Supplementary data

Methods: extended version

Mice. Wild-type Balb/c (BALB/cOlaHsd) and C57BL/6 (C57BL/6JOlaHsd) mice were purchased from Envigo. Ubi-GFP C57BL/6-Tg(UBC-GFP)30Scha/J mice (004353) and B6;129-Cd3e^{tm1Lov}/J mice (004177) were obtained from the Jackson Laboratory. K5-TOM mice were obtained by crossing of KRT5-Cre^{ERT2} knock-in mice [1] with Rosa26-tdTomato mice on mixed background (originally Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze/}$J mice – 007909, Jackson Laboratory). B6.Cg-Commd10$^{Tg(Vav1-icre)A2Kio}$/J (008610, Jackson laboratory) were crossed to Rosa26-tdTomato mice to obtain VAV1-TOM mice. LysM-TOM mice were created by crossing of B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J (004781, Jackson Laboratory) with Rosa26-tdTomato mice. B6.129P2(C)-Cx3cr1$^{tm2.1(cre/ERT2)Jung}$/J (020940, Jackson Laboratory) were bred with Rosa26-tdTomato mice and B6.129P2-Gt(ROSA)26Sor$^{tm1(DTA)Lky}$/J (009669, Jackson Laboratory) to generate Cx3cR1-TOM and Cx3cR1-DTA mice, respectively. Tamoxifen citrate containing mouse chow at a treatment dosage of 400mg/kg (TD.55125, Envigo) was used to activate the CreERT2 protein, thereby inducing Cre recombinase activity in K5-TOM, Cx3cR1^{creER} -TOM and Cx3cR1^{creER}-DTA. The duration of treatment is indicated within each experiment. Mice were either sacrificed by CO_2 asphyxiation or by total exsanguination and excision of the heart under deep isoflurane sedation combined to intraperitoneal anaesthesia (xylazine 10% and ketamine 5% in phosphate-buffered saline (PBS)).

Heterotopic Tracheal Transplantation model. HTT was performed by adapting the murine model developed by Hertz et al. (19) and has already been used in our laboratory as described by Lemaitre et al [2]. Briefly, donor hearts and lungs were exposed though extended median sternotomy, thymus and heart were dissected away. The trachea was separated from the

oesophagus by blunt dissection, excised from the first tracheal ring to the main bronchi and placed in 0.9% sodium chloride until transplantation. Recipient mice were anaesthetized with a mixture of xylazine 10% and ketamine 5% in PBS. A 3 mm incision was made through the dermis posteriorly, at the cervico-thoracic junction, and two small subcutaneous pouch (1.5 x 0.5 cm) were created by blunt dissection on the right and left side of the incision. Each recipient received two tracheas (one for each pouch) in order to reduce the total number of mice needed for the experiments.

Tacrolimus treatment and blood concentration. HTT recipient mice received an intraperitoneal injection of 1 mg/kg/day of tacrolimus (FK506, ab120223, Abcam) from the day of transplantation until harvesting. The control group received vehicle only (DMSO 1.125%, Tween80 4%, PEG400 5.5% in PBS). All injections were performed under gaseous anaesthesia (isoflurane 3%) in order to avoid any damage to the dorsal grafts during mice manipulation and reduce the stress induced by daily injections. Blood was taken by terminal cardiac puncture under deep anaesthesia at three hours post tacrolimus or vehicle injection (C3). Whole blood FK506 concentrations were determined by liquid chromatography-mass spectrometry (LC-MS) at Erasme Hospital (ULB, Brussels) according to validated clinical methods.

Histological assessment. All samples were fixed in 4% formaldehyde at 4°C. Paraffin embedding of samples and staining of 4 μm sections with Haematoxylin-Eosin (HE) and Masson's trichrome (MT) were performed by DIAPath-CMMI (Gosselies). Images of murine samples at 100x total magnification were acquired using an HD camera on an Olympus BX43 microscope. Scans of human lungs were obtained with the NanoZoomer 2.0-HT (Hamamatsu) and analysed with NDP.view v2.7.47 software. The histopathological score for epithelial loss and lumen occlusion in murine allografts was established using ImageJ software. Total luminal area was calculated from the inner edge of tracheal cartilages. The proportion of epithelial loss was estimated based on the perimeter of the lamina reticularis.

