After 24 hours, the media was collected, centrifuged to remove cell debris, and NO levels were measured using the Griess reagent system kit (Promega).

**Nitric oxide measurement by FACS:** Neutrophils isolated from the blood of WT or KO LLC-tumour bearing mice were co-cultured for 4 hours with LLC sh*Met*, washed and resuspended in 20 mM HEPES-PBS, and incubated for 10 minutes with 5 $\mu$ M DAF-FM diacetate (Molecular probes) in absence or presence of mouse HGF (100 ng/ml) at 37°C. Cells were then washed and analysed by FACS.

**Lung cancer patients:** We enrolled 4 non-small cell lung carcinoma-patients; exclusion criteria were: previous history of oncological, chronic inflammatory, or autoimmune diseases within 10 years prior to this study. The protocol was approved by the Ethics Committee of the University Hospitals Gasthuisberg (Leuven), and all subjects consented prior to study participation.

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- 37 Shaked, Y. *et al.* Rapid chemotherapy-induced acute endothelial progenitor cell mobilization: implications for antiangiogenic drugs as chemosensitizing agents. *Cancer Cell* **14**, 263-273 (2008).

## SUPPLEMENTARY TABLES

**Supplementary Table 1: Vector copy number (VCN) in WT and *Met* KO HSCs.**

WT		<i>Met</i> KO	
Mrp8:Empty	Mrp8:Met	Mrp8:Empty	Mrp8:Met
1,40 ± 0,13	1,79 ± 0,18	1,88 ± 0,12	1,92 ± 0,19

The data represent the number of integrated lentiviral vector (LV) copies per cell genome (vector copy number, VCN ± s.e.m.) of HIV-gag (contained by both Mrp8:Empty and Mrp8:Met LVs) in HSPCs, collected 9 days after in vitro transduction ( $n=4$  technical replicates). See Methods for technical details.

**Supplementary Table 2. List of all the shRNA sequences purchased from Sigma, used in this study.**

shRNA	TRC number	Sequence
Mouse <i>Met</i> (1)	TRCN0000023530	CCGGGCACGACAAATACGTTGAAATCTCGAGATTTCAA CGTATTTGTCGTGCTTTTT
Mouse <i>Met</i> (2)	TRCN0000023529	CCGGCGGGATTCTTTCCAAACACTTCTCGAGAAGTGTTT GGAAAGAATCCCGTTTTT
Mouse <i>Met</i> (3)	TRCN0000023531	CCGGCCCCGACGTGAACACATTTGATCTCGAGATCAAAT GTGTTACGTCGGGTTTTT
Mouse <i>Met</i> (4)	TRCN0000023533	CCGGCCTTGAATAGAATCACAGATACTCGAGTATCTGTG ATTCTATTCAAGGTTTTT
Human <i>TNFA</i> (1)	TRCN0000003757	CCGGCTGTAGCCCATGTTGTAGCAACTCGAGTTGCTACA ACATGGGCTACAGTTTTT
Human <i>TNFA</i> (2)	TRCN0000003758	CCGGGCAGGTCTACTTTGGGATCATCTCGAGATGATCCC AAAGTAGACCTGCTTTTT
Human <i>TNFA</i> (3)	TRCN0000003759	CCGGCAGGTCTACTTTGGGATCATTCTCGAGAATGATCC CAAAGTAGACCTGTTTTT
Scramble	SHC002V	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGC TCTTCATCTTGTTGTTTTT

