Online Data Supplement

The Axl receptor tyrosine kinase is a discriminator of macrophage function in the inflamed lung.

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This file contains supplementary materials and methods.

Analysis of TAM receptor expression on murine bone marrow-derived macrophages (BMDMs): Cells from the bone marrow were incubated with M-CSF (20 ng/ml) or GM-CSF (20 ng/ml) (Invivogen, Toulouse, France) in RPMI 1640 medium containing 20% FCS (Gibco, Paisley, UK), 100 u/ml penicillin, 100 µg/ml streptomycin and 20mM Hepes (all from Sigma-Aldrich, Dorset, UK) for 3 d at 37 ^{o}C. Fresh medium with either M-CSF or GM-CSF was added for a further 4-7 days. In some experiments washed cells were further incubated with Gas6 (R&D systems, Abingdon, UK), IFNα (e-Bioscience, Hatfield, UK) or p(I:C) (Invivogen, Toulouse, France).

Differentiation of human monocyte-derived macrophages: Peripheral blood from healthy individuals was obtained from the National Health Services-Blood and Transplant (NHS Blood Bank), PBMCs isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare, Buckinghamshire, UK) and monocytes sorted using human CD14 MicroBeads (Miltenyi Biotec, Surrey, UK) by magnetic-activated cell sorting. Monocytes were plated at

$5x10^5$ cells/ml in RPMI 1640 medium supplemented with 10% FCS, 100 u/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (Sigma-Aldrich) and 50 ng/ml M-CSF (Peprotech, Rocky Hill, NJ, USA), and cultured for 7 days prior to stimulation with IFNα (R&D systems, Abingdon, UK) or polyinosinic:polycytidylic acid (p(I:C)) (Invivogen).

Human alveolar macrophage isolation and culture: 3 patients undergoing lung surgery for suspected or confirmed cancer were recruited. Lung tissue distant from the tumor site was removed before perfusing with 0.1 M NaCl for macrophage isolation. The retrieved fluid was centrifuged at 400g for 10 min, and the cell pellet was resuspended in RPMI 1640. Cell suspension was floated over a Ficoll-Paque gradient (GE Healthcare) and centrifuged at 400g for 30 min. The mononuclear cells at the Ficoll interface were extracted and resuspended in RPMI growth media. Viable macrophages were isolated and counted using trypan blue exclusion. Alveolar macrophages were seeded in 24-well plates at $4x10^5$ cells per well and cultured in RPMI 1640 supplemented with 10% FCS, 100 u/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine. Cells were either left untreated or were stimulated with p(I:C) (1 µg/ml) for 4 h prior to lysis for RNA extraction. All patients provided written, informed consent, and the study was approved by the local ethics committee. Patient demographics (median (range)): age: 62 (58-80); gender: two females, one male; smoking history (pack year): 47 (19-50) years; FEV: 2.47 (2.39-2.63); FEV 1% (forced expiratory volume in one second) predicted: 96 (89-106.2).

Immunofluorescent staining of lung section: Formalin fixed lung tissues were embedded in paraffin. 8 µm paraffin sections were de-paraffinised and rehydrated in xylene followed by descending concentrations (down to 50%) of ethanol and finally submerging in water. Antigen retrieval was performed by boiling slides in 10 mM Tris buffer pH 9.0 containing 1 mM EDTA and 0.05% Tween 20 for 20 min and cooling in water for 10 min. Slides were

then blocked with Tris buffered saline (TBS) containing 1% BSA and 10% normal serum for two hours at RT, washed with TBS containing 0.025% Triton X-100, stained with Axl (H-124) and MerTK (F-18) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4^0C and bound antibody detected with either Alexa Fluor 488 or 564 secondary antibodies. Coverslips were applied with prolong gold mounting medium with DAPI (Life Technologies, Paisley, UK) and were examined with an Olympus BX51 fluorescent microscope using a 40x/ 0.75 UPlan Fln objective and captured using a Coolsnap ES camera (Photometrics, Tucson, AZ, USA) through MetaVue Software (Molecular Devices, Sunnyvale, CA, USA). Specific band pass filter sets for DAPI, FITC and Texas Red were used to prevent bleed through from one channel to the next. Images were then processed and analysed using ImageJ (http://rsb.info.nih.gov/ij).

