**STAR\* METHODS**

**KEY RESOURCES TABLE**

**LEAD CONTACT AND MATERIALS AVAILABILITY**

Further information and requests for resources and reagents should be directed to, and will fulfilled by, the Lead Contact, Thomas Marichal (t.marichal@uliege.be).

**METHOD DETAILS**

***Mice***

The following mice were used in this study; CD45.2 WT (Janvier Labs), CD45.1 WT (Shen et al., 1985)(The Jackson Laboratory, Strain #002014), *Cx3cr1Gfp/+* (Jung et al., 2000)(The Jackson Laboratory, Strain #005582), *Tmem119Cre/+* (generated by Marichal Lab and Cyagen Bioscience, Santa Clara, CA, USA), Cx3cr1LSL-DTR/+ (Diehl et al., 2013)(The Jackson Laboratory, Strain #025629), *Nr4a1-/-* (Lee et al., 1995)(The Jackson Laboratory, Strain #006187), Maffl/fl (ref.)(kindly provided by …), Mafbfl/fl (developed by Bureau Lab and GIGA-Mouse Facility, University of Liège, Belgium), *Lyz2Cre/+* (Clausen et al., 1999)(The Jackson Laboratory, Strain #004781) and *Ms4a3Cre/+* (Lui et al., 2019)(kindly provided by Florent Ginhoux). All mice were used on a C57BL/6 background and a mix of male and female mice between 6 and 12 weeks of age were used for each experiment, unless otherwise stated. The mice were bred and housed under specific pathogen-free conditions at the GIGA Institute (Liège University, Belgium), maintained in a 12-h light-dark cycle, and had access to normal diet chow and water *ad libitum*. All animal experiments described in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Liège. The ‘Guide for the Care and Use of Laboratory Animals,’ prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, as well as European and local legislations, were followed carefully.

***Generation of IM-DTR mice***

*Tmem119Cre* knock-in mice in C57BL/6 background were generated using CRISPR/Cas-mediated genome engineering by Cyagen Bioscience (Santa Clara, CA, US). In brief, the *Tmem119* targeting vector was designed by cloning a genomic fragment encompassing exon 2 of the *Tmem119* gene from BAC clones RP23-187D5 and RP23-126P3. A *Cre-polyA* cassette was introduced in the *Tmem119* targeting vector upstream of the ATG start codon between a 2.1 kb 5′ homology arm and 2.1 kb 3′ homology arm. *Tmem119*-gRNA (protospacer, CAGGGGACCATGTTGAGCTATGG), *Cas9* mRNA and *Tmem119* targeting vector were co-injected into pronuclei of C57BL/6J one cell stage zygotes, followed by implantation of the zygotes into surrogate mothers to obtain targeted knock-in offspring. F0 knock-in founder animals were identified by PCR followed by sequence analysis. *Tmem119Cre/+* mice were then crossed to C57BL/6J wild type mice for at least for three generations. *Tmem119Cre/+* and *Cx3cr1LSL-DTR/+*mice were crossed to create *Tmem119Cre/+;Cx3cr1LSL-DTR/+*mice, referred as ‘IM-DTR’ mice. CD45.1/2 IM-DTR mice were generated by crossing IM-DTR mice to CD45.1 wild type mice.

***Generation of cMaf/Mafb DKO, cMaf SKO and Mafb SKO mice***

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***Chemicals treatments***

For IM depletion, IM-DTR mice were intraperitoneally (i.p.) injected with a single dose of 50 ng of DT (Sigma), unless otherwise stated. For EdU incorporation, mice were i.p. injected with 1mg/mouse of EdU 12–16 h before sacrifice. For CSF1R blocking, 100 mg/kg of PLX3397 was given by i.p. injection 24 h and 48 h post DT injection.

***Generation of bone marrow chimeras***

Eighteen-week-old CD45.2 or CD45.1/2 IM-DTR mice were anesthetized by intraperitoneal injection of Ketamine (75 mg/kg) and Xylazine (10 mg/kg). Lungs were protected with a 0.6-cm-thick lead cover and mice were lethally irradiated with two doses of 6 Gy 15 min apart. Once recovered from the anaesthesia, mice were reconstituted by intravenous (i.v.) administration of 10 × 106 bone marrow cells from congenic CD45.1 or CD45.2 WT mice. For mixed bone marrow chimeras, mice were injected i.v. with 10 × 106 bone marrow cells consisting of a 1:1 mix of bone marrow cells obtained from CD45.1 WT and CD45.2 *Nr4a1−/−* or *Ms4a3Cre/+ Mafbfl/fl* mice. From the day of irradiation, mice were treated for 4 weeks with 0.05 mg/mL of enrofloxacin (Baytril, Bayer) in drinking water. Chimerism was assessed by flow cytometry in the blood and the lung 5 weeks after irradiation.

