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Supplementary Information for

Targeting scavenger receptor MARCO on tumor-associated macrophages activates TRAIL dependent tumor cell killing by NK cells.

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This PDF file includes:

Figures S1 to S7 and supplementary methods S1

Other supplementary materials for this manuscript include the following:

Movie S1 and Movie S2

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Figure S1

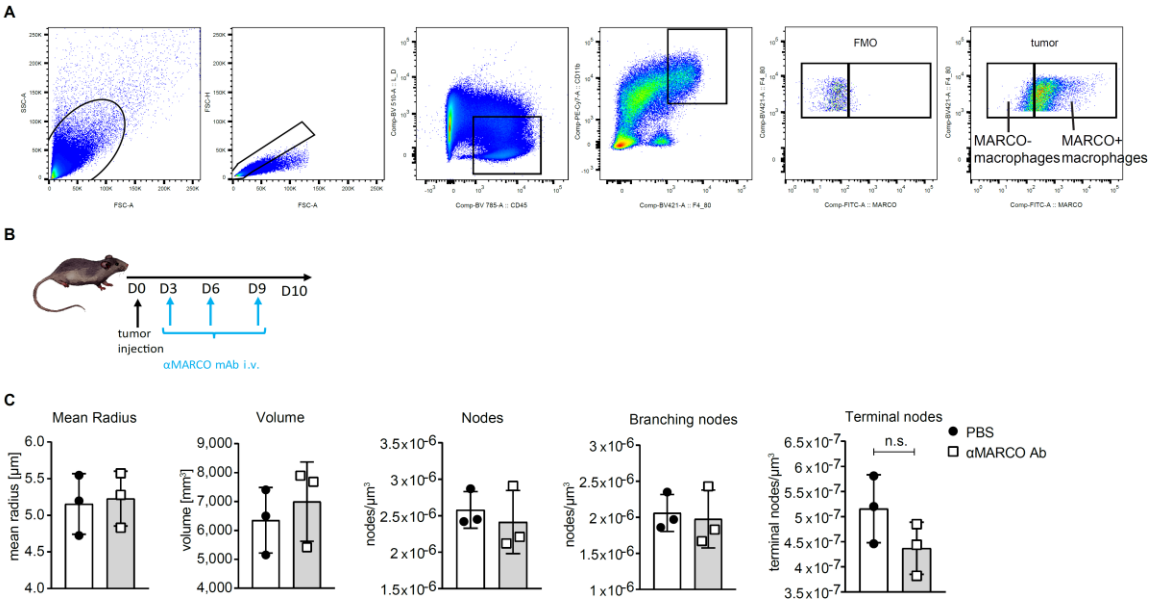
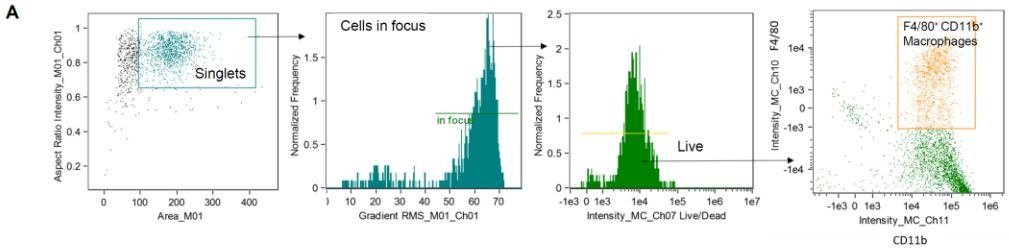
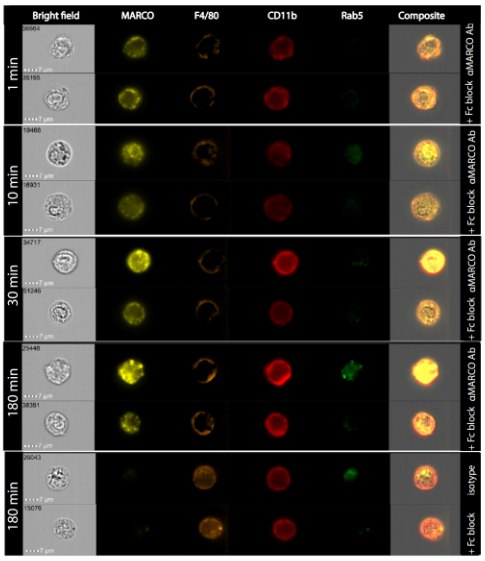


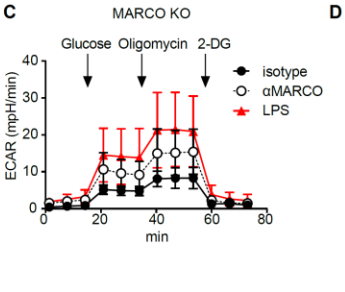
Fig. S1. MARCO$^+$ cells are in proximity to T cells in B16 tumors and targeting with anti-MARCO Ab does not influence tumor blood vessel size or node formation.

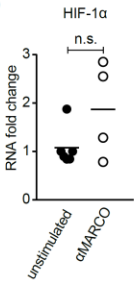
A) Flow cytometry gating strategy to define MARCO$^+$ macrophages inside B16 tumor on d10. Representative gating shown. n=9. B) Schematic of the B16 melanoma tumor model and Ab treatment schedule. C) CD31$^+$ vessel properties in B16 tumors on day 10 in PBS treated or anti-MARCO Ab treated mice analyzed by whole tumor imaging. n=3/group. Branching, terminal and total nodes are normalized to tumor size. Significance calculated by student`s t-test. Graphs show Mean+/- SD.

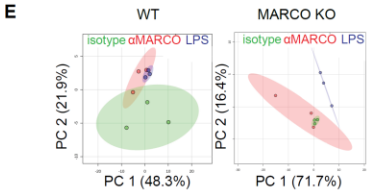
Figure S2


A
Singlets
Aspect Ratio Intensity_M01_Ch01
Area_M01
Cells in focus
in focus
Normalized Frequency
Gradient RMS_M01_Ch01
Live
Intensity_MC_Ch07 Live/Dead
F4/80+ CD11b+
Macrophages
Intensity_MC_Ch10 F4/80
Intensity_MC_Ch11
CD11b


B
Bright field
MARCO
F4/80
CD11b
Rab5
Composite
1 min
10 min
30 min
180 min
180 min
+ Fc block αMARCO Ab
+ Fc block αMARCO Ab
+ Fc block αMARCO Ab
+ Fc block αMARCO Ab
+ Fc block isotype


