**Monocytes can Proliferate in Vacant Tissue Niches**

**prior to Differentiation into Macrophages**

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**Summary**

Resident tissue macrophages (RTM) are differentiated immune cells populating distinct niches and exhibiting important tissue-supportive functions. RTM maintenance is thought to depend either on monocyte engraftment and differentiation, or on the self-renewal of mature RTM. Here, we discovered that monocytes can re-enter cell cycle and proliferate locally before their differentiation into RTM*.* We developed a mouse model of inducible lung interstitial macrophage (IM) depletion in which the vacant niche is repopulated by BM-derived monocytes giving rise to fully differentiated IM subsets. By performing time-course single-cell RNA-sequencing analyses of myeloid cells during niche refilling, we found that few Ly6C+ classical monocytes could self-renew locally in a CSF1R-dependent manner. We further showed that the transcription factor MafB restricted such proliferation and was essential to mediate RTM specification and identity in our model. Our data provide evidence that, in the mononuclear phagocyte system, self-renewal is not merely restricted to myeloid progenitor cells and mature macrophages, but is also a tightly regulated capability of mature monocytes developing into RTM *in vivo*.

**Introduction**

Resident tissue macrophages (RTM) are self-maintaining immune cells that are integral parts of mammalian tissues and exert important tissue-supportive functions. The relationships between RTM, blood monocytes and bone marrow (BM) progenitors were originally appreciated by van Furth and Cohn, who classified these cells in the “mononuclear phagocyte system” (MPS) (van Furth and Cohn, 1968; van Furth et al., 1972). The dogma that BM progenitors give rise to mature circulating monocytes differentiating into RTM has been challenged later by multiple reports showing that RTM populations can arise from embryonic yolk sac macrophages and fetal monocytes seeding the tissues before the establishment of definitive hematopoiesis, and are long-lived cells that can self-maintain in tissues with minimal contribution from monocytes (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015; Hashimoto et al., 2013; Yona et al., 2013). Nevertheless, several pieces of evidence still support the validity of the MPS model, as it has been demonstrated that, throughout adult life, monocytes can give rise to RTM in proportions that depend on the tissue accessibility and on the nature and extent of perturbations leading to RTM depletion (Blériot et al., 2020; Guilliams and Svedberg, 2021; Hume et al., 2019; Liu et al., 2019; Mould et al., 2021).

Regardless of origin, the niche of residence is thought to be an essential determinant of RTM identity and function (Blériot et al., 2020; Guilliams and Scott, 2017; Guilliams et al., 2020). In a given niche, RTM can respond to local trophic factors for their maintenance, such as colony stimulating factor 1 (CSF1), and are also imprinted by niche-derived signals triggering expression of specific transcription factors (TF) and differentiation programs, thereby tailoring a tissue-specific identity, i.e., a phenotypic and functional specialization that fulfills the functional needs of a given tissue (Gautier et al., 2012; Lavin et al., 2014, 2015; Schneider et al., 2014). Supporting this, distinct RTM precursors have been shown to be equally able to differentiate into transcriptionally similar RTM populations *in vivo* (van de Laar et al., 2016; Scott et al., 2016).

While the current paradigm proposes that the repopulation and maintenance of RTM niches can be achieved either via monocyte engraftment and differentiation, or via self-renewal of mature RTM (Guilliams et al., 2020; Hume et al., 2019), the slow turnover of RTM at steady-state and the lack of models allowing to capture rare events such as monocyte-to-RTM transitioning cells have hampered investigations of RTM dynamics *in vivo*. Lung interstitial macrophages (IM) represent an interesting model to study RTM dynamic, as we and others have shown that IM were long-lived RTM that were slowly replenished by BM-derived monocytes in adults and encompassed two functionally distinct subsets populating different niches (Chakarov et al., 2019; Gibbings et al., 2017; Sabatel et al., 2017; Schyns et al., 2018, 2019). Here, we developed a novel transgenic mouse model of diphteria toxin (DT)-inducible IM niche depletion that allowed us to capture the dynamics of events occurring during the repopulation of IM niches at the single cell resolution in an unprecedented way. We discovered that few Ly6C+ classical monocytes (cMo) could undergo a very subtle time- and MafB-restricted CSF1R-dependent proliferation in vacant tissue niches before their differentiation into IM subsets. Our data indicate that, in the MPS, self-renewal is not merely restricted to myeloid progenitor cells and mature macrophages, but is also a capability of mature cMo developing into RTM in vivo.

**Results**

**DT treatment efficiently targets lung IM in *Tmem119Cre;Cx3cr1LSL-DTR*mice**

Monocyte engraftment and differentiation into RTM is a rare event at steady-state, making it difficult to capture and analyze (Guilliams et al., 2020; Hume et al., 2019). The maintenance of lung IM obeys this rule, with less than one third of the embryonic IM pool replaced by cMo at the age of 8 months in mice (Sabatel et al., 2017; Schyns et al., 2019). Hence, to investigate the dynamics of IM development *in vivo*, we sought to accelerate lung IM differentiation by creating a vacant niche that would presumably be rapidly refilled, as shown for other RTM (Guilliams et al., 2020; Scott et al., 2016). To this end, we aimed to generate a transgenic model of DT-induced lung IM bolus depletion. We uploaded MPS cell population-derived microarray data from the ImmGen database (Heng et al., 2008) and our previous work (Sabatel et al., 2017) into the Gene Expression Commons platform (Seita et al., 2012) and aimed to define lung IM by the combined expression of two genes, as genes exclusively expressed by one RTM (sub)population among all host cells are extremely rare. We found elevated expression of Chemokine (C-X3-C motif) receptor 1 (*Cx3cr1*) in IM, as in many other cells from the MPS (Figures 1A and 1B; Figure S1A and S1B), as well as of Transmembrane protein 119 (*Tmem119*), which was also found to be upregulated in the microglia (Figure 1A), as expected (Bennett et al., 2016). Next, we generated C57BL/6 mutant mice expressing Cre recombinase under the control of endogenous *Tmem119* by inserting a Cre-polyA cassette upstream of the start codon of exon 2 by CRISPR/Cas9-mediated engineering, namely *Tmem119Cre* mice (Figure 1C), and we confirmed elevated expression of Cre recombinase protein in both CD206+ and CD206− IM subsets, but not in any of the other lung myeloid cell populations analyzed (Figure 1D). Next, we crossed *Tmem119Cre* with *Cx3cr1LSL-DTR*mice to create *Tmem119Cre;Cx3cr1LSL-DTR*mice, referred as ‘IM-DTR’ mice hereafter, in which cells expressing both *Cx3cr1* and *Tmem119* should express the diphtheria toxin receptor (DTR) and be sensitive to DT-induced death (Figure 1E).