***Adoptive transfer of bone marrow monocytes***

Bone marrow monocytes were isolated from congenic CD45.2 WT mice using Monocyte Isolation Kit (Miltenyi Biotec). Bone marrow monocyte (2 x 106 cells/mouse) were administered intravenously into CD45.1/2 IM-DTR mice, which had received 500 ng DT i.p. 24 h before monocyte transfer to deplete endogenous IM.

***Isolation of (tissue) leukocytes***

Blood was collected by retro-orbital plexus bleeding of terminally anaesthetized mice. Mice were then euthanized by cervical dislocation. Peritoneal lavage was obtained by injecting 10 mL HBSS into the peritoneal cavity and collecting the washout. Mice were then perfused with PBS via the left ventricle and lungs, brain, liver, spleen, intestine and colon were dissected. Lungs, brains, liver and spleen were cut into small pieces with razor blades, and digested for 1 h at 37 °C in HBSS containing 5% v/v of FBS (Gibco), 1 mg/mL collagenase A (Roche) and 0.05 mg/mL DNase I (Roche). After 45 min of digestion, the suspension was flushed using a 18 G needle to dissociate aggregates. PBS (Gibco) containing 10 mM of EDTA (Merck Millipore) was added to stop the digestion process and suspensions were filtered. Leukocytes from lungs, brain and liver were isolated using a density gradient (Percoll from GE Healthcare). …

***Isolation of leukocytes from intestine and colon***

Mouse intestine and colon were separated by cutting at 2 cm below pylorus and 2 cm above rectum, and cleaned of mesentery, Peyer’s patches and fat in pre-cold HBSS with 2% FBS. After removal of contains, both intestine and colon were open by a longitudinal cut and washed 3 times in pre-cold HBSS with 2% FBS.

To remove mucus and epithelial cells, intestine and colon were incubated with HBSS with 2% FBS and 1 mM dithiothreitol (DTT) for 20 min with shaking followed by vortex for 30 seconds, and subsequently incubated with HBSS with 2% FBS and 1.3mM ethylenediaminetetraacetic acid (EDTA) for 40 min and vortex for 30 seconds.

To release cells from tissue, intestine and colon were cut into ~1 mm pieces and incubated for 1 hour at 37 ˚C with RPMI with 2% FBS, 2 mg/mL collagenase IV (Sigma) and 40 U/mL DNase I (Roche). At the end of incubation, tissue was homogenized with a 19G syringe and filtered through a 70 µm strainer. Wash the passthrough with pre-cold PBS with 2% FBS and 0.78 EDTA. Cells were ready for staining after being pelleted by centrifuge of 1400 rpm 7 min.

***Flow cytometry***

Cells (0.5–5 x 106) were pre-incubated with Mouse BD Fc Block™ (BD biosciences) to block Fc receptors and stained with appropriate antibodies at 4°C in the dark for 30 min. For Ki-67 staining, extracellular-stained cells were permeabilized and stained using FITC Mouse Anti-Ki-67 Set (BD Biosciences). For EdU staining, extracellular-stained cells were permeabilized and stained using Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit (Thermo Fisher), according to manufacturer instructions. Cell viability was assessed using LIVE/DEAD Fixable Near-IR (775) stain (Thermo Fisher) and the cell suspensions were analyzed with a LSRFortessa (BD Biosciences). Results were analyzed using FlowJo software (Tree Star Inc.). Lung monocytes, IM and AM were sorted using an FACSAriaIII (BD Biosciences). The full list of antibodies used can be found in the Key Resources Table.

***Bulk RNA-seq***

IM-depleated mice were IM-DTR mice that had been treated with 50ng DT i.p. and sacrificed 14 days later. Control mice were the litermate of IM-DTR mice without DT treatment. On the date of experiment, mice were sacrificed by neck dislocation. Lung cells were released after fine slicing and incubation with collagenase I and DnaseI for 30 min at 37˚C, and purified by Percoll gradient-density centrifuge as described above. Lung mono-nuclear cells were stained for 30 min at 4 ˚C by incubating with the mixed antibodies: V500-CD45.2, PE-Cy7-CD11b, Viability Dye, PE-F4/80, BV421-CD64, BV786-CD11c, APC-CD206 and PE-CF594-Ly6C. Alveoloar macrophages (AM), CD206+ interstitial macrophages (CD206+ IM) and CD206- IM were FACS-sorted into Trizol in a BD FACSAria III. Total RNA was extracted with standard Trizol RNA extraction protocol. RNA quality and quantity were evaluated using a 2100 bioanalyzer (Agilent) and the Quant-iT™ RiboGreen™ RNA Assay Kit (ThermoFisher).