C
MARCO KO
Glucose
Oligomycin
2-DG
ECAR (mpH/min)
min
isotype
αMARCO
LPS


D
HIF-1α
n.s.
RNA fold change
unstimulated
αMARCO


E
WT
MARCO KO
isotype αMARCO LPS
PC 2 (21.9%)
PC 1 (48.3%)
PC 2 (16.4%)
PC 1 (71.7%)

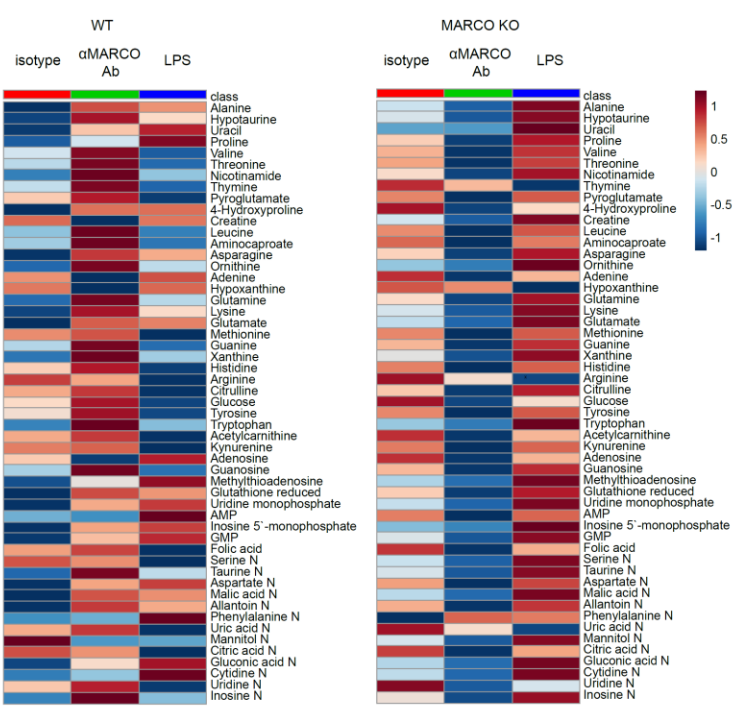

F
WT
MARCO KO
isotype
αMARCO Ab
LPS
class
Alanine
Hypotaurine
Uracil
Proline
Valine
Threonine
Nicotinamide
Thymine
Pyroglutamate
4-Hydroxyproline
Creatine
Leucine
Aminocaproate
Asparagine
Ornithine
Adenine
Hypoxanthine
Glutamine
Lysine
Glutamate
Methionine
Guanine
Xanthine
Histidine
Arginine
Citrulline
Glucose
Tyrosine
Tryptophan
Acetylcarnithine
Kynurenine
Adenosine
Guanosine
Methylthioadenosine
Glutathione reduced
Uridine monophosphate
AMP
Inosine 5`-monophosphate
GMP
Folic acid
Serine N
Taurine N
Aspartate N
Malic acid N
Allantoin N
Phenylalanine N
Uric acid N
Mannitol N
Citric acid N
Gluconic acid N
Cytidine N
Uridine N
Inosine N
1
0.5
0
-0.5
-1

Fig. S2. Targeting MARCO by monoclonal antibody results in metabolic changes within the MARCO expressing macrophages.

A) Gating strategy for the image stream experiments. Macrophages were defined as live CD11b and F4/80 double positive cells. Representative gating is shown. B) Representative images from peritoneal macrophages analyzed by image stream. CD11b+ F4/80+ cells were pre-stimulated with Fc block for 30 minutes before adding fluorescently labelled anti-MARCO Ab and stained for early endosomal marker Rab5. n=2 /stimulation. Representative for two independent experiments. C) Glycolytic rate of MARCO deficient peritoneal macrophages is not affected by 24 hours of anti-MARCO Ab treatment compared to the control group. Analyzed by Seahorse XFe96 Analyzer. Representative experiment out of three is shown. n= 3-6. D) Hypoxia induced factor- 1α (HIF-1α) is upregulated in peritoneal macrophages after 24 hours of anti-MARCO Ab treatment. Histogram shows RNA fold change, normalized to unstimulated cells. E) Principal component analysis (PCA) on 24 hours rat IgG1 isotype, anti-MARCO Ab or LPS treated WT and MARCO KO peritoneal macrophages. 53 metabolites were included and MetaboAnalyst 4.0 was used for analysis. n=3. F) Heatmaps showing metabolites of WT and MARCO KO peritoneal macrophages, treated for 24 hours with either rat IgG1 isotype, anti-MARCO Ab or LPS. Data were log transformed and auto-scaled prior to analysis. Heatmaps were created with MetaboAnalyst 4.0 using Euclidean distance measure and Ward clustering algorithm. n=3/group.