We treated IM-DTR mice with DT intraperitoneally (i.p.) and assessed the efficiency and specificity of IM depletion within the lung MPS 24 hours later by flow cytometry (Figure 2A). As compared to untreated IM-DTR, DT-treated IM-DTR mice were efficiently depleted in both IM subsets with the dose of 50 ng, while alveolar macrophages (AM) and Ly6C+ cMo were not affected by DT (Figures 2B-F). Of note, lung DC subsets were not sensitive to DT treatment (Figure 2G; Figure S1B) and no significant recruitment of granulocytes was triggered by DT (Figure 2H; Figure S1C), supporting that IM undergoing DT-mediated death were rapidly cleared and did not trigger overt inflammation. Finally, we evaluated the numbers of blood cMo, Ly6C− patrolling monocytes (pMo) and other RTM populations 24 hours post-DT. We found reductions in numbers of intestinal lamina propria macrophages, although it did not reach statistical significance, and no impact of DT was observed on microglia (Figures S1D-K). Hence, both IM subsets are efficiently and specifically depleted by DT in IM-DTR mice.

**Classical monocytes give rise to fully differentiated IM subsets in IM-DTR mice**

Next, to assess whether the empty IM niche is repopulated by newly differentiated IM, we performed time-course studies of lung monocyte and RTM numbers after DT treatment in IM-DTR and littermate controls. As shown in Figures 3A and 3B, IM depletion occurred as early as 12 hours post-DT. Two to three days after DT, IM numbers were still low in IM-DTR mice as compared to controls, and this was associated with a significant increase in numbers of infiltrating cMo (Figure 3B). From day 3 onwards, IM numbers gradually increased to reach levels similar as the ones in littermate controls 7 days post-DT (Figure 3B). The influx of cMo into the lungs of IM-DTR mice was preceded by a significant increase in lung and serum levels of monocyte chemoattractant protein 1 (MCP-1/ CCL2) (Figure 3C), supporting that cMo are attracted to the lung and contribute to IM niche refilling in our model.

To verify this assumption, we performed two sets of experiments. First, we analyzed lethally irradiated, thorax-protected CD45.2+ IM-DTR chimeric mice that were reconstituted with CD45.1+ BM cells (Figure 3D). DT treatment of chimeric IM-DTR mice triggered an IM-intrinsic increase in donor CD45.1 chimerism to levels similar to those observed in monocytes, demonstrating a major contribution of BM cells to IM niche replenishment (Figure 3D). Second, we transferred CD45.1+ Ly6C+ BM monocytes intravenously (i.v.) into IM-DTR mice one day post-DT and followed their fate in the lung, which showed progressive loss of Ly6C expression followed by the subsequent acquisition of CD64 (Figure 3E). Such pattern of differentiation was consistent with the existence of an NR4A1-dependent Ly6C− pMo intermediate (Hanna et al., 2011; Schyns et al., 2019). To test this, we generated BM competitive chimeras in thorax-protected CD45.1/2 IM-DTR mice engrafted with a 1:1 mix of CD45.1+ *Nr4a1+/+* and CD45.2+ *Nr4a1−/−* BM cells, and found that IM replenishment was entirely NR4A1-independent after DT treatment, ruling out the existence of a pMo intermediate stage (Figure 3F).

Finally, we compared native and repopulated IM subsets by bulk RNA-sequencing (RNA-seq) and found that repopulated IM were largely similar to native IM, with only 28 and 30 differentially expressed (DE) genes between native and refilled CD206+ and CD206− IM, respectively (log2 fold-change +/− 1 and adjusted *P* value < 0.05) (Figures S2A-C). Even though *Tmem119* mRNA levels were lower in refilled IM subsets as compared to native IM, refilled IM could still be efficiently re-depleted by DT (Figure S2D). Thus, similar to the steady-state situation, cMo give rise to fully differentiated IM subsets after DT treatment in IM-DTR mice, albeit in an accelerated way.

**IM development from monocyte is captured in real-time by single cell RNA-sequencing**

We next sought to capture the full pattern of monocyte-to-IM development at the single cell (sc) resolution. Lung monocytes and IM were isolated from 5 IM-DTR mice per time point and were subjected to sc droplet encapsulation with the 10X Genomics platform (Zheng et al., 2017), scRNA-seq and quality control filtering (Figure 4A). A total of 15,941 myeloid cells were analyzed and projected to global and time-specific Uniform Manifold Approximation and Projection (UMAP) plots (Figures 4B and 4C). Seven distinct cell clusters were identified, whose frequencies per time point are shown in Figure 4D. Dimensional reductions, gene expression, DE and Gene Ontology (GO) analyses can be explored online using this platform: <https://gigaimmunophysiology.shinyapps.io/IM-DTR_v2/>.

Based on a DE analysis, we identified clusters corresponding to Ly6C+ cMo (*Ccr2, Ly6c2*), Ly6C− pMo (*Ace, Nr4a1*), CD206− IM (*H2-Ab1, Cd74*) and CD206+ IM (*Lyve1, Mrc1*) (Figures 4E and 4F). Cluster 5 upregulated apoptosis-related genes (*Bax, Trp53, Tnf*), was almost uniquely present 12 hours post-DT and disappeared afterwards, likely representing DT-targeted native IM undergoing cell death, while Cluster 7 encompassed few contaminating *Zbtb46+* and *Ccr7*+ DCs (Figures 4E and 4F). Most interestingly, Cluster 6 encompassed cells that were predominantly present between 24 and 96 hours post-DT and made a tiny bridge between Ly6C+ cMo and a branching point leading to both IM subsets, which we named ‘transit’ cells (Figure 4B and 4C). RNA velocity analysis further confirmed that the fate of transit cells moved from cMo towards IM subsets (Figures 4G). Of note, no link was observed between Ly6C− pMo and IM, confirming the absence of a pMo intermediate (Figure 4G).