Human samples. Paraffin-embedded samples were obtained from lung explants of patients undergoing a second transplant for BOS. Only samples with histological confirmation of OB lesions were further analysed by immunofluorescence (n = 10 patients). Specimens showing healthy peripheral tissue from patients undergoing pulmonary resection for well delimited carcinoid tumor were used as controls (n = 7 patients). More information about clinical samples are shown in Supplementary information (Table S1).

Immunofluorescence and confocal analysis. *IF-Frozen.* All murine samples were fixed in 4% PFA for 2 hours, then washed in PBS three times for 15min, and cryoprotected in 30% sucrose/PBS overnight before embedding in OCT (Tissue-Tek). Afterwards, 10 μm cryosections were washed in PBS (2x 7min), permeabilized with 0.25% Triton X-100/PBS (2x 5min), then blocked for 3 hours at room temperature in an optimized blocking solution (PBS with 6.5% goat serum, 6.5% donkey serum, 5% bovine serum albumin, 5% horse serum, 0.1% Tween20), which was also used to dilute antibodies. Primary antibodies were incubated overnight at 4°C in humid chambers at the following dilutions: rabbit anti- human/mouse αSMA (1:100, polyclonal, Abcam, Ab5694), AF700 rat anti-mouse CD11b (1:50, M1/70, , BD, 557660), rat anti-mouse CD45 (1:100, I3/2.3, Abcam, ab25386), APC rat anti-mouse EpCAM/CD326 (1:50, G8.8, BD, 563478), BV750 rat anti-mouse F4/80 (1:50, T45-2342, BD, 747295), rabbit anti-mouse proCollagen1a1 (1:100, polyclonal, Invitrogen, PA5-35379). Sections were washed three times for 5min each in PBS, incubated with secondary antibodies solution for 2 hours at room temperature in the dark. The following conjugated antibodies were used: AF488 donkey anti-rabbit IgG (1:1000, A21206), AF594 donkey anti-rat IgG (1:500, A21209), AF594 donkey anti-rabbit IgG (1:1000, A21207), AF647 donkey anti-rabbit IgG (1:1000, A21247), AF647 goat anti-rat IgG (1:500, A31573), all purchased from ThermoFisher Scientific. All antibodies had been centrifuged at 1300 RCF for 2.5 minutes before using to get rid of eventual aggregates. The slides were then washed 10min in PBS, incubated with 0.1%

DAPI/PBS for 5min, rinsed with 20mM Teen20 in Tris Buffered Saline (TBS-T) for 10 minutes and mounted with 2.5% Dabco antifade reagent (D2522, Sigma-Aldrich) in Glycergel mounting medium (C0563, Agilent). All samples were protected from direct light exposure during the whole procedure to prevent the fading of spontaneous GFP and tdTomato signal. No anti-GFP or anti-tdTomato antibodies were used in order to avoid false positives from non-specific immunoglobulin binding.

IF-Paraffin. 4µm-thick paraffin-embedded sections from human lungs were deparaffinized with baths of xylene and ethanol, then washed in distilled water. Antigen retrieval was performed in 10mM Citric Acid Buffer (pH 6.0) heated in a microwave at 750W for 15 minutes. After washing in distilled water, all sections were permeabilized with 0.25% Triton X100/PBS (2x 5min), then incubated with optimized blocking solution (PBS with 10% donkey serum, 5% bovine serum albumin, 5% horse serum, 0.1% Tween20) for 2 hours. The same blocking solution was used to dilute antibodies, which had been centrifuged (1300 RCF for 2.5min) before use. Primary antibodies were applied over night at 4°C at the following dilutions: rabbit anti-human/mouse αSMA (1:50, polyclonal, Abcam, Ab5694), mouse anti-human CD45 (1:50, MEM-28, Abcam, ab8216), mouse anti-human CD68 (1:50, KP1, Abcam, ab955). Sections were washed three times in PBS, then stained with the following secondary antibodies for 2 hours at room temperature in the dark: AF488 donkey anti-rabbit IgG (1:500, A21206) and AF594 donkey anti mouse (1:500, A21203), both from ThermoFisher Scientific. After washing in PBS (5min) and TBS-T (5min) the slides were incubated with 0.1% Sudan Black in 70% isopropanol in the dark, then rinsed with distilled water two times for 5min. Nuclei were stained with 0.1% DAPI/PBS. After washing in TBS-T for 10min, all sections were mounted with 2.5% Dabco/Glycergel.