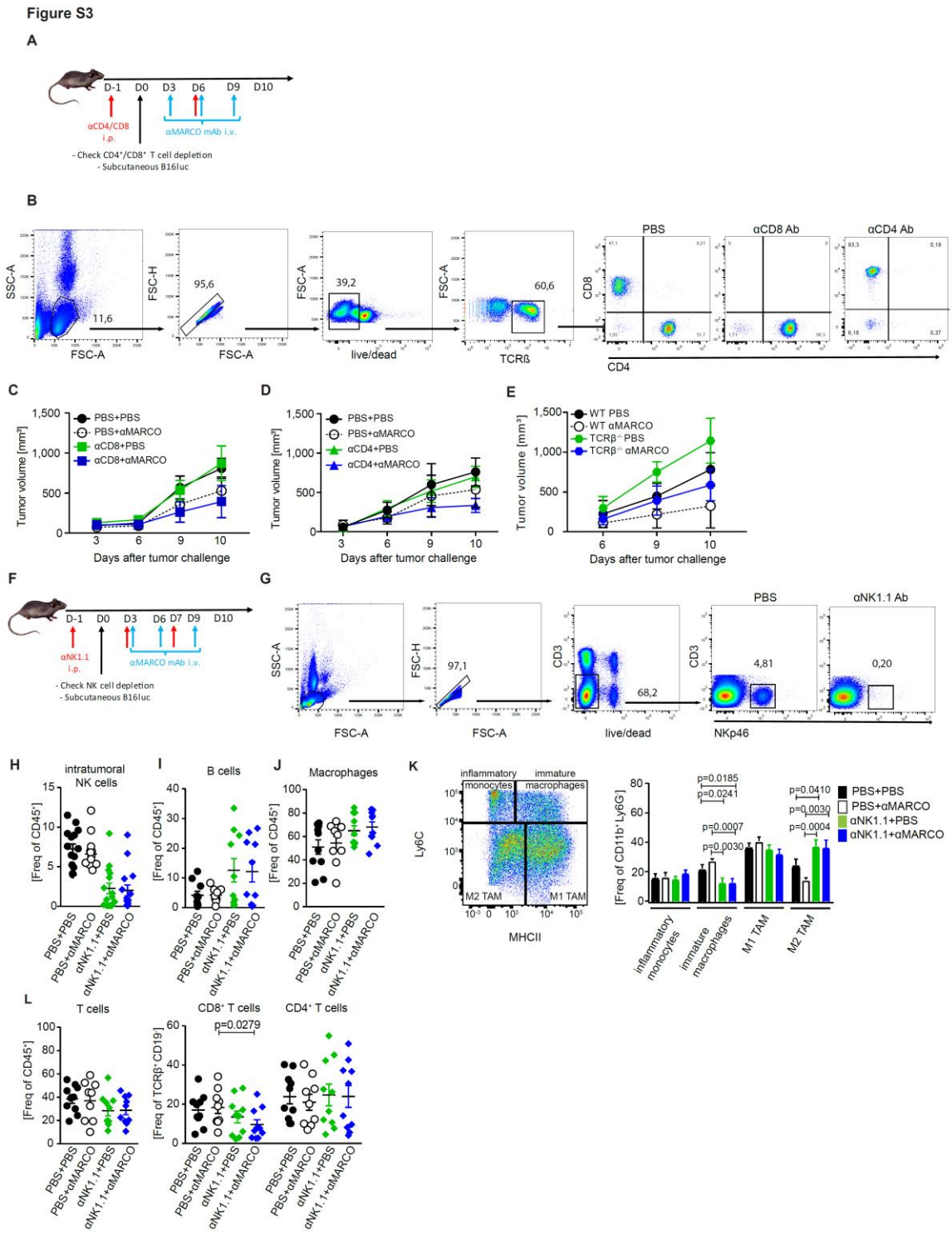


Fig. S3. Neither depletion of CD4+ or CD8+ cells nor TCR-b deficiency impairs anti-MARCO Ab mediated anti-cancer effect.

A) Schematic of the B16 model for T cell depletion experiments and their Ab administration. Depleting Ab were injected i.p. 24 hours before tumor cells inoculation. B) Gating strategy for evaluation of successful CD4+ and CD8+ T cell depletion in the blood. Depletion was confirmed

in the blood on day -1, 3 and 6 and inside the B16 tumor on day 10. Representative image shows day 3. C) B16 tumor growth curves measured by caliper in PBS or anti-MARCO Ab treated mice. Mice were either WT or depleted for CD8+ cells. D) B16 tumor growth curves measured by caliper in PBS or anti-MARCO Ab treated mice. Mice were either WT or depleted for CD4+ cells. E) B16 tumor growth curves measured by caliper in PBS or anti-MARCO Ab treated mice. Mice were either WT or deficient for TCR-β. F) Schematic of the B16 model for NK cell depletion experiments and their Ab administration. Depleting Ab were injected i.p. 24 hours before tumor cells inoculation. G) Gating strategy for evaluation of NK cell depletion in the blood. Depletion was confirmed in the blood on day -1, 3 and 6 and inside the B16 tumor on day 10. Representative image shows day 3. H-L) Frequency of NK cells (H), B cells (I), macrophages (J) and T cells (L) inside B16 tumors on day 10 in PBS or anti-MARCO Ab treated mice. Mice were either WT or depleted for NK cells. Macrophage subpopulations (K) were defined based on their Ly6C and MHCII expression. Representative flow cytometry gating to define different TAM populations: Pre-gating on CD45+ CD11b+ Ly6G- and based on MHCII and Ly6C expression distinction between inflammatory monocytes, immature macrophages, pro-inflammatory M1 and immunosuppressive M2 TAMs.Data are expressed as mean ± SEM. P values were calculated by Mann-Whitney test.

Figure S4

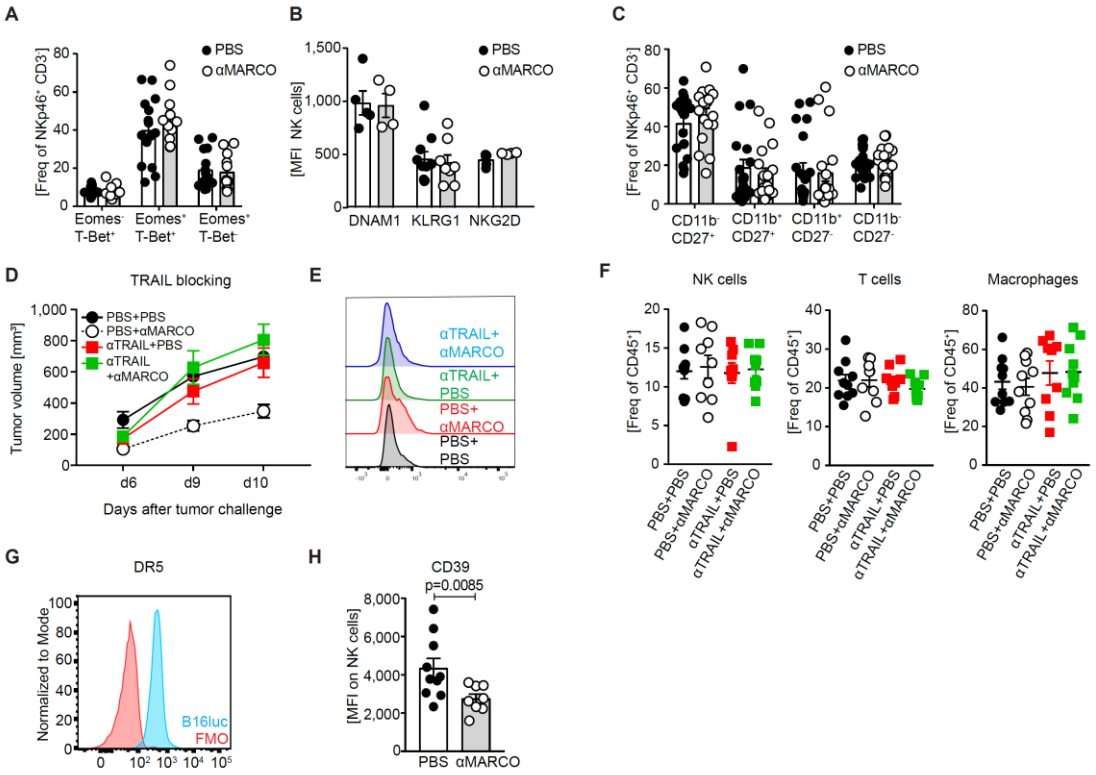


Fig. S4. Anti-MARCO Ab treatment does not affect activation or maturation of NK cells and neither NK cell depletion nor blocking of TRAIL changes immune cell composition inside the tumor.