One hundred nano-grams of RNA was used to generate the libraries using the Truseq stranded mRNA kit (Illumina). These libraries were sequenced on an Illumina Novaseq sequencer on a SP flow cell. Sequence alignment with the mouse genome (GRCm38), sequence counting and quality control were performed using the nf-core/rnaseq pipeline.

RNA-seq data were analyzed using R Bioconductor (3.5.1) and DESeq2 package (version 1.26.0) (Love et al., 2014). Briefly, differentially expressed (DE) genes between refilled and control IMs were calculated with DESeq2 package, and only genes with adjusted p < 0.05 were considered as significant DE genes.

***scRNA-seq***

To compare the de novo refilled IMs to intact IMs, the lung IMs from IM-DTR mice which had been treated for 96 hours with 50 ng DT (group “DT96h”) and those from the control littermates (group “No treatment”) were analyzed with single-cell RNAseq. Briefly, 5 mice from each group were sacrificed by neck dislocation and lung cells were released after fine slicing and incubation at 37 ˚C for 30 min with Dnase I and collagenase I. CD11b+ cells were purified with CD11b MicroBeads (Miltenyi) and stained with mixed antibodies V500-CD45.2, PE-Cy7-CD11b, Viability Dye, PE-F4/80, BV421-CD64, BV786-CD11c, APC-CD206 and PE-CF594-Ly6C. The lung IMs and monocytes were sorted by BD FACSAria III and pooled before 10X Chromium library construction.

To access the differentiation dynamics of de novo refilled IMs, the lung IMs and monocytes of IM-DTR mice treated with 50ng DT at three timepoints were collected, stained and sorted as described above. The lung IMs and monocytes were sorted from three groups of mice: group “DT12h” contains 5 IM-DTR mice which were treated with 50 ng DT 12 hours before sacrifice; group “DT24h” contains 5 IM-DTR mice which were treated with 50 ng DT 24 hours before sacrifice; group “DT48h” contains 5 IM-DTR mice which were treated with 50 ng DT 48 hours before sacrifice. Cells from each group were barcoded with different Biolegend anti-mouse Hastags before being pooled for library construction.

For library preparation, approximately 3000 cells per sample (for “DT96h” and “No treatment”) or 20 000 cells (for pooled “DT12h”, “DT24h” and “DT48h” sample) were suspended in PBS with 0.04% BSA and loaded into the Chromium Controller with a Chromium Next GEM Chip G (10X Genomics), in which they were partitioned and their polyA RNAs captured and barcoded by Single Cell 3’ v3.1 Gel Beads (10X Genomics). The cDNA were amplified and libraries compatible with Illumina sequencers were generated using Chromium Next GEM Single Cell 3ʹ GEM, Library & Gel Bead Kit v3.1 (10X Genomics). The libraries were sequenced on an Illumina NovaSeq sequencer with NovaSeq S1 100 cycle kit (Read1: 28 cycles, read2: 76 cycles, index1: 10 cycles, index2: 10 cycles) at a depth of 50 000 reads per cell.

The Cell Ranger (v3.0.2) application (10x Genomics) was then used to demultiplex the BCL files into FASTQ files (cellranger mkfastq), to perform alignment (to Cell Ranger human genome references 3.0.2 GRCm38/build 97), filtering, UMI counting and to produce gene-barcode matrices (cellranger count).

Filtered matrix files were used for further scRNAseq analyses with R Bioconductor (3.12) and Seurat (3.2.1)(Stuart et al., 2019). The cells from pooled “DT12h”, “DT24h” and “DT48h” sample were demultiplexed with the barcode detected in each cell.

Filtered matrices containing cell IDs and feature names in each sample were used to build a Seurat object. We performed a quality control by filtering out the cells with less than 200 detected genes, the genes detected in less than 3 cells and the cells exhibiting more than 10% of mitochondrial genes. Gene counts in each sample was normalized separately by default method “LogNormalize” with scale factor 10000 and log-transformation. Two thousands of highly variable features were identified with the “vst” method.

After merge cells from all samples, the contaminated cells were removed based on the expression of specific genes. Four clusters were identified in the remained cell using FindClusters functionand the differentially expressed genes were calculated using FindAllMarkers function (Seurat package).

***Gene ontology (GO enrichment analysis with differentially expressed (DE)***

The DE gene lists for enrichment analyses were calculated using Seurat function FindMarkers with only.pos = TRUE in order to output only positively regulated genes. Thresholds logfc.threshold of 0.2 and adjusted p of 0.01 were applied to filter the gene lists. Gene Ontology enrichment analyses were made using enrichGO functions from clusterProfiler package (Wu et al., 2021) with default arguments. Only biology process (BP) terms of ontology were showed in final results.