Immunohistochemistry. Immunohistochemical stainings of allografts from Cx3cR1^{CREER}-DTA recipients and controls with Anti-CD3ε monoclonal antibody (clone 2GV6, Roche 05 278

422 001) were performed by DIAPath-CMMI (Gosselies). Four-µm-thick sections were subjected to standard IHC on Ventana discovery XT (Ventana, Roche Diagnostics, Belgium) using the DISCOVERY ChromoMap DAB Kit according to manufacturer's recommendations. Briefly, the formalin-fixed-paraffin-embedded tissues sections, mounted on Superfrost slides, were deparaffinised and rehydrated. Antigen retrieval was performed for 36min using Cell Conditioning 1 (Roche Diagnostics). The slides were incubated with the rabbit monoclonal anti-CD3 antibody for 12min (RTU antibody, clone 2GV6 from Ventana, Roche Diagnostics, Belgium), then washed and incubated for 12min with the anti-rabbit IgG DAB detection system (RTU, DISCOVERY OmniMap anti-Rb, Ventana, Roche Diagnostics, Belgium). Immunostainings were detected by incubation with diaminobenzidine and hydrogen peroxide. All immunohistochemistry slides were counterstained with Gill's hematoxylin, dehydrated and mounted. For each staining, an external positive control was included as well as a negative control (omitting the primary antibody).

Confocal analysis. All images were acquired with a Zeiss LSM 710 confocal microscope in Best Signal mode in order to reduce the risk of signal overspilling and false colocalisation. Pictures were analysed and manually quantified using Zen 2.3 lite Blue software in grid mode (150% digital zoom). For colocalisation of multiple signals, the number of positive cells for each channel was assessed sequentially in each square of the grid by displaying DAPI staining with one channel at the time in order to better identify live cells expressing multiple markers. For each channel, the same values of black and white were employed to all samples in the same experiment. GFP-negative and tdTomato-negative sections were used as negative controls in Ubi-GFP, K5-TOM, VAV1-TOM, LysM-TOM and Cx3cR1-TOM experiments. Furthermore, for all experiments samples incubated only with secondary antibodies or with DAPI were used as negative controls as well. This allowed to exclude non-specific staining due to secondary antibodies or fluorescent tissue background. For murine samples, tile scan acquisition with

autofocus mode (200x total magnification with 0.6 digital zoom per tile) was performed in order to acquire the whole airway in one picture, except for allografts shown in Figure 1 and 2, for which four separate fields (200x, 0.6 digital zoom) overall including the whole section were acquired. Quantification of co-localizing signals was assessed exclusively in the luminal area, delimited by the inner edges of tracheal cartilages. Human lungs quantification was carried out on four random fields (200x, 0.6 digital zoom) per sample. Were excluded from cell count: 1) αSMA^+ smooth muscle cells; 2) cells with apoptotic or no visible nucleus (showed by DAPI staining); 3) non-specific cell-surface signals detected in channels used for cytoplasmic markers; 4) cytoplasmic signals from surface-only markers, often caused by necrotic debris and red blood cells.

Immunohistochemical quantification. Digitization of tissue sections stained with anti-CD3ε antibodies were performed using the Hamamatsu Nanozoomer® (zoom 20x). Image processing and analysis were performed using Visiomorph DP 2020.1 (Visiopharm, Hoersholm, Denmark) to determine the positively and negatively stained cells and calculate the fraction of positively stained cells over the whole surface of the lumen.

Flow cytometry. Tracheal grafts were minced and incubated at 37°C (5% CO_2) for 2 hours with 2mg/ml Collagenase A (Merck) in 5% FCS/RPMI and filtered on a 100µm strainer. The two allografts harvested from the same recipient were pooled together to increase the total number of cells analysed for each sample. Single bone marrow cells were dissociated with a syringe. Spleens were mechanically separated into single-cell suspensions. All samples were incubated with ACK for RBC lysis, stained to exclude dead cells (Live/Dead Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation, Life Technologies or ViaKrome 808 fixable viability Dye, for infrared 808 nm excitation, Beckman Coulter), incubated with rat anti-mouse CD16/CD32 (BD 553141, clone 2.4G2, 1:100) and labelled for surface markers for 30min at 4°C in the dark. After fixation and permeabilization with CytoFix/CytoPerm (BD Biosciences)