A) Expression of transcription factor Eomes and T-Bet by B16 intra-tumoral NK cells. n= 10-14. Summary of two independent experiments. B) Expression of different activation markers by B16 intra-tumoral NK cells. MFI is shown. n= 4-8. C) Maturation of B16 intra-tumoral NK cells on day 10 defined by CD11b and CD27 expression. n= 12-15. Summary of three independent experiments. D) B16 tumor growth curves measured by caliper in PBS or anti-MARCO Ab treated mice. Mice were either WT or injected with TRAIL blocking Ab prior tumor inoculation. E) Representative TRAIL expression on NK cells in B16 tumor on day 10 in the TRAIL blocking experiment. Mice were PBS or anti-MARCO Ab treated. F) Frequency of NK cells, T cells and macrophages in B16 tumors on day 10 in PBS or anti-MARCO Ab treated mice. WT mice were injected with vehicle or TRAIL blocking Ab. G) Histogram shows DR5 expression on in vitro-cultured B16 cells. DR5 is the ligand for TRAIL. H) Expression of CD39 on B16 intra-tumoral NK cells after anti-MARCO Ab treatment. All flow cytometry data show intra-tumoral cells on day 10 except Supplementary Figure 4G which shows DR5 expression on in vitro- cultured B16 tumor cell line. NK cells always defined as CD45+ CD3- NKp46+ cells. Data are expressed as mean ± SEM. P values were calculated by Mann-Whitney test.

Figure S5

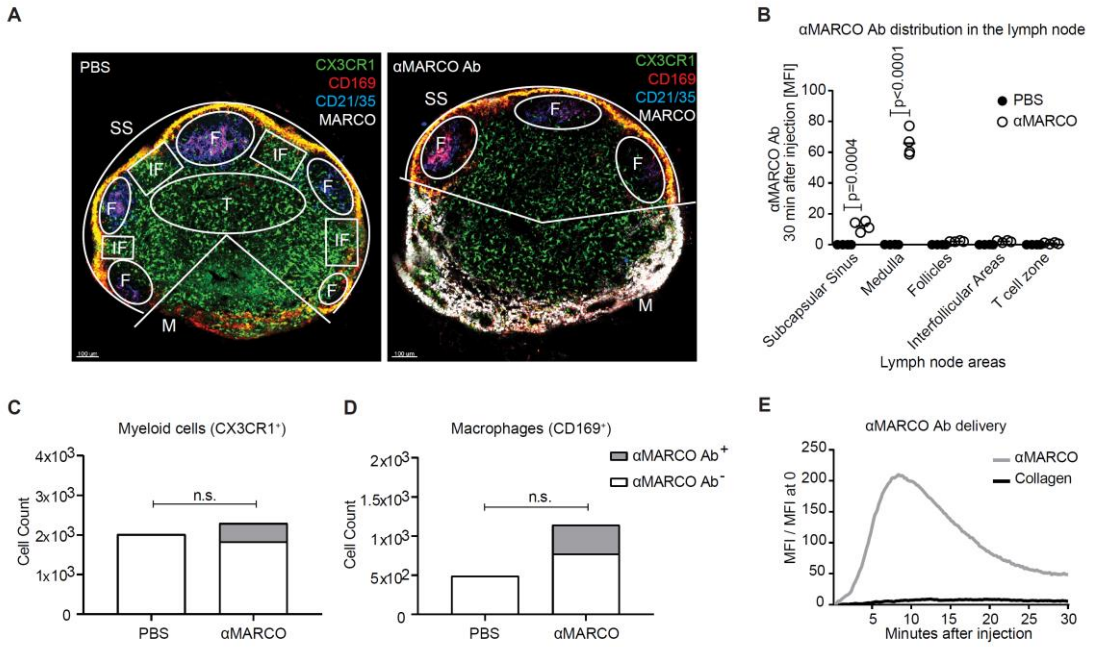


Fig. S5. Anti-MARCO Ab delivery to the popliteal lymph node and combinatorial treatment of B16 bearing mice with anti-PD-1 Ab and anti-MARCO Ab does just slightly change the immune composition in the tumor.

A) Confocal reconstructions of popliteal LNs from mice treated, respectively, with, PBS and anti-MARCO Ab. Lymph node regions (SS = subcapsular sinus, F = follicules, M = medulla, IF = interfollicular regions, T = T cells zone) are highlighted. B) Quantification of mean fluorescence intensity (MFI) of anti-MARCO Ab in the different lymph node regions. Data are Mean. C-D) Quantification of anti-MARCO Ab positive and anti-MARCO Ab negative cells in, respectively, myeloid cells (CX3CR1+) (C) and macrophages (CD169+) (D) (n = 4). Data are Mean ± SD. E) Quantification of total anti-MARCO Ab MFI in the LN in time. Anti-MARCO Ab MFI is normalized to background (MFI before anti-MARCO Ab injection). MFI= Mean fluorescence intensity, P values calculated by unpaired t- test.

Figure S6

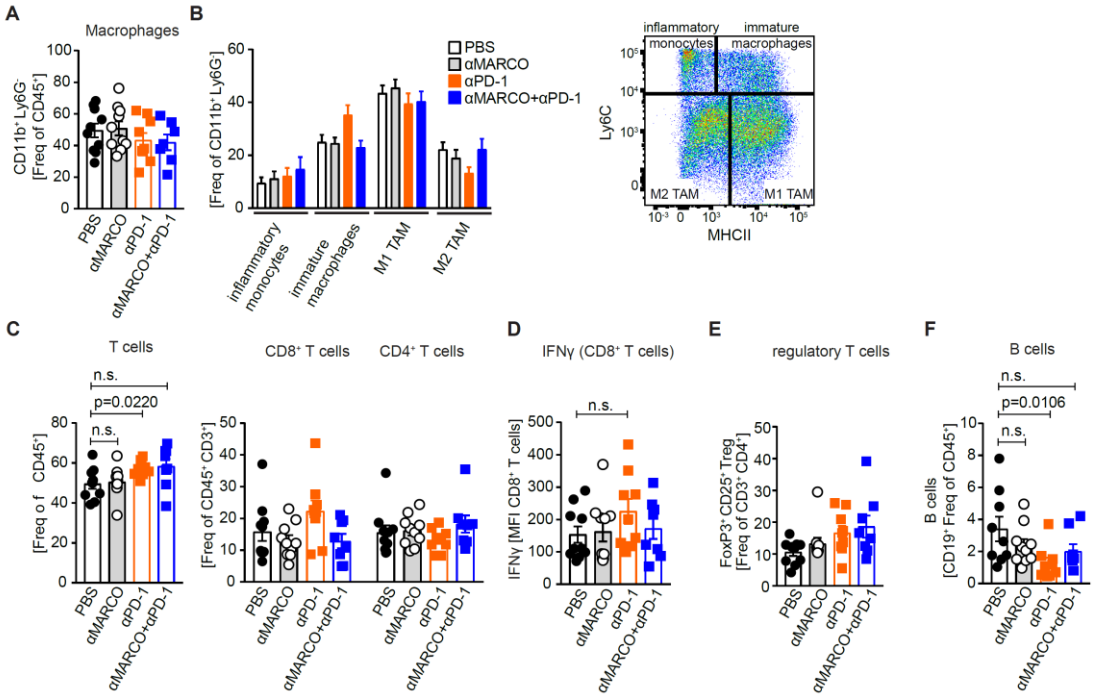


Fig. S6. Combinatorial treatment of B16 bearing mice with anti-PD-1 Ab and anti-MARCO Ab does only slightly change the immune composition in the tumor.

