

Supplemental Methods

Double Immunofluorescence. Tissues were sectioned in OCT, and post-fixed with acetone. Washes were with 0.1% BSA/PBS and non-specific antibody blocking with avidin and biotin (Vector Laboratories). The first primary antibody (as detailed above) was incubated overnight at 4°C. Species-specific biotinylated secondary antibodies (Vectastain ABC Kit, Vector) were incubated for 30min at RT. Texas red Avidin D or Fluorescein Avidin D (Vector) was then incubated for 30min. This process was repeated for the second primary antibody. Sections mounted with fluorescence mounting media with DAPI (Vectashield, Vector), and visualized as above.

Antibody Targeting. For blockade of the $\alpha 4$ subunit of VLA-4, mice were injected intravenously day 4, 8 and 12 with rat anti-mouse antibody to CD49d (clone R1-2, IgG_{2b}K, 200 μ g, BD Biosciences Pharmingen) animals were sacrificed on day 14 for evaluation of cluster formation. To target metastasis at a later stage of tumour development the anti- $\alpha 4$ antibody was injected day 6, 10, 14, 18 and 22, and animals were sacrificed on day 24. All groups were compared to rat anti-mouse IgG_{2a}K isotype control (KLH/G2a1-1, Southern Biotech) administered in the same schedule as the experimental groups.

Selective bone marrow transplantation. Rosa-26 mice received adeno-VEGF₁₆₅ (AdVEGF) on day 0, 4, and 8ⁱ. On day 12, BM isolated and labelled with biotinylated murine VEGFR1 antibody and anti-biotin magnetic beads (Miltenyi Biotec) was separated using MACS (Miltenyi Biotec). Purity of VEGFR1⁺ BM was 95% following three serial passages. The negative selection population represented non-R1 cells⁵. WT mice transplanted after irradiation with the selective BM as described above. Id3 KO (Id1^{+/-}Id3^{-/-}) mice were transplanted with selective Id3 competent VEGFR1⁺ BM with intravenous injections of 10⁵ cells every three days for a total of 23 days. Control animals were given VEGFR1⁺ cells without a tumour.

Fluorescent tumour transfection. B16 and LLC cells (1x10⁵) were resuspended into serum free media with lentiviral vector supernatant containing the DsRed reporter

gene or GFP gene. Concentrated viral constructs with titers of $1-2 \times 10^8$ infectious particles per ml (multiplicity of infection is 50) were used to infect 2×10^6 cells as previously describedⁱⁱ. Flow cytometric analysis was performed to confirm degree of fluorescence. C57Bl/6 mice were inoculated with 2×10^6 LLC/GFP⁺ or B16/DsRed⁺ cells.

MMP-9 KO. Mice were obtained from Jackson laboratory with C57Bl6 background.

In vitro aggregation assays. On day 14, BM-derived VEGFR1⁺ cells were isolated from mice implanted with B16 cells. R1⁺ cells (5×10^5) were stained fluorescent red (PKH26-GL, Sigma) and cultured on 0.2% gelatin in M199 media supplemented with 10% FCS, and incubated with rhVEGF (10 ng/mL, R&D Systems), anti-VEGFR1 (10 µg/ml, mF-1, ImClone), or anti-α4 (CD49d, PS_2, 20 µg/ml, Southern Biotech) for 14 hours. Also studies were performed with pre-incubation of VEGFR1⁺ cells with rhVEGF, anti-VEGFR1, anti-α4 for 1 hour prior to incubation with tumour cells for fourteen hours. The R1⁺ cells were co-cultured with B-16 or LLC tumour cells, labelled fluorescent green (PKH2-GL, Sigma)ⁱⁱⁱ and analysed for aggregation and proliferation.

Migration Assays. Migration of B16 or LLC tumour cells to VEGFR1⁺ cells was performed using 12 µm pore transwells as previously described^{iv}. Fluorescently labelled tumour cells (PKH2-GL, Sigma) were seeded at 1×10^5 cells into upper chamber and 1×10^5 VEGFR1 cells were plated into lower chamber in serum free media. There was no concentration gradient between the upper and lower chambers. Analysis was performed every 6 hours with direct visualization and manual counting of cells per 200x field using an inverted fluorescent microscope (Nikon Eclipse TE 2000-U).

Flow Cytometry. Peripheral blood mononuclear cells were incubated with fluorescently conjugated monoclonal antibodies CD11b (M1/70, PE anti-mouse, BD Pharmingen), Sca-1 (E13-161.7, PE and FITC anti-mouse Ly-6A/E, Becton

Dickinson) and VEGFR1 (clone mF-1, FITC, ImClone Systems) as described⁹. For cKit flow analysis after a single cell suspension was obtained, cells were stained directly with perCP cKit and then placed in PBS without fixation and analyzed on a Coulter FC500 cytometer.