**Classical monocytes proliferate locally in a CSF1R-dependent way before differentiating into macrophages**

Next, we applied Monocle sc trajectory analysis (Trapnell et al., 2014) to the scRNA-seq data encompassing cMo, transit cells and both IM subsets and identified two main trajectories, both starting from Ly6C+ cMo, moving across transit cells until a branching point, and then bifurcating towards either CD206− or CD206+ IM subsets, in line with our real-time analysis (Figure 5A). DE genes that were similarly regulated in both IM subsets trajectories were analyzed along pseudotime using tradeSeq (Van den Berge et al., 2020) and encompassed 3 main classes of genes (Figure 5B). First, cMo downregulated expression of genes enriched in cellular extravasation, leukocyte migration and chemotaxis, in line with their tissue recruitment (Figure 5B; Figure S3A)*.* Second, cMo underwent an unexpected time-restricted transient upregulation of genes associated with cell proliferation, such as *Ube2c, Aurkb, Racgap1, Cdk1, Ccnb2* and *Mki67* (Figures 5B and 5C). Such proliferative profile was corresponding to transit cells, as attested by their elevated G2/M cell cycle score, indicative of DNA replication, growth and mitosis, respectively (Figure 5D). Such state was then followed by increased expression of genes enriched in cell adhesion, supporting cell engraftment into their niche, as well as in known IM functional features (Figure 5B; Figure S3B) (Chakarov et al., 2019; Schyns et al., 2019). So far, these data are consistent with the hypothesis that cMo, once in a vacant niche, can re-enter cell cycle and expand before differentiating into IM subsets.

To formally test this possibility, we generated thorax-protected, lethally-irradiated CD45.2 IM-DTR chimeric mice engrafted with CD45.1+ donor BM cells. We created a vacant IM niche by DT treatment and injected EdU i.p. 16 hours before the analysis of lung myeloid cells performed 0, 2 and 5 days post-DT (Figure 5E). Notably, we found an incorporation of EdU that was significantly increased donor CD45.1+ cells repopulating the IM gate at day 2 post-DT as compared to day 0, i.e. at a time where transit cells are present in our time-course scRNA-seq analyses (Figure 5E). Importantly, such EdU signal was substantially higher in donor cells as compared to host cells at day 2, and was significantly decreased in donor CD45.1+ IM at day 5 as compared to day 2 (Figure 5E). Altogether, these data support that extravasating cMo proliferate rapidly after extravasation, and that such proliferation precedes the differentiation into macrophages.

Given the crucial role of CSF1 receptor (CSF1-R) signalling in the homeostasis of the MPS, including in the regulation of cell proliferation (Guilliams et al., 2020; Hume et al., 2019; Jenkins and Hume, 2014; Tushinski et al., 1982), we assessed its contribution to the proliferation of repopulating IM in DT-injected, EdU-pulsed IM-DTR mice by treating them with pexidartinib (PLX3397, i.e., a CSF1-R inhibitor). Interestingly, we found that IM-specific EdU incorporation was almost completely abrogated 3 days post-DT in PLX3397-treated mice as compared to vehicle-treated counterparts (Figure 5F), demonstrating that the proliferation was dependent on CSF1-R.

**MafB restricts proliferation and mediates IM development *in vivo***

Next, we sought to gain insights into the transcriptional control of the balance between transient monocyte expansion and macrophage differentiation. To this end, we applied the SCENIC algorithm (Aibar et al., 2017) to our scRNA-seq data to map gene regulatory networks and predict TF activities in single cells (Figure 6A). Strikingly, MafB was one of the TFs whose activity score was very high in IM, especially in CD206+ IM, as compared to cMo (Figure 6A). This observation was particularly interesting to us, since MafB has been shown to restrict CSF1-dependent proliferation of myeloid progenitor cells *in vivo* (Sarrazin et al., 2009), as well as the self-renewal ability of macrophages *in vitro* (Aziz et al., 2009). Of note, TradeSeq analysis of *Mafb* expression showed that the transient upregulation of the cycling gene *Mki67* was followed by an increase in *Mafb* expression in both trajectories of CD206+ and CD206− IM subset development (Figure 6B), suggesting that the activation of MafB might restrict proliferation and facilitate IM development from monocytes in our model. Of note, intracellular staining for MafB protein in lung myeloid cells revealed an elevated expression of MafB in lung IM subsets as compared to cMo, pMo, AM and DCs (Figure 6C). Thus, we generated C57BL/6 *Mafb* floxed mice (*Mafbfl/fl*) and crossed them with mice expressing Cre recombinase constitutively under the control of the lysozyme M promoter (*Lyz2Cre*) or the Ms4a3 promoter (*Ms4a3Cre*) to generate mice with myeloid-restricted *Mafb* deficiency. To assess whether MafB was mediating IM development from cMo *in vivo*, we generated BM competitive chimeras with thorax-protected CD45.1/2 IM-DTR mice engrafted with a 1:1 mix of BM cells from CD45.1+ wild-type and CD45.2+ *Ms4a3Cre*;*Mafbfl/fl* mice. We evaluated the chimerism of lung myeloid cells 7 days after DT treatment and found that myeloid-restricted *Mafb* deficiency strongly impaired the ability of cMo to repopulate the niches of both IM subsets (Figure 6D). Next, we analyzed lung IM from *Lyz2Cre*;*Mafbfl/fl* and littermate control mice for MafB expression, abundance and proliferative potential. We found that MafB protein was absent in IM from *Lyz2Cre*;*Mafbfl/fl* mice (Figure 6E), and that IM were almost completely absent in *Lyz2Cre*;*Mafbfl/fl* mice, while numbers of cMo and pMo were not affected (Figures 6F and 6G). Interestingly, Ki67 staining revealed an increased proliferative ability of the few IM present in *Lyz2Cre*;*Mafbfl/fl* mice as compared to controls (Figure 6H). Altogether, these data are concordant with the hypothesis that MafB regulates IM development before their differentiation into one or the other subsets and can restrict the proliferative potential of IM precursors in the tissue.