A) Frequency of macrophages in B16 tumors on day 12 in PBS, anti-MARCO Ab, anti-PD-1 Ab or anti-PD-1 and anti-MARCO Ab treated mice. Total macrophages defined as CD45+ Ly6G- CD11b+ cells. B) Subpopulation of macrophages were defined by CD45+ Ly6G- CD11b+ and further MHCII and Ly6C expression. C) Frequency of T cells in B16 tumors on day 12 in PBS, anti-MARCO Ab, anti-PD-1 Ab or anti-PD-1 and anti-MARCO Ab treated mice. Total T cells defined as CD45+ CD3+ cells, subpopulations were defined by further CD4 or CD8 expression. D) Interferon- γ (IFNγ) expression in CD8+ T cells in B16 tumors on day 12 in PBS, anti-MARCO Ab, anti-PD-1 Ab or anti-PD-1 and anti-MARCO Ab treated mice. E) Histogram shows frequency of regulatory T cells from CD4+ T cells in B16 tumors on day 12 in PBS, anti-MARCO Ab, anti-PD-1 Ab or anti-PD-1 and anti-MARCO Ab treated mice. Regulatory T cells were defined by CD45+ CD3+ CD4+ CD25+ FoxP3+ expression. F) Histogram shows frequency of B cells in B16 tumors on day 12 in PBS, anti-MARCO Ab, anti-PD-1 Ab or anti-PD-1 and anti-MARCO Ab treated mice. B cells were defined as CD45+ CD3- CD19+ cells. For all histograms n= 8-10, data pooled from two independent experiments. MFI= Mean fluorescence intensity. Data are expressed as mean ± SEM. P values calculated by Mann-Whitney test.

Figure S7

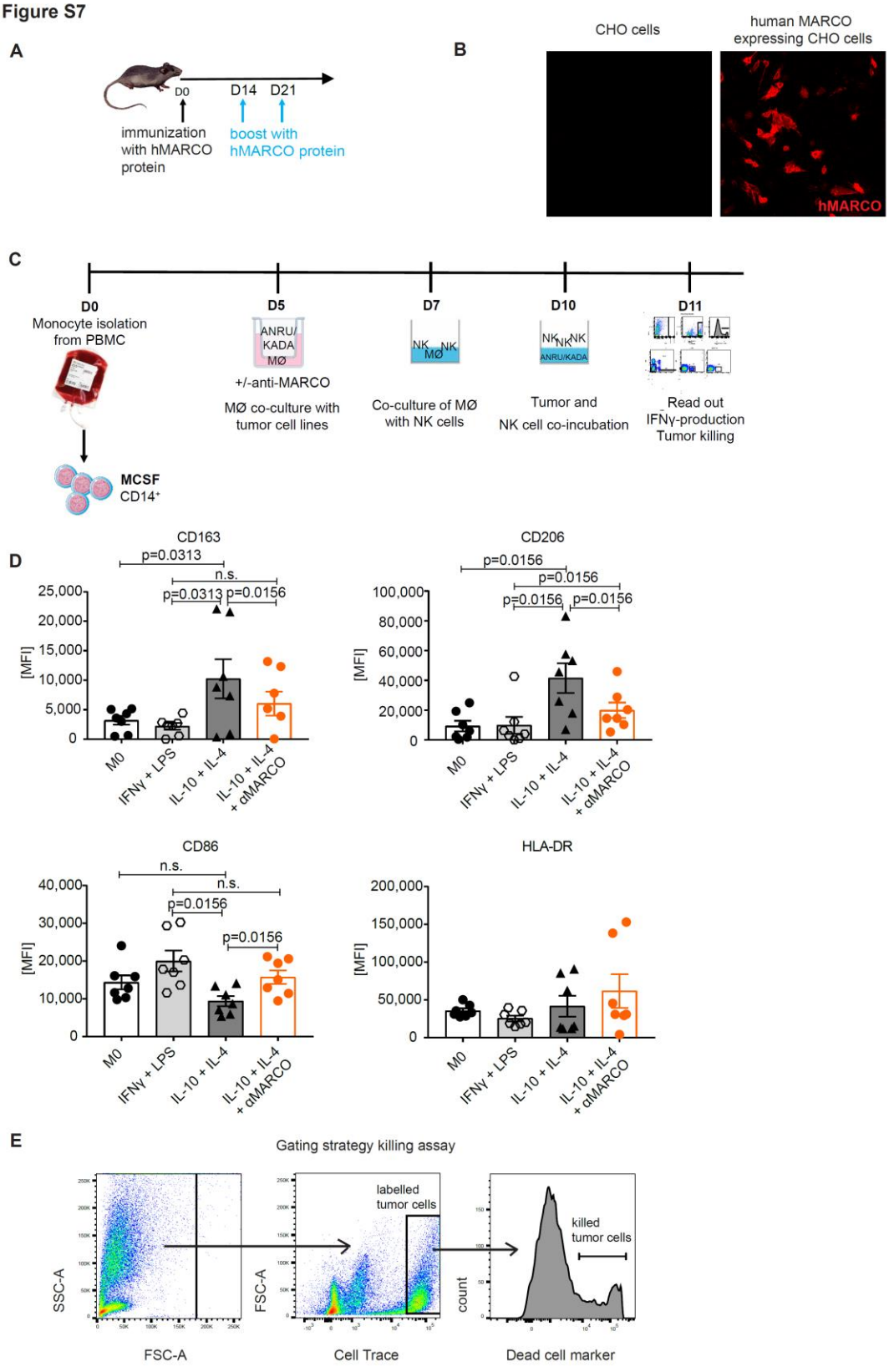

A
D0
D14
D21
immunization with hMARCO protein
boost with hMARCO protein
B
CHO cells
human MARCO expressing CHO cells
hMARCO
C
D0
Monocyte isolation from PBMC
MCSF
CD14+
D5
ANRU/KADA
MØ
+/-anti-MARCO
MØ co-culture with tumor cell lines
D7
NK
MØ
Co-culture of MØ with NK cells
D10
ANRU/KADA
Tumor and NK cell co-incubation
D11
Read out IFNγ-production Tumor killing
D
CD163
p=0.0313
n.s.
p=0.0313
p=0.0156
[MFI]
M0
IFNγ + LPS
IL-10 + IL-4
IL-10 + IL-4 + αMARCO
CD206
p=0.0156
p=0.0156
p=0.0156
p=0.0156
CD86
n.s.
n.s.
p=0.0156
p=0.0156
HLA-DR
E
Gating strategy killing assay
SSC-A
FSC-A
labelled tumor cells
FSC-A
Cell Trace
killed tumor cells
count
Dead cell marker