***Single-cell RNA velocity estimation***

For each sample, the counts for unspliced- and ambiguous transcripts were calculated from CellRanger output using velocyto command-line tool (http://velocyto.org) (la Manno et al., 2018) and saved in loom files. The single-cell RNA velocities were estimated using scVelo toolkit (https://scvelo.readthedocs.io) (Bergen et al., 2020). Briefly, the loom files were used as input for scVelo analysis. Genes with minimum 20 of both unspliced and spliced counts and on the top list of 2000 genes were filtered, normalized and log transformed (scv.pp.filter\_and\_normalize with default parameters). Thirty principal components (PCs) and 30 neighbors obtained from euclidean distances in PCA space were used for computing first-/second-order moments for each cell. We used generalized dynamical modeling to recover the full splicing kinetics of spliced genes and the single-cell RNA velocities were plotted with the same cluster labels and embedding as in figure 4a.

***Single-cell regulatory network inference and clustering (SCENIC) analysis***

To find the potential active transcription factors (TF), only Ly6C+ classical monocytes (cMo), CD206- IM, CD206+ IM cells were subjected to SCENIC analysis using SCENIC package (Aibar et al., 2017). The normalized counts, nFeature\_RNA, nCount\_RNA in merged Seurat object were used to initial SCENIC analysis. The genes expressed with a value of 3 in 0.5% of the cells and detected in 1% of the cells were kept for following SCENIC analysis. Co-expression network analysis was made with GENIE3 in the SCENIC package.

To demonstrate the SCENIC results, the results of 3.4\_regulonsAUC were added to the metadata of Seurat object for that regulon AUC scores could be plot using FeaturePlot function. The top 50 regulons with highest variance were showed in the heatmap with their Z-scores.

***Monocle, TradeSeq and pseudotime analysis across IM differentiation***

To access the regulation of gene expression across IM differentiation, only Ly6C+ classical monocytes (cMo), CD206- IM, CD206+ IM cells were subjected to Monocle (Trapnell et al., 2014) analysis. The Monocle CDS object were built with counts and metadata from Seurat object and convert using SeuratWrappers package. Cells were clustered with cluster\_cells function using calculated UMAP coordination and resolution of 0.51E-3. The trajectory and pseudotime of IM differentiation were construct using learn\_graph and order\_cells functions. The DE genes across trajectory were calculated using Moran’s I test (graph\_test function) and only the genes with q\_value of 0 and Morans\_I over 0.25 were kept as significant DE genes and subject to further analyses.

To compare the expression patterns of DE genes across pseudotime, the counts matrix, pseudotime and cell weights calculated above were then used as input in fitGAM function (TradeSeq package (van den Berge et al.)). The association of average expression of each gene with pseudotime was tested using associationTest and the DE genes between CD206+ and CD206- IM differentiation were calculated with diffEndTest function. The value of the estimated smoother on a grid of pseudotimes was for each of DE genes using predictSmooth. The DE genes with waldStat > 70 and |logFC| > 2 were annotated as “changed genes” which meant their expression patterns were different in CD206+ and CD206- IM differentiation trajectories, while the rest of DE genes were considered as “unchanged genes” which meant the expression patterns were similar in two differentiation trajectories. Finally, the scaled estimated smoothers calculated by predictSmooth were used to build heatmap with ComplexHeatmap package (Gu et al., 2016).

***Gene signature scoring***

The IM and cMo specific gene signatures were calculated with previously published scRNAseq data (Schyns et al., 2019) by comparing either IM or cMo population to all other cell types in the dataset using FindMarker function (Seurat). The genes with logFC > 1 and only positively regulated ones were considered as IM or cMo signature. The signatures were then used to calculate the scores for each cells for both Control and Mafb-KO samples with VISION package (Jones et al. 2021). The scores were stored in Seurat object and plotted with Seurat package.

***MCP-1/CCL2 quantification***

Mice were sacrificed at indicated time points after DT administration. Blood was collected and lungs were dissected from PBS-perfused mice. Blood samples were left undisturbed for 30–45 min at RT to allow clot formation. The serum was separated from the blood clot by centrifugation for 10 min at 2000 x g at 4°C. Serum was stored at −80°C. Dissected lungs were snap frozen and homogenized in 360 µL ice cold lysis buffer (40 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10% glycerol and cOmplete™ Protease Inhibitor Cocktail (Sigma)) using a tissue homogenizer (IKA) with the addition of 1% Igepal (…) after homogenization. Samples were then rotated for 20 min at 4°C, followed by a centrifugation to pellet debris. Protein concentration of cleared lysates was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher), according to manufacturer instructions. Cleared lysates were stored at −80°C. CCL2 levels in serum and lung homogenates were determined using MCP-1/CCL2 Mouse Uncoated ELISA Kit (Thermo Fisher), according to manufacturer instructions.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**DATA AND CODE AVAILABILITY**

* Single-cell RNA-seq and bulk RNA-seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. The DOI is listed in the key resources table.
* All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table.
* Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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