## SUPPLEMENTARY NOTES

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**SUPPLEMENTARY FIGURES**

**Supplementary Fig. 1**

Fig. 3e

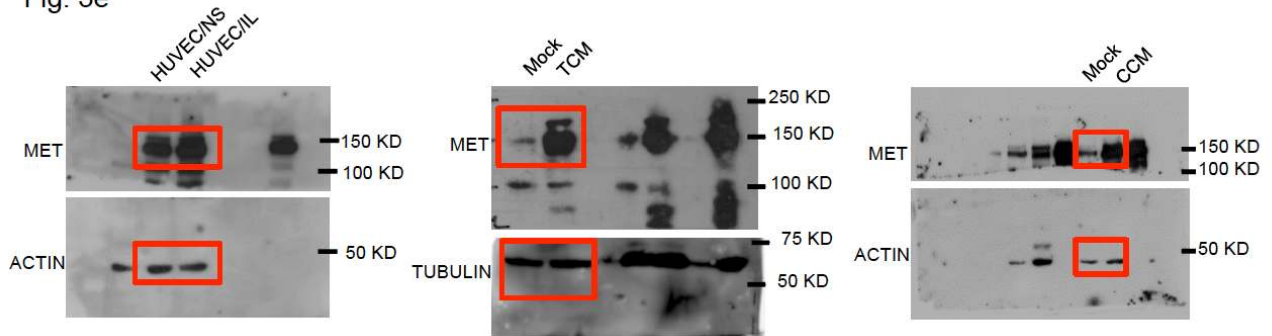


Fig. 3g

