SUPPLEMENTARY MATERIALS AND METHODS

Tumor models

3LL-R Lewis lung carcinoma cells were harvested and single-cell suspensions of 3 x 10^6 in 200 µl of PBS were injected subcutaneously into the right flank of syngeneic C57Bl/6 mice. Tumor volumes were calculated using the formula: $V = \pi \times [d^2 \times D] / 6$, where d is the minor tumor axis and D is the major tumor axis.

Intrathoracic injections were performed following a previously reported protocol with minor modifications (1). Briefly, 5 x 10^5 3LL-R carcinoma cells were harvested and resuspended together with 25 µg Matrigel in 50 µl PBS. Cell suspensions and injection kit were kept on ice until injection. Mice were anesthetized and were placed in the left lateral decubitus position. One-ml tuberculin syringes with 30-gauge hypodermic needles were used to inject the cell inoculum percutaneously into the right lateral thorax, at the lateral dorsal axillary line, approximately 1.5 cm above the lower rib line just below the inferior border of the scapula. The needle was quickly advanced approximately 6 mm into the thorax and was quickly removed after the injection. After tumor injection, the mouse was turned to the right lateral decubitus position. At day 7, mice were sacrificed and lung tissue and lung tumors were removed.

Morphologic analysis

Cytospins were obtained by spinning down cells on precoated microscope slides (Thermo Shandon), fixing with methanol and staining with May-

Grünwald and Giemsa dye for 5 and 15 minutes, respectively. Images were obtained with a Zeiss LSM 710 NLO confocal microscope (using ZEN 2009 software) at 40/0.65 magnification.

Mixed Leukocyte Reaction assays

For Mixed Leukocyte Reaction (MLR) assays, T cells were purified from Balb/c splenocytes, by first immunodepleting $CD11c^+$ and $CD19^+$ cells using a MACS LD column with anti-CD11c and anti-CD19 microbeads (Miltenyi biotech) and subsequently positively selecting $CD4^+$ or $CD8^+$ T cells using anti-CD4 or anti-CD8 microbeads (Miltenyi biotech). $2x10^5$ purified Balb/c T cells were cultured in round-bottom 96-well plates with different concentrations of TAM or TADC sorted from 13-days old 3LL-R tumors or C57Bl/6 splenic cDC. Three days later, ^{3}H-thymidine was added and cell proliferation was measured after another 18h culture as counts per minute (cpm) on a Wallac 1450 Liquid Scintillation Counter.

In vivo labeling of blood monocytes

Latex labeling of blood monocytes was performed as described earlier (2, 3). Briefly, to label $Ly6C^{lo}$ monocytes and track their infiltration in tumors, mice were injected intravenously with 250µl of 0.5 µm fluoresbrite yellow-green microspheres (Polysciences) diluted 1:25 in PBS. 24 hours later, mice received sc 3LL-R injections. To label and track $Ly6C^{hi}$ monocytes, mice were injected iv with 250 µl of clodronate liposomes. 18 hours later, mice received iv latex injection and 24 hours later a sc 3LL-R injection. Six days after 3LL-R injection, mice were sacrificed and tumor single-cell suspensions were

analysed by flow cytometry. Clodronate was a gift from Roche and was incorporated into liposomes as previously described (4).

Bromodeoxyuridine labeling and Ki67 stainings

Tumor-bearing mice were given an intraperitoneal injection of 1mg BrdU followed by continuous BrdU administration in the drinking water (0.8 mg/ml, Sigma). Tumors were collected after consecutive time points and BrdU intracellular stainings were performed following the manufacturer's instructions (BD Biosciences). PE-labeled anti-Ki67 or matching isotype controls (BD Biosciences) was added together with FITC-labeled anti-BrdU in the final step of the intracellular staining protocol.

Hypoxia assessment and pO_2 measurements

For hypoxia stainings, tumor-bearing mice were injected with 80 mg/kg body weight pimonidazole (Hypoxyprobe-1, HP-1, HPI Inc.) and 2 hours later tumors were collected. For immunohistochemistry, tumors were fixed for 6h in 4% paraformaldehyde, rehydrated overnight in 20% sucrose and frozen in liquid nitrogen. 5µm sections were first incubated 30' with 10% normal donkey serum (Jackson ImmunoResearch Laboratories) to block aspecific binding sites. For F4/80, MHC II and anti-HP-1 triple stainings, sections were: (1) incubated overnight with purified rat anti-MHC-II and purified rabbit anti-HP-1, (2) incubated 1h with F(ab')2 donkey anti-Rat/Cy3 and F(ab')2 donkey anti-rabbit/Cy5, (3) remaining anti-rat binding sites were blocked with 5% normal rat serum (Jackson ImmunoResearch Laboratories), (4) incubated 1h with rat anti-F4/80/alexa-fluor488. Sections were mounted with fluorescent mounting medium (Dako). Pictures were acquired with a Plan-Neofluar 10x/0.30 or

Plan-Neofluar 20x/0,50 (Carl Zeiss) objective on a Zeiss Axioplan 2 microscope (Carl Zeiss) equipped with an Orca-R2 camera (Hamamatsu) and Smartcapture 3 software (Digital Scientific UK). For flow cytometric HP-1 measurements, tumor single-cell suspensions were made, and cells were fixed and permeabilized using the BD Biosciences Fix/Perm kit. Finally, rat anti-HP1/FITC was added for 30' at 37°C.