**MafB and c-Maf differentially control lung IM identity**

Finally, we explored to what extent the identity of the remaining IM was impacted by MafB deficiency in *Lyz2Cre*;*Mafbfl/fl* mice. In parallel, we sought to investigate the contribution of myeloid-restricted c-Maf to IM maintenance and identity. Indeed, MafB and c-Maf are b-ZIP TFs belonging to the same family of large Maf proteins (Hamada et al., 2020) and can cooperate together in some contexts, such as the regulation macrophage self-renewal (Aziz et al., 2009; Molawi and Sieweke, 2013). Moreover, we found that Maf activity (Figure 6A), as well as mRNA and protein expression, were higher in IM, especially in CD206+ IM, as compared to cMo (Figures S4A-C). However, as opposed to the situation seen in *Lyz2Cre*;*Mafbfl/fl* mice, we found that IM numbers were not affected by c-Maf deficiency (Figures S4D and S4E).

Hence, we performed scRNA-seq analysis of lung Mo and IM in *Lyz2Cre*;*Mafbfl/fl*, *Lyz2Cre*;*Maffl/fl* and control mice. As compared to the lungs of control mice encompassing cMo, pMo and both IM subsets, lungs of *Lyz2Cre*;*Mafbfl/fl* mice were virtually devoid of lung IM, whereas a transcriptionally distinct cluster of cells appeared instead (Figures 7A-C), supporting that the few “IM” present in *Lyz2Cre*;*Mafbfl/fl* mice have a completely different transcriptional profile. Of note, we found 216 DE genes (log2 fold-change +/− 0.5 and adjusted *P* value < 5.10−2) between IM from control and those from *Lyz2Cre*;*Mafbfl/fl* mice (Figures 7D). Strikingly, expression of prototypical RTM- and IM-identity genes (*Mrc1, Adgre1, Pf4, Tmem176a, Tmem176b, Tmem119, Apoe, C1q, Mafb, Cd63*) (Schyns et al., 2019) was severely affected by myeloid-restricted *Mafb* deficiency (Figure 7E). Impaired expression of the prototypical RTM markers CD64 and MertK was also observed at the protein level in IM from *Lyz2Cre*;*Mafbfl/fl* mice as compared to littermate control mice (Figure 7F). In addition, the profile of myeloid cells appearing in MafB-deficient mice was enriched in similar biological responses as those found in the beginning of the cMo-to-IM trajectory (Figure 7G, compare with “Class 1” in Figure 5B). The cMo signature was also significantly higher in MafB-deficient cells as compared to controls, while the IM signature was lower (Figure 7H). These data support a severe impairment of IM development and identity in the absence of MafB.

Based on scRNA-seq data, differences between lung myeloid cells from control and *Lyz2Cre*;*Maffl/fl* mice were, however, more subtle, as we only found a few DE genes between control and c-Maf-deficient IM (Figures S4F-H). *Folr2* was among the significantly downregulated genes in c-Maf-deficient IM (Figure S4H), suggesting that the identity of the CD206+ IM subset was regulated by c-Maf (Schyns et al., 2019). While bulk IM numbers (Figure S4E) and MerTK expression (Figure S4I) were not affected by c-Maf deficiency, we found decreased levels of CD206 expression within IM (Figure S4I), concordant with the scRNA-seq findings and a recent report showing c-Maf dependency for CD206+ perivascular RTM (Moura Silva et al., 2021). Thus, while Mafb is a master regulator of IM development and identity, the role of c-Maf seems to be less important and restricted to one subset of IM, i.e., CD206+ IM.

**Discussion**

Here, we showed that mature classical monocytes have the ability to proliferate locally in vacant RTM niches before undergoing differentiation into distinct RTM subsets, thus shedding new light on monocyte stemness and the complex regulation of myeloid cell proliferation and differentiation in peripheral tissues. A novel model of lung RTM niche depletion and refill was developed, which allowed us to uncover a transient CSF1R-dependent proliferation of extravasating Ly6C+ cMo that would arguably be difficult to capture in a steady-state setting. We provided evidence that the TF MafB was an important regulator of this process, as MafB-deficient cells exhibited an increased proliferation potential and a competitive disadvantage in repopulating a vacant tissue niche. Moreover, we found that the development and identity of both IM subsets were severely impaired in myeloid-restricted MafB-deficient mice, while myeloid-restricted c-Maf deficiency only triggered discrete changes that were specific to the CD206+ IM subset.

The IM-DTR model was devised based on a novel intersectional strategy defining lung RTM by the combined expression of *Cx3cr1* and *Tmem119,* a marker that was previously considered to be microglia-specific (Bennett et al., 2016). Of note, our study emphasizes some underappreciated similarities between lung IM and the microglia, despite obvious differences in terms of origins and differential requirements for the local trophic factors IL-34 and CSF1 for their maintenance (Chakarov et al., 2019; Ginhoux et al., 2010; Greter et al., 2012). First, both IM subsets and the microglia are Tmem119+ CX3CR1hi RTM populations, even though microglia numbers were not affected under the experimental conditions tested here. Second, we found that both IM subsets required MafB for their terminal differentiation and maintenance, which has also been shown to be the case for microglia (Matcovitch-Natan et al., 2016). Third, both IM and the microglia exert important tissue-supportive homeostatic functions, respectively in the lung (Bedoret et al., 2009; Chakarov et al., 2019; Sabatel et al., 2017; Schyns et al., 2018) and in the brain (Borst et al., 2021; Matcovitch-Natan et al., 2016). Whether these observations reflect similarities in their niche, in niche-imprinting signals or in their interactions with nerves remains an open and interesting question for future research.