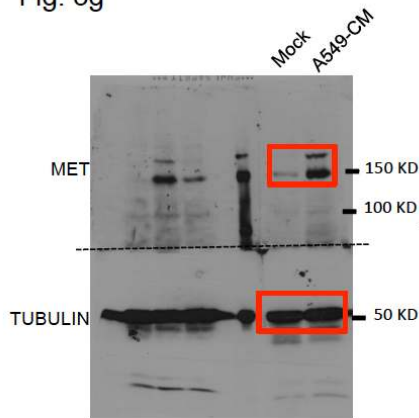


Fig. 3h

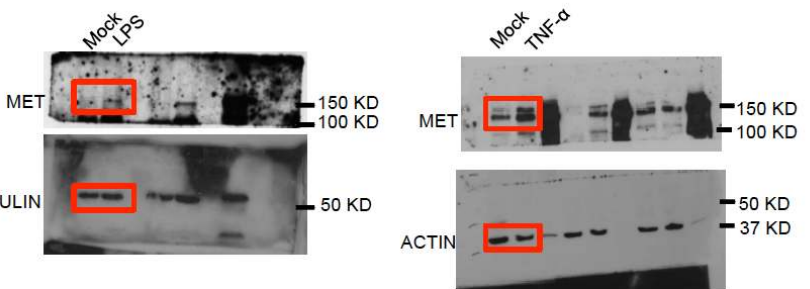


Fig. 3h

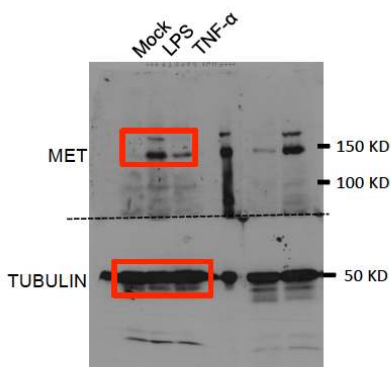
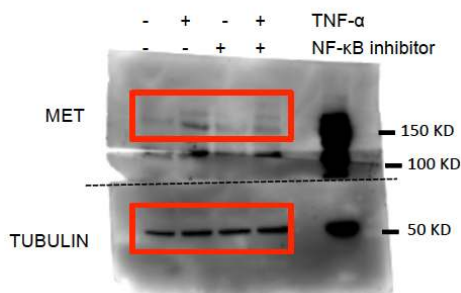
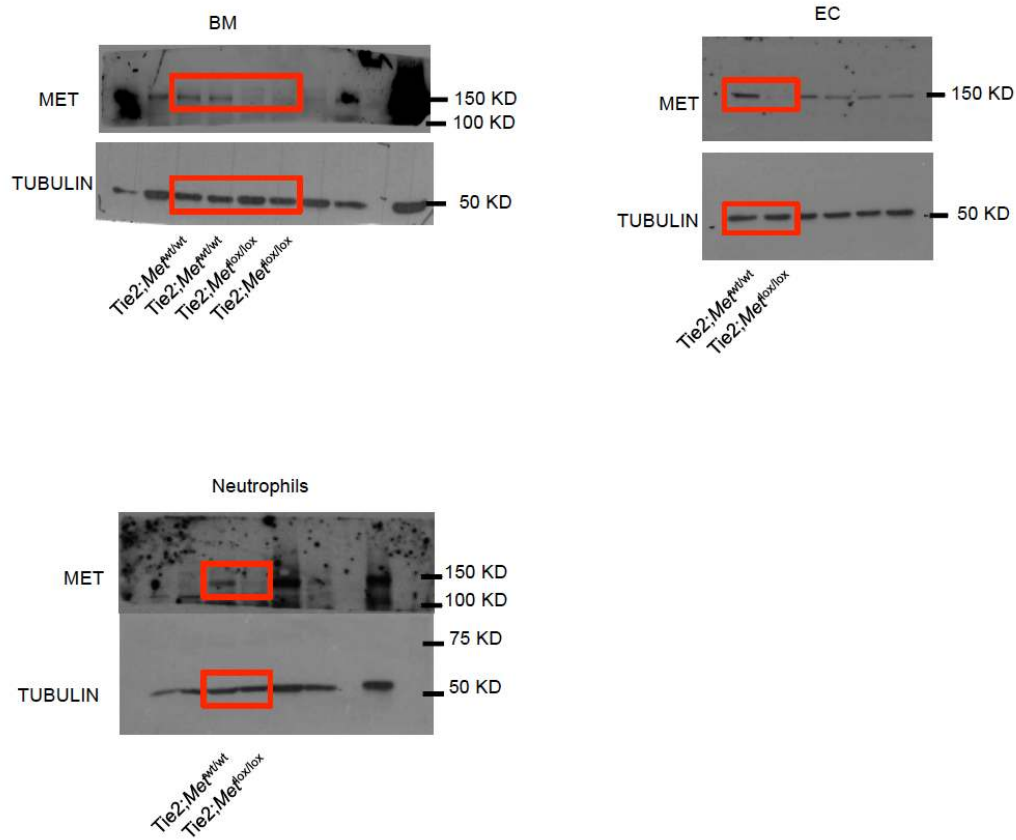


