SUPPLEMENTARY MATERIALS AND METHODS

Tumor models and cell cultures

3LL-R Lewis lung carcinoma cells or TS/A breast carcinoma 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 or Balb/c respectively.

3LL-R cells were generated in house from C57BL/6 Lewis Lung carcinoma as previously described (1). TS/A cells were a kind gift of Vincenzo Bronte (University of Verona, Verona, Italy).

3LL-R and TS/A cell lines were maintained in Roswell Park Memorial Institute-1640 medium (RPMI; Sigma) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco), 300 µg/ml L-glutamine (Gibco), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco) and monthly tested for the presence of mycoplasma.

For TAM cultures, this medium was supplemented with 1mM non-essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen) and 0.02 mM 2-mercapto ethanol (Invitrogen).

Measurement of IL-34, M-CSF, GM-CSF and CCL2 production

Briefly, for M-CSF, GM-CSF and CCL2 96-well plates (NUNC Maxisorp, eBioscience) were coated overnight with capture antibodies for the respective cytokines using coating buffer (8.34 g $NaHCO_3$ in 1L H_2O). All incubations were at room temperature protected from light. The following day, wells were blocked with 300 µl blocking buffer (PBS 1% BSA) for two hours. Subsequently, plates were washed and serial standard dilutions and samples were loaded on the 96-well plate and incubated for two hours. After washing, 100 µl of detection antibody diluted in blocking buffer was added for

one hour. After washing, streptavidin-HRP was added and plates were incubated for 20 minutes. Substrate solution (TMB Substrate Reagent Set; BD OptEIA^{TM}) was added and plates were incubated for 15 minutes, after which 2N H_2SO_4 was added to stop the color-reaction. Plates were read on a microplate-reader at 450 nm. Optical densities of the standard curve were plotted using a linear fit and analyzed with Microplate Manager. For IL-34, pre-coated plates were used (Quantikine ELISA kit R&D systems). A similar protocol was followed, using the reagents provided in the kit.

Intravital Imaging

To assess the proportion of intravascular and extravascular monocytes in tumors, 3LL-R tumor-bearing CX3CR1-GFP$^{+/-}$ reporter mice were anesthetized followed by tracheotomy and controlled respiration with 2.5% isoflurane in 100% O_2. Tumors were surgically exposed and carefully dissected from the surrounding connective tissue, preventing operation induced tissue traumata, followed by extracorporal immobilization of the tumor on a custom-made stage and embedding in 3% PBS agarose. Subsequently, mice were injected intravenously with 300 µg isotype Ab or α-M-CSFR Ab and with 1µg Evans blue (Sigma Aldrich) per gram body weight to visualize the blood vessels. Intravital microscopy was performed using a LaVision multiphoton laser-scanning microscope (BioTec, Biele feld, Germany) and images were analyzed using Imaris 7.7 (Bitplane, Zurich, Switzerland).

Bromodeoxyuridine labeling

To assess the amount of $BrdU^+$ cells, tumor single-cell suspension were pre-incubated for 20 min with rat anti-mouse CD16/CD32 (2.4G2, BD Biosciences) to prevent aspecific binding and subsequently with fluorescently-labeled Ab for extracellular cell-surface markers for 20 min on melting ice protected from light. After washing with staining buffer, cells were fixed for 25 min on melting ice with 100 μl Cytofix/Cytoperm buffer (BD Bioscences). Cells were washed with Perm-wash buffer (BD Biosciences) and incubated for 10 min with 100 µl Cytofix/Cytoperm PLUS buffer (BD Biosciences) on melting ice. Cells were again washed with Perm-wash and incubated for 5 additional minutes with Cytofix/Cytoperm buffer on melting ice. To ensure an optimal working of the BrdU labeling, cells were incubated for 1h at 37°C with 30 μg DNAse (Worthinton) diluted in PBS. After washing with Perm-wash buffer, cells were incubated with anti-BrdU Ab diluted in Perm-wash (1:50) for 20 min at room temperature. Cells were washed with Perm-wash and BrdU signals were measured via flow cytometry.

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 $5x10^4$ MHC-II^{lo} or MHC-II^{hi} TAM subsets sorted from 14-days old 3LL-R tumor-bearing mice, treated with either isotype or α-M-CSFR every

two days from day 0 past tumor inoculation until sacrifice (day 14 p.i). 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.

SUPPLEMENTARY REFERENCES

1. Remels LM, De Baetselier PC. Characterization of 3LL-tumor variants generated by in vitro macrophage-mediated selection. International journal of cancer Journal international du cancer 1987;39(3):343-52.

List of abbreviations

BM: Bone marrow

BrdU: bromodeoxyuridine

CCL: C-C chemokine ligand

GFP: Green fluorescent portein

GM-CSF(R): Granulocyte-macrophage colony stimulating factor (receptor)

Imm: immature

Iv: intravascular

M-CSF(R): Macrophage colony stimulating factor (receptor)

MFI: Median Fluorescence Intensity

MLR: mixed leukocyte reaction

Mo: monocytes

MΦ: macrophage

TAM: tumor-associated macrophage

TADC: tumor-associated dendritic cell