The current dogma proposes that, in the MPS, self-renewal is limited to myeloid progenitor cells and, under some circumstances, to mature RTM. As a corollary, RTM maintenance is thought to be achieved either via the self-renewal of fully differentiated RTM, or via the recruitment and engraftment of monocytes that differentiate into RTM in a tissue-specific manner (Blériot et al., 2020; Guilliams et al., 2020; Hashimoto et al., 2013; Hume et al., 2019; Jenkins and Hume, 2014; Lavin et al., 2015; Molawi and Sieweke, 2013; Yona et al., 2013). Our results challenge this notion and show that mature cMo can also self-renew in vacant tissue niches to contribute to RTM development *in vivo*. Upon DT-triggered lung IM niche depletion in IM-DTR mice, we showed that local expansion of cMo was transient, restricted to few extravasating Ly6C+ cMo and depended on CSF1R-dependent signaling pathways. The data are consistent with the idea that a limited number of blood monocytes can give rise to a larger number of RTM in tissues through a sequence of events involving first a proliferation in response to local CSF1R ligands, followed by the activation of common and subset-specific RTM signatures. While the relative contribution of the CSF1R ligands CSF-1 and IL-34 to cMo proliferation remains to be determined, reports supporting that IM maintenance requires CSF-1 rather than IL-34 would be consistent with a preferential contribution of the CSF1/CSF1-R axis to cMo self-renewal (Greter et al., 2012; Ural et al., 2020; Wang et al., 2012). In this context, the amount of locally available CSF1 could be a major regulator cMo self-renewal (Guilliams et al., 2020). Local CSF1 levels can be influenced by multiple factors, including the secretion rate by structural cells, which can be increased in response to inflammation and RTM death-related factors, the extent of depletion of CSF1-consuming RTM within the niche, and the subsequent consumption of CSF1 by recruited and expanded cMo (Bonnardel et al., 2019; Guilliams et al., 2020; Tushinski et al., 1982).

In our model, local expansion of cMo occurred before the branching towards one or the other IM subset. Whether proliferating monocytes are already primed and committed in the BM to differentiate into one specific RTM subset, as shown for DCs (Schlitzer et al., 2015), or have an equal capacity to give rise to distinct RTM populations, remains to be determined. The ongoing development of methods combining scRNA-seq with a lineage barcode to track clones of cells across time should help address this interesting question (Weinreb et al., 2020).

We found that the TF MafB had its expression and predicted activity increased right after the local expansion of cMo in both trajectories of IM subset development. Interestingly, MafB has been shown to restrict CSF1-dependent proliferation in myeloid progenitor cells (Kelly et al., 2000; Sarrazin et al., 2009), as well as the self-renewal ability of differentiated macrophages (Aziz et al., 2009), linking MafB activity with CSF1 responsiveness and the balance between self-renewal and differentiation. Our data are consistent with the hypothesis that MafB is also required to restrict monocyte proliferation and drive their differentiation in peripheral tissues. Indeed, we showed that myeloid-restricted *Mafb*-deficient BM cells were unable to give rise to any of the two differentiated IM subsets in a competitive chimera setting. In addition, we found a virtual absence of bone fide IM in myeloid-restricted *Mafb*-deficient mice but rather detected few cells falling into the gate of “IM” that seemed to be blocked in an undifferentiated pre-macrophage transcriptional state, and exhibited an increase proliferation potential and a severely impaired RTM identity. Our data are also consistent with other reports showing that MafB can directly regulate expression of *Adgre* (coding for F4/80) (Moriguchi et al., 2006) and genes coding for the complement component C1q complex (Hamada et al., 2020; Tran et al., 2017).

Our results clearly emphasize a differential requirement for MafB and c-Maf in lung RTM *in vivo*, as c-Maf deficiency was uniquely associated with changes that were restricted to the CD206+ IM subset. Of note, a recent report demonstrated that c-Maf regulated perivascular RTM phenotypes across different tissues (Moura Silva et al., 2021). While lung IM were not specifically analyzed in this report, our data complement the findings of Moura Silva and colleagues, as c-Maf-dependent CD206+ IM have previously been shown to be preferentially associated with the vasculature (Chakarov et al., 2019).

In conclusion, the breakthrough discovery that mature monocytes are endowed with the ability to self-renew in vacant niches to give rise to RTM suggests that more attention should be given to this process in clinically relevant conditions associated with dysregulated myeloid responses and the appearance of MafB+ monocyte-derived macrophages populations, such as in the context of lung diseases like Covid-19 (Bost et al., 2020; Silvin et al., 2020; Vega et al., 2020) or pulmonary fibrosis (Aran et al., 2019; Misharin et al., 2017). It also supports the idea that systemic measurements of monocyte responses (performed typically in the blood) might not appropriately reflect the actual immune responses and immunopathology occurring in peripheral tissues. Further understanding the molecular basis underlying CSF1-R-dependent monocyte self-renewal in peripheral organs will be crucial to be able to manipulate such pathways for preventive or therapeutic purposes.

**Author contributions**

T.M. conceived, supervised and secured funding for the project; S.H., Q.B., D.V., W.P. and T.M. designed the experiments; S.H., Q.B. and D.V. did most of the experiments, compiled the data and prepared the figures. W.P., J.S., C.L., L.F. and C.R. were implicated in experiments related to the analysis of myeloid-restricted *Maf* and *Mafb*-deficient mice; Q.B. performed all the bioinformatic analyses with the help of D.V.; D.P. and F.B. generated *Mafbfl/fl* mice; P.M. helped with all *in vivo*-related experiments; F.B. was involved in the generation of IM-DTR mice; T.M. write the manuscript with the help of S.H., Q.B. and D.V.; all authors provided feedback on the manuscript.

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**Declaration of Interest**

The authors have no conflict of interest to declare.