Fig. 3k

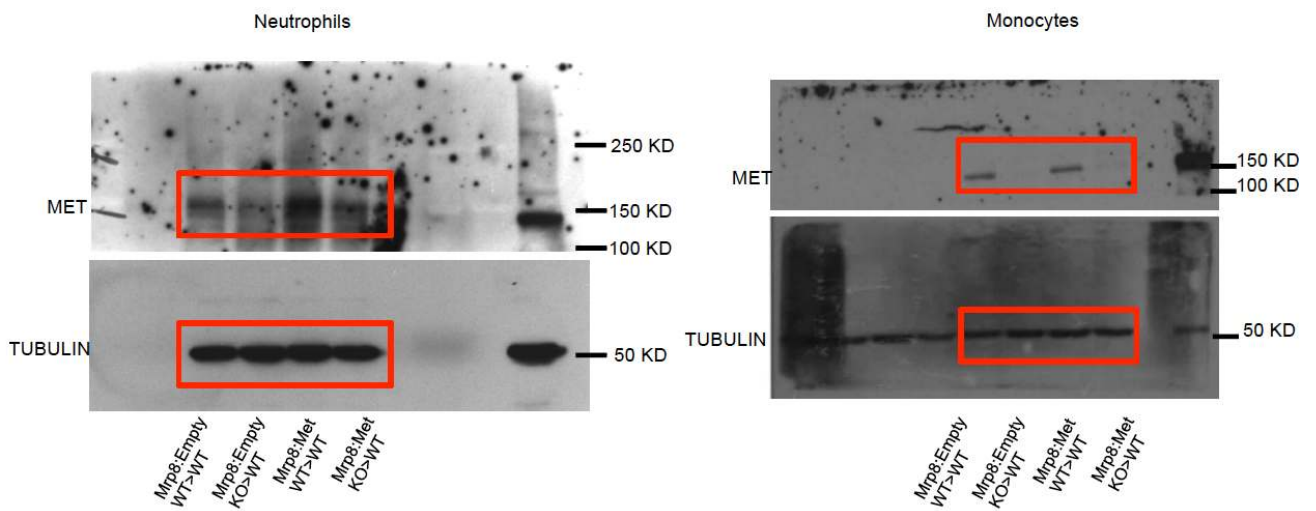


*Continues*

Extended Data Fig. 2a

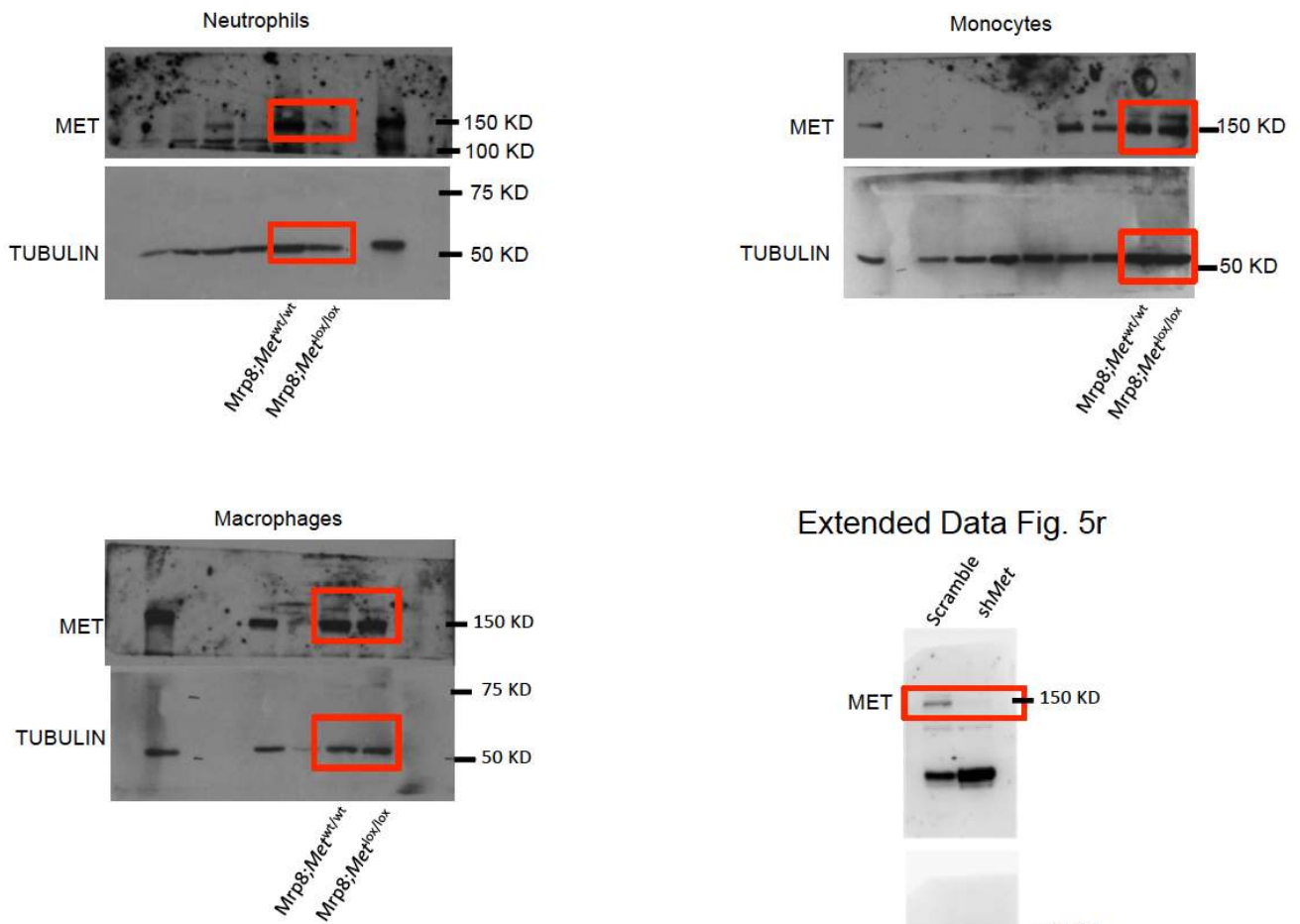


Extended Data Fig. 4a



Continues

### Extended Data Fig. 4d



### Supplementary Figure 1. Full-size scans of uncropped western blot images

Uncropped, full-size scans of Western blots shown in Fig. 3e,g,h,k; Extended Data Fig. 2a; Extended Data Fig. 4a,d; Extended Data Fig. 5r as indicated. Molecular weight markers are indicated. Red boxes highlight the lanes that are displayed in the corresponding Figures.