Fig. S7. Production of anti- human MARCO antibodies and targeting human MARCO by monoclonal antibodies affects human macrophage polarization. A) Scheme of the production of mouse anti- human MARCO Ab. hMARCO= human MARCO. B) Representative image of in-house produced mouse anti-human MARCO Ab binding to CHO cells that have been transfected to express human MARCO. CHO= Chinese hamster ovary cells. C) Scheme of the human co-culture system. D) The effect of anti-MARCO Ab treatment on human immune suppressive macrophages and their marker expression. In addition to IL-4+ IL-10, treated macrophages have been incubated with anti-MARCO Ab. CD163 and CD206 are markers for immune suppressive macrophages, CD86 and HLR-DR for pro-inflammatory cells. n=6-7. E) Representative gating strategy for the killing assay that is described in Fig 4h-i. Tumor cells were labelled with CellTrace before the assay to track them and after the assay stained with dead cell marker to stain for dead cells. Cells that were double positive were counted as killed tumor cells. MFI= Mean fluorescence intensity. Data are expressed as mean ± SEM. P values calculated by Wilcoxon test.

Movies S1-2 (separate files). Movies showing movement tracks of NK cells (green) interacting with B16 melanoma cells (red) before (Movie S1) and after (Movie S2) administration of anti-MARCO (ED31) antibodies. Collagen is depicted in blue and macrophages in white (CD169).

SI Methods:

Primary cells from humans and mouse.

The primary melanoma cell lines were generated from 3 different melanoma patients (acronym ANRU, KADA and MAT02 p11). Melanoma patients KADA (male, 36 years old when operated/included), ANRU (male, 77 years old when operated/included) (both described in (1)) and MAT02 p11 (male, 61 years old when operated/included) were diagnosed and operated for stage III or stage IV melanoma, respectively. The protocol for patient participation was approved by the local Ethics Committee (Dno. 2011/143-32/1 and 2015/1862-32) and the Institutional Review Board. All patients signed a written informed consent in accordance with the Declaration of Helsinki. The resected tumors were used for establishment of tumor cell lines by mechanical separation of tumor tissue. Tumor cells were cultured in RPMI (LifeTechnologies) supplemented with 20% FBS, penicillin (100 U/ml; LifeTechnologies) and streptomycin (100 µg/ml; LifeTechnologies). Tumor cells were frequently monitored for growth and medium was changed/cultures expanded when needed. For cells from mice the animals were euthanized using CO_2. The abdominal skin was cut using a scissor and gently pulled back leaving the peritoneal cavity intact. The peritoneum was flushed with 5ml ice-cold PBS and gently massaged. Peritoneal macrophages were then isolated using the Macrophage Isolation Kit (Peritoneum) (Miltenyi) following the manufacturer`s protocol. The only change was that FcR blocking reagent was replaced with buffer to prevent blocking of Fc receptors as we previously showed that MARCO signaling is FcγRIIb dependent (Georgoudaki et al., 2016). Peritoneal macrophages were maintained in DMEM (HyClone) supplemented with 10% FBS (HyClone), 1% Penicillin-Streptamycin (PenStrep; HyClone), 1% 2-mercaptoethanol (Invitrogen). Human cells, monocytes and NK cells, were isolated from healthy donor peripheral blood mononuclear cells (PBMCs) provided by the Stockholm Blood Bank by Ficoll- Paque method. Human monocytes were cultured in RPMI (HyClone) supplemented with 10% FBS, penicillin (100 U/ml; LifeTechnologies) and streptomycin (100 µg/ml; LifeTechnologies) for 6 days with 100 ng/ml M-CSF (Peprotech) prior to overnight polarization towards either pro-inflammatory (20 ng/ml IFNγ (Peprotech) + 20 ng/ml LPS (Invivogen)) or immune suppressive (20 ng/ml IL-4 + 20 ng/ml IL-10, both RnD systems)

macrophages and, where stated, in the presence of anti-MARCO Ab (10 µg/ml). On day 3 fresh medium supplemented with M-CSF was added. For co-culture experiments, human $CD56^+$ $CD3^-$ NK cells were isolated from PBMCs via negative selection (Miltenyi) and cultured in RPMI supplemented with 10% FBS, penicillin (100 U/ml; LifeTechnologies) and streptomycin (100 µg/ml; LifeTechnologies).

Treatment, blocking and depletion *in vivo.*

On day 3, 6 and 9, mice were injected intravenously (i.v.) with 100µg of anti-MARCO mAb (rat IgG1, clone ED31) and/or 180µg anti-PD-1 (BioXCell, rat IgG2a, clone RMP1-14) or 75µg anti-PD-L1 Ab (BioXCell, rat IgG2b, clone 10F.9G2). Tumor size was measured manually every second to third day using a Caliper (AgnoTho`s AB). On day 10 (or day 12 for combinatorial treatment experiments) of the experimental setup, the animals were sacrificed, and tumors were collected for further analyses. Mice of the same age and sex were pooled accordingly, for most experiments female mice were used except for the B16-luc tumor experiment in TCRβ-/- mice (male), P2X7R knockout and FcγRIII knockout mice (pooled data from males and females). For all depletion experiments, either 500µg of the depleting antibodies in PBS or PBS as a control were injected intraperitoneally (i.p.) 24h prior to tumor cell transplantation and repeated every fourth to seventh day. $CD4^+$ T cell depletion: mouse IgG2a, clone GK1.5; $CD8^+$ T cell depletion: rat IgG2b, clone YTS169.4; NK cell depletion: mouse IgG2a, clone PK136 (all from BioXCell). To confirm the depletion, blood samples were collected from the tail vein into 0.5 M EDTA (final dilution in the tube 1:10; Life Technologies) and analyzed in flow cytometry. For TRAIL blocking *in vivo*, 500µg of anti-TRAIL antibody (clone N2B2) was injected i.p. 24h prior to tumor cell injection and repeated every fourth day. Blocking of TRAIL has been described elsewhere (2).