cells were stained for intracytoplasmic markers for 20 minutes in the dark. All cytometric analysis were performed on a Cytoflex-LX cytometer and analysed with FlowJo10 software, except for VAV1-TOM bone marrows for which a Cyan-LX cytometer was used. Combined anti-mouse CD19, CD3ε and NK1.1 staining was used for lineage (LIN) negative selection of B cells, T cells and NK cells in some experiments. Anti-mouse CD11b (AF700, M1/70, 1:100, 557660), CD11c (APC, HL3, 1:100, 550261), CD19 (APC-Cy7, 1D3, 1:100, 557655; PE-Cy7, 1D3, 1:100, 552854), CD3ε (APC-Cy7, 145-2C11, 1:100, 55759), CD45 (BV421, 30-F11, 1:200, 563890; BV650 30-F11, 1:200, 563410; BV510, 30-F11, 1:200, 563891), CD64 (AF647, X54-5/7.1, 1:100, 558939), Ly6C (FITC, AL-21, 1:100, 553104; BV421, AL-21, 1:100, 562727), Ly6G (PerCp-Cy5.5, 1A8, 1:100, 560602; BV650, 1A8, 1:100, 740554), F4/80 (BV750, T45-2342, 1:50,747295), NK1.1 (APC-Cy7, PK136, 1:100, 560618; PerCP-Cy5.5, 1:100, 551114) were purchased from BD Bioscience. Anti-mouse EpCAM/CD326 (APC-Cy7, G8-8, 1:100, 118218), CD68 (BV711, FA-11, 1:50, 137029) and Cx3cR1 (PE-Cy7, SA011F11, 1:100, 149016) were obtained from Biolegend. Anti-mouse αSMA (FITC, 1A4, 1:50, F3777) was purchased from Sigma-Aldrich.

FACS sorting and scRNA-seq analysis. Single-cell suspension from ten BALB/c-to-LysM-TOM allografts and one LysM-TOM spleen were obtained as per flow cytometry protocol. According to 10X Genomics sample preparation protocol all samples were additionally filtered on a 40µm strainer to avoid 10X microfluidic channel clotting. Cells were stained to exclude dead cells (Live/Dead, Life Technologies) and tdTomato^{+} cells were sorted on a BD FACSAria III using tdTomato^{-} allografts as controls. Microscope assessment after Trypan blue staining allowed to count viable cells following FACS sorting. Cell preparations were centrifuged at 500 RCF for 7 min and pellets were resuspended in 10%FCS/RPMI medium. Cells were loaded on the Chromium Controller (10x Genomics) and Single-cell RNA-seq libraries were prepared using the Chromium Single Cell 3′ v2 Reagent Kit (10x Genomics) according to manufacturer's

protocol. Libraries were loaded to an Illumina Novaseq (Brightcore platform, http://www.brightcore.be/), and Cell Ranger (10x Genomics) functions mkfastq and count were used to demultiplex the sequencing data and generate gene-barcode matrices. We regressed out mitochondrial and ribosomal genes using SCTransform function with default parameters from Seurat R package (version 3.1.2)[3]. Contaminant cells (T and B cells) were removed from the analyses. Principal component analysis was performed with default parameters and used in UMAP analysis to identify clusters. We applied FindAllMarkers function to identify differentially expressed genes in each cluster as compared to all other clusters (Wilcoxon Rank Sum test). The differentially expressed genes were used to annotate cells populations which were subject to differential expression analysis between the two organs. AUCell R package [4] was used to identify cells with an active gene set (i.e. gene signatures) in our single-cell RNA-seq data. AUCell uses the "Area Under the Curve" (AUC) to determine if a critical subset of the input gene signature is upregulated within the expressed genes for each cell.

Statistical analysis. GraphPad Prism software v6.01 was used for statistical analysis. Comparisons between two data sets were performed using Mann-Whitney non parametric t-test. Kruskal-Wallis test with Dunn's multiple comparisons test were used when comparing more than two groups. Correlation analyses were done by using Spearman's correlation test.

Study approval. All animal work was carried out in compliance with and after approval by the institutional Animal Care and local committee for Animal Welfare from the Biopark ULB Charleroi (BUC) (P2016/93, #LA1500474). All human *ex vivo* analysis were approved by the ethics committee of Erasme Hospital, ULB, Brussels (P2019/377) and were in accordance with the guidelines outlined by the board. Human samples were obtained from Erasme Hospital biobank (BE_BERA1; Biobanque Hôpital Erasme-ULB (BERA); BE_NBWB1; Biothèque Wallonie Bruxelles (BWB); BBMRI-ERIC).

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