**Main Figure Titles and Legends**

**Figure 1. Intersectional strategy to target lung interstitial RTM (IM) *in vivo*** (A) (top) Heatmap showing gene activity in the indicated myeloid cell populations, inferred from microarray data uploaded on Gene Expression Commons platform; (bottom) Bar graphs showing gene activities of *Cx3cr1* and *Tmem119*. (B) *Cx3cr1* mRNA expression assessed by flow cytometry in lung myeloid cell populations of *Cx3cr1GFP/+* and control *Cx3cr1+/+* mice: (top) Representative flow cytometry histograms and (bottom) normalized MFI. (C) Strategy to generate *Tmem119Cre* transgenic mice by CRISPR-Cas9 targeting. (D) Cre recombinase protein expression assessed by flow cytometry in lung myeloid cell populations of *Tmem119Cre/+* and control *Tmem119+/+* mice: (top) Representative flow cytometry histograms and (bottom) normalized MFI. (E) *Cx3cr1LSL-DTR/+* were crossed with *Tmem119Cre/+* mice to generate ‘IM-DTR’ mice. (B,D) Data show mean +/− SEM and individual values (n=3 mice). See Figures S1A and S1B for gating strategies. Alv, alveolar; BL, blood; BM, bone marrow; cDC1/2, type 1/2 conventional DC; CNS, central nervous system; DC, dendritic cell; LN, lymph node; Lu, Lung; MFI, mean fluorescence intensity; Mo, monocyte; Mac, macrophage; PC, peritoneal cavity; SI, small intestine lamina propria; SLN, skin-draining lymph nodes; SP, spleen red pulp.

**Figure 2. Efficiency and specificity of DT-induced IM depletion in IM-DTR mice.** (A) Experimental outline. (B) Representative UMAP density plot of lung single live CD45+ CD11b+ or CD11c+ mononuclear cells analyzed by flow cytometry. (C) Representative UMAP heatmap plots depicting expression levels of the indicated surface markers in lung myeloid cells, as in B. (D) Representative UMAP plot showing the lung myeloid cell populations analyzed 24 hours after 50 ng DT treatment (+ DT) or no treatment (− DT) in IM-DTR mice, as in B. Insets indicate % of bulk IM within CD45+ CD11b+ or CD11c+ mononuclear cells. (E) Representative contour plot of (top) Ly6C and CD64 expression within lung single live AM-excluded CD45+SSCloCD11b+ cells, or (bottom) F4/80 and CD11c expression within lung single live CD45+ cells from non-treated (− DT) and DT-treated (+ DT) IM-DTR mice. Insets indicated % of the indicated cell populations within the parent gate. (F) Absolute numbers of the indicated lung myeloid cell populations quantified by flow cytometry in IM-DTR mice injected 24 hours before with increasing amounts of DT. Horizontal dotted lines represent the average number of cells in non-injected counterparts. (G-H) Absolute numbers of (G) lung DC subsets and (H) granulocytes quantified by flow cytometry in IM-DTR mice injected 24 hours before with 50 ng DT. (F-H) Data show mean +/− SEM and are pooled from (F) 2-4 independent experiments (n= 6-15/group); (G-H) 2 independent experiments (n=8/group), each symbol representing individual mice. *P* values were calculated using a two-way analysis of variance (ANOVA) with Tukey’s post hoc test. \*, *P*<0.05; \*\*, *P*<0.01; \*\*\*, *P*<0.001; \*\*\*\*, *P*<0.0001; ns, not significant. cDC1/2, type 1/2 conventional DC; Eos, eosinophils; Neu, neutrophils.

**Figure 3. A vacant IM niche is rapidly repopulated by classical monocyte-derived IM.** (A) Representative contour plot of Ly6C and CD64 expression within lung single live AM-excluded CD45+SSCloCD11b+ cells at different time points post-DT injection (50 ng i.p.) in IM-DTR mice. Insets indicate the % of cells within the indicated gates. (B) Absolute numbers of the indicated lung myeloid cell populations quantified by flow cytometry at different time points post-DT injection in IM-DTR and littermate control mice. (C) Lung and serum levels of CCL2 assessed by ELISA at different time points post-DT injection in IM-DTR and littermate control mice. (D) BM contribution to IM niche refilling is evaluated in lethally-irradiated, thorax-protected CD45.2+ IM-DTR chimeric mice transplanted with CD45.1+ BM cells: (top) experimental outline; (bottom left) representative CD45.1 and CD45.2 contour plots within blood leukocytes, lung AM and IM 2 days after DT; (bottom right) Refilling rate as a measure of donor chimerism in the indicated cell populations and time points after DT. (E) BM-derived CD45.1+ cMo can give rise to lung IM when transferred i.v. into DT-treated CD45.1/2+ IM-DTR mice: (top) Experimental outline; (bottom) Representative contour plot of Ly6C and CD64 expression within lung single live AM-excluded CD45.1+SSCloCD11b+ cells at different time points post-DT injection in CD45.1/2+ IM-DTR mice. Arrows estimate the direction of monocyte-to-IM differentiation (F) IM niche refilling is independent of Nr4a1. Bar graph showing % of CD45.1/2+ host, CD45.2+ *Nr4a1-/-* and CD45.1+ *Nr4a1+/+* donor chimerism in the indicated cell populations 7 days post-DT in lethally-irradiated, thorax-protected CD45.1/2+ IM-DTR chimeric mice transplanted with a 1:1 mix of CD45.2+ *Nr4a1-/-* and CD45.1+ *Nr4a1+/+* BM cells 4 weeks before. (B-D, F) Data show mean ± SEM and are pooled from (B, D, F) at least two independent experiments (n=5-10/time point) or (C) two independent experiments (n=4-5/time point). Individual values are also shown in C. (B, C) *P* values were calculated by two-way ANOVA with Tukey’s post test. In (F), *P* values compare donor CD45.1+ *Nr4a1+/+* chimerism between cell populations and were calculated using a one-way ANOVA with Tukey’s post hoc test. \*, *P*<0.05; \*\*, *P*<0.01; \*\*\*, *P*<0.001; \*\*\*\*, *P*<0.0001; ns, not significant.