Tumor sample preparation and flow cytometry.

For intravital microscopy experiments, 10^6 B16.F1-mCherry mouse melanoma cells (from the IRB stock) were implanted s.c. in the right footpad of anesthetized Ncr1-GFP mice (isoflurane 3 %) in 20 µL of sterile PBS. Primary tumor volume was daily measured. When both the tumor diameters were > 8 mm (approximately 256 mm^3 volume), 2P-IVM of metastasized pLN was performed. The tumor volume was calculated using the following formula: 0.5236 x major diameter x minor diameter. For other experiments, tumors were collected in cold PBS on day 10 (or day 12 for the combinatorial treatments), mashed with soft pressure using a 5ml syringe plug (BD) and passed through a 100µm filter strainer (Corning). After washing thoroughly with PBS, erythrocytes were lysed using 1x ammonium-chloride-potassium buffer (1x ACK; 155 mM NH_4Cl, 10 mM $KHCO_3$ 0.1 mM EDTA)/sample for 4 min at RT. Finally, the cells were resuspended in FACS buffer (PBS supplemented with 0.5% BSA and 2mM EDTA) and single cell suspensions were stained for flow cytometry. Single- cell suspensions were prepared, and erythrocytes were lysed using 1x ACK as described above. Non-specific antibody binding was blocked with anti-CD16/32 (Fc Block, Biolegend) and for discrimination of live-dead cells, samples were incubated with Fixable Dead Cell Stain Kit (Life Technologies) before surface staining. For intracellular stainings, the Transcription Factor Staining Buffer Set (eBioscience, Invitrogen) was used and the manufacturer`s protocol was followed. All samples were analyzed using a BD LSR Fortessa X-20 cytometer and analyzed with FlowJo software.

3D blood vessel staining (iDISCO).

B16-luc tumors were collected on day 10 after being treated with anti-MARCO Ab or PBS control (100µg i.v. on day 3, 6 and 9). They were fixed in 4% PFA overnight at 4°C followed by 1h at RT before being washed three times with PBS at RT (30 min each). To fit the dimensions of the light-sheet microscope, tumors were trimmed to a proper sample size of 8mm x 30mm x 6mm. Next, the tumor samples were dehydrated in upgraded series of methanol (Merck) in distilled H_2O (20%, 40%, 60%, 80% and 100% twice, 1h each) at RT, chilled for 30 min at 4°C and bleached in freshly prepared 5% hydrogen peroxide (Sigma) in methanol at 4°C overnight. After the bleaching, samples were rehydrated in the reverse downgraded series of methanol in H2O (80%, 60%, 40%, 20%, PBS, 1h each) at

RT and washed in PBS supplemented with 0,2% Triton-X-100 (Sigma; PTx2) twice for 1h at RT. Tumors were permeabilized in 0,3 M glycine (Sigma) and 2% DMSO (Sigma-Aldrich) in PTx2 at 37°C for 2 days and blocked in PTx2 supplemented with 6% goat serum (Dako) and 10% DMSO (Sigma) at 37°C for another 2 days. Next, the samples were stained with the primary Armenian hamster monoclonal anti-CD31 Ab (clone 2H8, Millipore) in PBS supplemented with 0,2% Tween-20 (Sigma-Aldrich), 10 µg/ml heparin (Sigma-Aldrich; PTwH), 5% DMSO and 3% goat serum for 10 days at 37°C to ensure penetration into the core of the tumor. After this, the samples were washed four times in PTwH, 1-2 hours each and once overnight at RT with gentle shaking. The secondary Alexa Fluor 647-conjugated goat polyclonal anti-hamster IgG Ab (clone: poly4055, Biolegend) was incubated in PTwH with 3% goat serum for 7 days at 37°C. After washing and dehydrating as described above, samples were incubated with dichloromethane (Sigma-Aldrich), two times for 15 min each, at RT before they were cleared with dibenzyl ether (DBE, Sigma-Aldrich) overnight at RT. Before imaging, the DBE was renewed. Antibody incubation steps were done while rotating the samples; fixing, blocking and washing was performed while gently shaken except the incubation with DBE where no movement took place. Light-sheet microscopy: Cleared tissues were acquired by using UltramicroscopeII from LaVision Biotec. 0.63X magnification, 2X objective lens, 30% laser power of 647 nm laser, minimum light sheet thickness (5 µm) and dynamic horizontal focus were used for acquisition. Image resolution is isotropic of 4.8 µm. Image analysis: Acquired images (16bit) were stitched using TeraStitcher (Bria A and Iannello G, BMC Bioinfomatics, 2012). Image analysis was done similarly according to published methods (3) but with modifications. Briefly, after conversion of images into 8bit, images were processed with ImageJ using unsharp mask and a series of Integral image filters (Normalize local contrast) to normalize local intensity difference within samples. We further processed images with Amira (Thermo Fisher) with modules of Non-local Means Filter and Background Detection Correction with default parameter. The vessels were segmented by using global thresholding. Bright dots which have intensity more than 40000 in 16bit images were judged as secondary antibody artifact and removed. We manually removed surface area where signal intensity was higher than inside. After the refining analysis area and removing dots, vessels were skeletonized and analyzed with Auto Skeleton module in Amira (FEI)

software with default setting. The parameters regarding vessel structure such as vessel volume, vessel thickness etc. were obtained with Spatial Graph Statistics module in Amira.

Intravital microscopy: image analysis.

Immunohistology and distribution analysis: 30 minutes after ED31 injection, mice were euthanized by CO_2 inhalation and pLN harvested and fixed in 4 % PFA at 4 °C for 4-6 h. Organs were embedded in 4 % low gelling agarose (Sigma-Aldrich) and 50µm sections were cut with Leica VT1200S vibratome (Leica Microsystems). Fc receptors were blocked (anti-CD16/32, Biolegend, 1 %) and sections stained with anti-CD169-PE (3D6.112, Biolegend, 1 %) and anti-CD21/35-PB (7E9 , Biolegend, 1 %), in 0.05 % Tween-20 and 0.5 % BSA PBS (Sigma-Aldrich) for two days at 4 °C, shaking. Immunofluorescence confocal microscopy was performed using a Leica TCS SP5 confocal microscope (Leica Microsystems). Micrographs were acquired in sequential scans and merged to obtain a multicolor image. Images were processed using ImageJ – Fiji 43. Maximum intensities were Z-projected for each channel. Specific ROIs were selected for each LN region, namely subcapsular sinus (SCS), interfollicular areas (IFA), follicles (F), T-cell zone (TZ) and medulla (M). ED31 mean fluorescent intensity (MFI) was then calculated for each area. 2photon intravital microscopy (2P-IVM) images acquisition and analysis: Deep tissue imaging was performed on a customized up-right two-photon platform (TrimScope, LaVision BioTec). Two-photon probe excitation and tissue second-harmonic generation (SHG) were obtained with a set of two tunable Ti:sapphire lasers (Chamaleon Ultra I, Chamaleon Ultra II, Coherent) and an optical parametric oscillator that emits in the range of 1,010 to 1,340 nm (Chamaleon Compact OPO, Coherent), with output wavelength in the range of 690–1,080 nm. pLN were exposed for imaging as previously described. In ED31 drainage studies, the antibody was injected in the footpad after the exposure of the pLN, then image acquisition immediately started. The delivery of ED31 to the pLN was quantified by measuring the mean fluorescence intensity (MFI) over time. More precisely, the fluorescence intensity of labelled ED31 was measured using the Surface function of Imaris software (Oxford Instruments). The intensity at time 0 was used for normalization, while the intensity of collagen, which was visible in a separate channel, was used as constant reference for comparison. In NK activation studies, image acquisition was