Tumor oxygen tension (pO_2) was measured using charcoal powder (100 mg; X0670-1; EM Science) as the oxygen-sensitive probe. Calibration curves were made by measuring the EPR line width as a function of the pO2 (5). Charcoals were injected into 700 $mm^3 \pm 200$ mm^3 tumors and oxygen tension was measured after 24h by using an EPR spectrometer (Magnetech) with a low-frequency microwave bridge operating at 1.1 GHz and extended loop resonator.

Tumor perfusion and vessel coverage measurements

For serial sections cut at 7 µm thickness, tissue samples were immediately frozen in OCT compound or fixed in 2% paraformaldehyde overnight at 4°C, dehydrated and embedded in paraffin. Paraffin slides were first rehydrated to further proceed with antigen retrieval in citrate solution (DAKO). Cryo-sections were thawed in water and fixed in 100% methanol. If necessary, 0.3% H_2O_2 was added to the methanol to block endogenous peroxidases. The sections were blocked with the appropriate serum (DAKO) and incubated overnight with the following antibodies: rat anti-CD31, rabbit anti-FITC, goat anti-CD105 or rabbit anti-NG2. Appropriate secondary antibodies were used: Alexa 488 or 568 conjugated secondary antibodies and, when necessary, Tyramide

Signaling Amplification (Perkin Elmer, Life Sciences) was performed according to the manufacturer's instructions. ProLong Gold mounting medium with DAPI (Invitrogen) was used. Microscopic analysis was done with a Zeiss Axioplan microscope and KS300 image analysis software, or with an Olympus BX41 microscope and CellSense imaging software. Perfused tumor vessels were counted on tumor cryosections from mice injected intravenously with 0.05 mg FITC-conjugated lectin (Lycopersicon esculentum; Vector Laboratories).

Chorioallantoic membrane angiogenesis assays

Chorioallantoic membrane (CAM) assays were performed as described earlier (6). Briefly, fertilized white leghorn chicken eggs (Wyverkens, Halle, Belgium) were incubated at 37°C for 3 days prior to removing 3 ml of albumen to detach the shell from the developing CAM. Next, a window was made in the eggshell to expose the CAM. At day 9, sterile absorbable gelatin sponges (1–2 mm^3; Hospithera, Brussels, Belgium) were impregnated with $5x10^4$ sorted TAM subsets and placed on the CAM. Sponges were also loaded with PBS/0.1% BSA (1 mg/ml, <50 µg/embryo) as negative control and with recombinant human VEGF-A165 (100 µg/ml, <5 µg/embryo) as positive control. At day 13, membranes were fixed with 4% paraformaldehyde and the area around the implants was analyzed using a Zeiss Lumar V.12 stereomicroscope with NeoLumar S 1.5x objective (15x magnification). Digital images were captured using an AxioCam MRc5 and processed with Axiovision 4.5 Software (Zeiss). To determine the number of blood vessels, a grid containing three concentric circles with diameters of 4, 5, and 6 mm was

positioned on the surface of the CAM and all vessels radiating from the sample spot and intersecting the circles were counted under a stereomicroscope.

Protein extraction and immunoblot:

Protein extraction was performed using whole cell lysate buffer (20mM Hepes, 250mM NaCl, 1 mM EDTA and 1% IGEPAL (Sigma-Aldrich)) containing Complete Protease Inhibitor (Roche Diagnostics). Used antibodies are listed in Supplementary Table 1. The protein concentration was determined using a BCA Protein Assay (Pierce Biotechnology Inc.). The SDS-PAGE gels (Invitrogen) of GLUT-1 and GLUT-3 were transferred to a nitrocellulose membrane Hybond-C Extra (Amersham), while a PVDF-membrane (Thermo scientific) was used for the detection of VEGF-A. Signal was detected using the ECL system (Amersham Biosciences) according to the manufacturer's instructions. Densitometric analysis was performed using ImageJ v1.45r software (http://rsbweb.nih.gov/ij/).

List of abbreviations

BrdU: bromodeoxyuridine

HIF: hypoxia-inducible transcription factors

HP-1: hypoxiprobe-1, pimonidazole

Imm: immature

MLR: mixed leukocyte reaction

Mo: monocytes

MΦ: macrophage

PHD2: prolyl hydroxylase domain 2

TAM: tumor-associated macrophage

TADC: tumor-associated dendritic cell

SUPPLEMENTARY REFERENCES

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