**Figure 4. Time-course scRNA-seq analyses of IM niche refilling reveal discrete transitioning cells.** (A) Experimental design of scRNA-seq experiments. (B) Three-dimensional UMAP plot depicting the transcriptional identity of lung myeloid cells merged from IM-DTR mice injected with DT 0, 12, 24, 48 and 96 hours before the analysis (n = 5 pooled mice per time point). (C) Three-dimensional UMAP plot from the 5 separate time points post-DT, as in B. (B, C) Inset indicates the number of cells analyzed. (D) Histogram showing frequency of each cluster at each time point post-DT. (E) Heatmap depicting the 20 most upregulated genes within each cluster. (F) Dot plots showing average expression of the indicated genes and percentages of cells expressing the genes within each cluster. (G) Prevalent pattern of RNA velocities substantiated by arrows and visualized on the same UMAP plot as shown in A. Right panel shows a higher magnification of the area depicted by a black line in the left panel.

**Figure 5. cMo can proliferate in a vacant niche via CSF1R-dependent mechanisms.** (A) Two-dimensional UMAP plot depicting the transcriptional identity and cell trajectories evaluated by Monocle analysis from single myeloid cells merged from IM-DTR mice injected with DT 0, 12, 24, 48 and 96 hours before the analysis (n = 3 pooled mice per time point). (B) Heatmap plot depicting the DE genes along pseudotime evaluated by tradeSeq in the common trajectory starting from Ly6C+ cMo and ending in CD206- and CD206+ IM subsets. DE genes are divided into 3 classes, and the main biological responses enriched in each class are represented on the right. (C) Gene expression of the indicated Class 2 genes along pseudotime evaluated by tradeSeq in both trajectories leading either to CD206− or CD206+ IM subsets. (D) (top) UMAP feature plot depicting lung myeloid cells as in A according to their G2/M cell cycle score. (bottom) G2/M cell cycle score of single cells within each cluster, as depicted by violin plots (height: score; width: abundance of cells). (E) Assessment of donor and host cell proliferation during niche refilling in DT-injected IM-DTR mice: (top) Experimental outline; (bottom left) Representative flow cytometry histograms of EdU incorporation in cMo and IM at days 0, 2 and 5 post-DT treatment in IM-DTR chimeric mice. Insets indicate the % of EdU+ cells within host CD45.2+ and donor CD45.1+ cells; (bottom right) Bar graph showing the % of EdU+ cells in host and donor cMo and IM at the indicated time points post-DT in IM-DTR chimeric mice. (F) Assessment of proliferation 3 days post-DT in IM-DTR mice treated or not with a CSF1-R antagonist: (left) Experimental outline; (middle) Representative contour plot of FSC and EdU signal within lung IM. Insets indicate the percentage of EdU+ cells; (right) Bar graph showing the % of EdU+ cells within IM. (E, F) Data show mean +/− SEM and individual values and are pooled from 2 independent experiments (n= 5-8/group). (D, E, F) *P* values were calculated using a (D) one-way or (E) two-way ANOVA with Tukey’s post hoc tests, and (F) with a two-tailed Student’s *t* test. \*\*, *P*<0.01; \*\*\*, *P*<0.001; ns, not significant.

**Figure 6. MafB restricts self-renewal and mediates IM development from monocytes.** (A) Heatmap depicting predicted TF activities across lung myeloid single cells merged from IM-DTR mice injected with DT 0, 12, 24, 48 and 96 hours before the scRNA-seq analysis (n = 5 pooled mice per time point), assessed by SCENIC algorithm. (B) Transcript expression of *Mki67* and *Mafb* along pseudotime evaluated by tradeSeq in both trajectories leading either to CD206− or CD206+ IM subsets. (C) MafB protein expression in lung myeloid cells: (top) Representative flow cytometry histograms of (red) MafB protein and (grey) isotype intracellular staining in the indicated cell populations; (bottom) bar graphs showing normalized MFI, with *P* values comparing bulk IM vs. every other population, or CD206+ IM vs. CD206− IM. (D) Requirement of MafB for IM development from cMo is evaluated in lethally-irradiated, thorax-protected CD45.1/2+ IM-DTR mice transplanted with a 1:1 mix of CD45.1+ wild-type and CD45.2+ *Ms4a3Cre;Mafbfl/fl* BM cells: (top) Experimental outline; (bottom left) Representative CD45.1 and CD45.2 contour plots within lung Ly6C+ cMo and bulk IM 7 days after DT; insets indicate % of cells within the parent gate; (bottom right) Bar graph showing % of CD45.1/2+ host, CD45.1+WT and CD45.2+ *Ms4a3Cre;Mafbfl/fl* donor chimerism in the indicated cell populations. (E) Efficiency of *Mafb* depletion within IM of *Lyz2Cre;Mafbfl/fl* mice evaluated by MafB protein intracellular staining and flow cytometry. Data are representative of one of five mice analyzed, each of them giving similar results. (F) Representative UMAP density plot of lung single live CD45+ CD11b+ or CD11c+ mononuclear cells analyzed by flow cytometry in *Lyz2Cre;Mafbfl/fl* and *Mafbfl/fl* littermate controls. Insets indicate % of bulk IM within CD45+CD11b+ or CD11c+ mononuclear cells. (G) Absolute numbers of lung Ly6C+ cMo, Ly6C- pMo and IM quantified by flow cytometry in *Lyz2Cre;Mafbfl/fl* and *Mafbfl/fl* littermate controls. (H) Proliferative potential of IM in *Lyz2Cre;Mafbfl/fl* and *Mafbfl/fl* littermate controls: (left) Representative contour plots of FSC and Ki67 staining; insets indicate the % of Ki67+ cells within IM; (right) Bar graph showing the % of Ki67+ cells within IM. (C, D, G, H) Data show mean +/− SEM and (C, G, H) individual values and are pooled from 2-3 independent experiments (n=7-9/group). In D, *P* values compare % of donor CD45.1+ WT chimerism. *P* values were calculated using (C, D) a one-way ANOVA with Tukey’s post-hoc test (for bulk IM) or a two-tailed Student’s *t* test (for IM subsets), (G) a two- way ANOVA with Tukey’s post-hoc test and (H) a two-tailed Student’s *t* test. \*\*\*, *P* < 0.001; \*\*\*\*, *P* < 0.0001; ns, not significant.