RNA extraction and quantitative (q)PCR: Total RNA was extracted using RNeasy micro kit (QIAGEN, Manchester, UK), quantified with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and equivalent amounts of RNA were reverse transcribed using High-Capacity RNA-to-cDNA Kit (Life Technologies). qPCR reactions were performed using pre-designed TaqMan expression assays and TaqMan Fast Universal PCR Master Mix (both from Life Technologies) on a QuantStudio 12K Flex PCR system (Life Technologies). Relative mRNA expression was calculated based on the ΔΔCT method using QuantStudio 12K Flex Software v1.1.1 (Life Technologies). Expression of Hprt (Mm01545399_m1) or average expression of Hprt and Gapdh (Mm99999915_g1) was used to calculate relative mRNA levels of Axl (Mm00437221_m1), Mertk (Mm00434920_m1) and Gas6 (Mm00490378_m1) in mouse cells, while Gapdh (Hs02758991_g1) expression was used to calculate relative expression of Axl (Hs01064444_m1) in human monocyte-derived macrophages. In primary human alveolar macrophages relative Axl expression was determined by SybrGreen (Applied Biosystems, Paisley, UK) qPCR using the following pairs of primers: Axl (fwd: 5'-CATGAAACATGGAGACCTAC-3'; rev: 5'-

ATCTCTTGGTACTCAGATACTC-3'), GAPDH (fwd: 5'-ACAGTTGCCATGTAGACC 3'; rev: 5'-TTTTTGGTTGAGCACAGG-3') (all from Sigma-Aldrich).

Western blotting: Protein lysates were prepared in RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (all from Sigma-Aldrich, Dorset, UK) and protein concentrations determined using a BCA Protein Assay Kit (Thermo Scientific). Lysates containing equivalent amounts of protein were resolved by electrophoresis on 4-15% mini-Protean TGX precast gels (BIO-RAD, Hercules, CA, USA), and proteins transferred to nitrocellulose membranes (BIO-RAD) using a semi-dry Trans-Blot turbo transfer system (BIO-RAD). Membranes were washed in PBS containing 0.05% Tween-20 (Sigma-Aldrich) (PBS/T), blocked in 2% milk (BIO-RAD) in PBS/T, and incubated overnight at 4°C with antibodies recognising Axl (M20) or actin (both from Santa Cruz Biotechnology) diluted in PBS/T containing 1% bovine serum albumin. Following extensive washing in PBS/T, membranes were incubated with HRP-conjugated anti-rabbit or anti-goat immunoglobulin antibodies (Dako, Cambridge, UK), developed using a Clarity Western ECL Substrate (BIO-RAD) and visualised with a ChemiDoc MP Imaging System (BIO-RAD).

Analysis of nucleosome release: Nucleosome release was assessed using Cell Death Detection ELISA Plus (Roche Diagnostics, Mannheim, Germany) as per the manufacturers' instructions. Briefly, BAL supernatants were mixed with biotynylated anti-histone antibodies and horseradish peroxidase-conjugated anti-DNA antibodies and incubated on a streptavidin-coated plate for 2 h at RT. After extensive washing samples were incubated with substrate solution containing 2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonate] and nucleosome content was determined by optical density measurement at 405 nm.

Detection of apoptotic cells: Annexin V/Dead Cell Apoptosis kit (Life Technologies) was used to determine early and late apoptotic cells in the BAL. Cells were resuspended in Annexin-binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$ at pH 7.4), and stained with Alexa Fluor 488-Annexin V and propidium iodide (PI) for 15 mins at room temperature. Samples were then washed and immediately analysed by flow cytometry.

Uptake of apoptotic thymocytes by airway macrophages: Thymocytes were incubated with 2.0 μM dexamethasone in order to induce apoptosis and labelled with pHrodo-SE (Life Technologies) for 30 min at RT. Apoptotic cells or apoptotic cells with Cytochalasin D at 5 μg/ml (Sigma-Aldrich) were added at 10:1 ratio (apoptotic cells:macrophages) to adhered wild type or $Axl^{-/-}$ airway macrophages for 1 h. Macrophages were then detached by incubation with trypsin for 10 min at 37^{o}C and analysed on a Canto II. pHrodo-labelled thymocytes become fluorescent when efferocytosed and detectable within the PE channel.