Supplementary Figure 1. Tumour and VEGFR2⁺ cells arrive after VEGFR1⁺ HPCs. **a.** Flow cytometric analysis of day 8,14,18, and 23 delineating the populations of GFP⁺ BMDCs and DsRed B16 Melanoma tumour cells in the lung (n=30). **b.** VEGFR1 (red) with CD34 (green) (middle panel) Frequency of co-expression of VEGFR1 and these hematopoietic markers (right graph). **c.** Analysis of CD117⁺ progenitor cells and fluorescent GFP⁺-LLC tumour cells in the lung by flow cytometry reveals the presence of these cells prior to tumour cell arrival represented by day 12, 14, 18 and 27. **d.** Bone marrow derived VEGFR2⁺ circulating endothelial progenitors arrive at the premetastatic niche after the VEGFR1⁺ hematopoietic progenitors. Double immunofluorescence of VEGFR1 (red) with VEGFR2 (green) (left panel). VEGFR1 (red) with CD31/PECAM (green) (middle panel) (arrows indicate endothelial cells). Double immunofluorescence of VEGFR2⁺ cells with GFP signifying bone marrow origin of these cells within the established and growing metastatic niche (right panel). **e.** Graph illustrates timing of arrival of VEGFR-2⁺ cells to the metastatic niche which coincides with tumour cell arrival as seen in lung tissue from animals with day 16 and day 24 tumours (cells/cluster/100x field) (n=6). Scale bars: **b**20 μ m; **d** left, middle left 20 μ m, right 10 μ m.

Supplementary Figure 2. VEGFR1 antibody inhibits metastasis in B16 tumours. B16 tumours (right panels) in mice treated with neutralizing antibody to VEGFR1 and/or VEGFR2. Closed arrows in spleen of WT show viable melanoma along with inset, while open arrows reveal necrosis and melanin granules. Arrows in anti-R1 (anti-VEGFR1) show a typical germinal center in spleen (blue tissue is lymphocytes or blue pulp, red tissue is red blood cells or

red pulp). Inset in anti-R1 +anti-R2 group represents isolated metastasis in the lung of one animal. Scale bars: 20 μm : insets WT spleen 8 μm , anti-R1+anti-R2 66 μm .

Supplementary Figure 3. Inhibition of VLA-4 and MMP-9 inhibits metastasis. B16 melanoma induces fibronectin expression in the lung. Images represent lung tissue 14 days after LLC implantation in WT mice treated with anti-VLA-4 antibodies (**a**) (β -gal with eosin counterstain, $n=8$, $p<0.01$ by Student's t test), MMP9^{-/-} mice (**b**) (VEGFR1 DAB with haematoxylin counterstain $n=6$). The WT mice given VLA-4 antibodies or in MMP-9 deficient mice have reduced β -gal and VEGFR1 cellular cluster formation represented in table (**c**) below images quantifying clusters or metastasis/100x field ($p<0.01$ by Student's t test). **d.** Fibronectin expression in lung tissue at day 3 and 14 after B16 melanoma tumour implantation. Arrows in day 14 represent fibronectin expression in a cluster site. Scale bars: **a** 80 μm , **d** left, middle panels 40 μm , right 8 μm .

Supplementary Figure 4. Bone marrow-derived VEGFR1⁺ cells attract tumour cells. **a.** VEGFR1⁺ cells (red) isolated from BM and co-cultured with B16 tumour cells (green) form aggregates and proliferate, this effect was lost with antibody treatment to VEGFR1 (middle panel) or VLA-4 (right panel). **b.** Transwell migration assay demonstrate increased migration of B16 tumour cells to VEGFR1⁺ selected population (in triplicates, $p<0.001\%$ for R1 compared to non R1 and media). **c.** SDF-1 (CXCL12) immunofluorescent staining of a VEGFR1⁺ cellular cluster at low and high magnification. Co-immunofluorescence of SDF-1 α and VEGFR1 with VEGFR1⁺ cellular clusters staining positive for SDF-1 α expression **d.** LLC and B16 tumour cells express CXCR4. Scale bars: **a** 50 μm , inset 120 μm , **c** left and right panel 40 μm , middle 8 μm **d** 20 μm .

Supplemental Figure 5. Tumours vary in their chemokine profile and fibronectin expression pattern. **a.** WT mice treated with LLC conditioned media (LCM) induce fibronectin expression in the liver (left panel) and lung (middle panel) compared to media controls (left and middle panel insets) (n=12). LCM supports β -gal⁺ BM clusters in the lung (right panel, TB=terminal bronchiole and BV= bronchial vein) (n=6). **b.** Mice treated with MCM develop enhanced fibronectin expression in the liver (left panel) and intestine (middle panel) as well as kidney, testis, lung (data not shown) with media control for fibronectin expression in intestine (middle panel inset) (n=12). WT mice with β -gal⁺ BM treated with MCM exhibit adjacent cluster formation localizing to the fibronectin enriched areas. (right panel) (n=6). **c.** ELISA levels for VEGF and PlGF in tumour-derived plasma for both LLC and B16 melanoma obtained from animals with day 14 tumour (in triplicates *p<0.05 for tumour-derived plasma compared to WT plasma, by one way ANOVA). Scale bars: **a,b** left and middle panels 40 μ m, insets 35.2 μ m, right panel 20 μ m.

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ⁱⁱ Rivella, S., May, C., Chadburn, A., Riviere, I., & Sadelain, M. A novel murine model of Cooley anemia and its rescue by lentiviral-mediated human beta-globin gene transfer. *Blood.* **101**. 2932-9 (2002).

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