performed in metastasized pLN before and after ED31 footpad injection, providing an internal negative control. Manual tracking of NK cells was performed using the Surface function of Imaris. In all 2P-IVM experiments, mice were euthanized by CO_2 inhalation at the end of image acquisition.

Metabolomics: Sample, data collection and analysis.

Sample extraction and quality control preparation: Sample extraction was performed on all samples in one day. Eppendorf tubes containing cell pellet samples (total number: 18; WT: 9, KO: 9) were thawed on ice (30-40 mins). Next, 750 µL of ice-cold MeOH was added to each Eppendorf and sonicated for 5 mins. Samples were then vortexed for 15 secs and allowed to equilibrate for 15 mins at room temperature. Afterwards, samples were centrifuged at 13000 g for 10 mins at 4°C. Once complete, 400 µL of the supernatant was removed and added to the Ultrafree® -MC Centrifugal filter devices (Merck Millipore Ltd., Cork, Ireland) and centrifuged at 10000 g for 4 mins at 4°C. Finally, 100 µlof each sample was aliquoted into separate LC-MS vials. The rest of the samples were pooled (80 µl) to use as a MS injection quality control (QC). LC-MS grade water was purchased from Sigma-Aldrich (St. Louis, USA). Formic acid (Optima® LC/MS), and acetonitrile (Optima ®-LC/MS), were purchased from Fisher-Scientific (Loughborough, UK). The internal lock masses (purine and HP-0921), and tune mix for calibrating the TOF-MS (ESI-low concentration tuning mix) were purchased from Agilent Technologies (Santa Clara, USA). LC-HRMS experiments were performed on a 1290 Infinity II ultra-highperformance liquid chromatography (UHPLC) system coupled to a 6550 iFunnel quadrupole-time of flight (Q-TOF) mass spectrometer equipped with a dual AJS electrospray ionization source (Agilent Technologies, Santa Clara, CA, USA). Polar metabolites were separated on a SeQuant® ZIC®-HILIC (Merck, Darmstadt, Germany) column 100 Å (100 mm × 2.1 mm, 3.5 µm particle size) coupled to a guard column (20 mm × 2.1 mm, 3.5 µm particle size) and an inline-filter. Mobile phases consisted of 0.1 % formic acid in water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B). The elution gradient used was as follows: isocratic step at 95 % B for 1.5 min, 95 % B to 40 % B in 12 mins and maintained at 40 % B for 2 mins, then decreasing to 25 % B at 14.2 mins and maintained for 2.8 mins, then returned to initial conditions over 1 min, and the column was equilibrated at initial conditions for 7

mins. The flow rate was 0.3 ml min-1, injection volume was 2 µl, and the column oven was maintained at 25 °C. Two independent injections were run for positive and negative acquisition modes. The Q-TOF MS system was calibrated and tuned according to the protocols recommended by the manufacturer. Nitrogen (purity > 99.9990 %) was used as a sheath gas and drying gas at a flow of 8 L min-1 and 15 L min-1, respectively. The drying and sheath gas temperature were set at 250 °C, with the nebulizer pressure at 35 psi and voltage 3000 V (+/- for positive and negative ionization mode, respectively). The fragmentor voltage was set at 380 V. The acquisition was obtained with a mass range of 50-1200 m/z, where full scan high-resolution data were acquired at three alternating collision energies (0 eV, 10 eV and 30 eV). The data acquisition rate was 6 scans sec^{-1}. Between 16 and 25 mins, LC flow was diverted to the waste and for further details regarding the acquisition methodology, data processing and metabolite confirmation please see Naz et al. and Daskalaki et al. (4, 5). For the analysis samples were randomized, and QCs injected before, in the middle, and right at the end of the sequence. Methanol blank samples were injected at the beginning, and end of the sequence. Spin-filtered methanol was also injected in duplicate at the beginning of the sequencing following the methanol system blanks. Positive and negative raw LC-HRMS files were independently processed with an in-house developed PCDL library for polar metabolites (n = 225) using Profinder version B.06 (Agilent Technologies). Proper identification of reported compounds (total number = 53; 40 from positive mode and 13 from negative) was assessed by accurate mass and retention time (AMRT) plus fragment identification at two collision energies (10 and 30 eV) using an in-house generated database as previously described [4]. The relative peak areas of 53 metabolites were analyzed with principal component analysis (PCA) and heatmaps using the online program Metaboanalyst 4.0 (6). The data were also pre-processed in Metaboanalyst: Data were log transformed and auto-scaled (mean-centered and divided by the standard deviation of each variable) prior to analysis. The heatmap was created with Euclidean distance measure and Ward clustering algorithm.

Anti-human MARCO antibody production.

Seven to eight weeks old female MARCO knockout mice were immunized i.p. with 50µg recombinant human MARCO protein (hMARCO, RnD Systems) in 1:1 ratio of PBS and

Imject Alum (Thermo Scientific). On day 14 and 21 mice were injected with hMARCO protein to boost the immune response. The production of anti-human MARCO Ab producing hybridomas was performed according to the manufacturer protocol (ClonaCell-HY Hybridoma Cloning kit, Stemcell Technologies). Briefly, splenocytes were fused with myeloma cells and formed colonies were disrupted into single cell colonies into 96- well plates. From there, individual colonies were tested for MARCO specific antibody production by sandwich ELISA (Weiland, 1978) and positive clones were expanded and further tested for their binding capacity to hMARCO expressing cells by flow cytometry and immunofluorescence staining. These cells were Chinese hamster ovary (CHO) cells that were transfected to express the human MARCO protein using the pcDNA3 plasmid (Invitrogen). Positive clones were expanded, supernatant collected, and antibodies isolated by standard protein purification method using G- protein – specific separation columns (GE Healthcare).

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