**Figure 7. IM identity is severely impaired in myeloid-restricted *Mafb*-deficient mice.** (A) UMAP plot depicting the transcriptional identity of lung myeloid cells from *Lyz2Cre;Mafbfl/fl* mice and littermate controls (n = 5 pooled mice per group). (B) UMAP feature plot representing single cell expression of *Mrc1* and *Lyve1* in lung myeloid cells merged from *Lyz2Cre;Mafbfl/fl* mice and littermate controls, identifying the CD206+ IM subset. (C) Histogram showing frequency of each cluster in *Lyz2Cre;Mafbfl/fl* mice and littermate controls. (D) Volcano plot depicting the DE genes between the IM and MafB-KO-enriched clusters. Transcripts significantly upregulated in IM and MafB-KO-enriched cells are colored in green and orange, respectively (log2 fold-change +/− 0.5 and adjusted *P* value < 0.05). (E) Expression levels of the indicated genes within the IM and MafB-KO-enriched clusters, as depicted by violin plots (height: expression; width: abundance of cells). (F) Surface expression levels of CD64 and MerTK in lung AM and IM, quantified by flow cytometry in *Lyz2Cre;Mafbfl/fl* andlittermate controls. (G) GO enrichment analysis performed on the upregulated genes in MafB-KO-enriched cells as compared to the IM cluster. (H) IM- and cMo-signature scores within the cMo, IM and MafB-KO-enriched clusters, as depicted by violin plots (height: scores; width: abundance of cells). (E) Adjusted *P* values obtained from the DE analysis of scRNA-seq data using Seurat are shown. (F) Data show mean +/− SEM and individual values and are pooled from 2 independent experiments (n = 6-7 / group).(F, G, H) *P* values were calculated using (F) a two-way ANOVA with Tukey’s post hoc test, (G) a two-tailed Mann-Whitney U-test with Benjamini-Hochberg false discovery rate (FDR) correction, or (H) a one-way ANOVA with Tukey’s post hoc test. \*\*\*, *P* < 0.001; \*\*\*\*, *P* < 0.0001; ns, not significant. MFI, mean fluorescence intensity.

**Supplemental Figure Titles and Legends**

**Figure S1, related to main Figures 1 and 2. Gating strategies used to delineate Mo, RTM and granulocytes and abundance of Mo and RTM in DT-injected IM-DTR mice.** (A-J) Flow cytometry gating strategy used to delineate (A) lung AM, cMo, pMo, bulk IM, CD206+ and CD206- IM; (B) lung DC subsets; (C) lung eosinophils (Eos) and neutrophils (Neu); (D) blood Ly6C+ cMo and Ly6C− pMo; (E) small peritoneal macrophages (SPM) and large peritoneal macrophages (LPM); (F) spleen red pulp macrophages (RPM); (G) Liver Kupffer cells (KC); (H) small intestinal lamina propria macrophages (SI LPM); (I) colonic lamina propria macrophages (C LPM); (J) brain microglia (MG). (K) Cell numbers of the indicated cell populations quantified by flow cytometry in IM-DTR mice injected with 50 ng DT 24 hours before and compared relatively to those of non-injected IM-DTR mice. Data show mean +/− SEM and are pooled from 2-3 independent experiments, each symbol representing individual mice (n= 8-10/group). *P* values were calculated using a two-way analysis of variance (ANOVA) with Tukey’s post hoc test. ns, not significant. cDC1/2, type 1/2 conventional DC.

**Figure S2, related to main Figure 3. Repopulated IM subsets are largely similar to native IM 14 days post-DT treatment in IM-DTR mice.** (A) Experimental outline for panels B and C. (B) Principal Component (PC) Analysis plot comparing Ly6C+ cMo, AM, CD206+ and CD206− IM from untreated IM-DTR mice, as well as CD206+ and CD206- IM from DT-injected IM-DTR mice 14 days post-DT (n= 3 pooled mice per replicate, 3 replicates per condition). Percentages indicate the variability explained by each PC component. (C) Volcano plots depicting the DE genes between native and repopulated (left) CD206− and (right) CD206+ IM. Transcripts significantly upregulated in native and repopulated IM subsets are colored in blue and red, respectively (log2 fold-change +/− 1 and adjusted *P* value < 10−2). (D) Bar graph showing lung IM numbers assessed by flow cytometry in IM-DTR mice treated or not with DT at days 0 and 14, and analyzed 24 hours after the last DT treatment (day 15). Data show mean +/− SEM and individual mice and are pooled from 2 independent experiments (n= 7-8/group). *P* values were calculated using a one-way ANOVA with Tukey’s post-hoc test. \*\*, *P* < 0.01; ns, not significant.

**Figure S3, related to main Figure 5. Trajectory-based differential expression analysis of cMo developing into IM subsets.** Gene expression of the indicated (A) Class 1 and (B) Class 3 genes along pseudotime evaluated by tradeSeq in both trajectories leading either to CD206− or CD206+ IM subsets in DT-injected IM-DTR mice.

**Figure S4, related to main Figure 7. c-Maf specifically controls the identity of the CD206+ IM subset.** (A) Heatmap plot depicting the DE genes along pseudotime evaluated by tradeSeq in the subset-specific trajectories starting from Ly6C+ cMo and ending in either CD206- or CD206+ IM subsets. (B) Gene expression of the indicated subset-specific genes along pseudotime evaluated by tradeSeq in both trajectories leading either to CD206− or CD206+ IM subsets. (C) c-Maf protein expression in lung myeloid cells: (top) Representative flow cytometry histograms of (red) c-Maf protein and (grey) isotype intracellular staining in the indicated cell populations; (bottom) Bar graph showing normalized MFI, with *P* values comparing bulk IM vs. every other population, or CD206+ IM vs. CD206− IM. (D) Efficiency of *Maf* depletion within IM of *Lyz2Cre;Maffl/fl* mice evaluated by c-Maf protein intracellular staining and flow cytometry. Data are representative of one of five mice analyzed, each of them giving similar results. (E) Absolute numbers of lung Ly6C+ cMo, Ly6C− pMo and IM quantified by flow cytometry in *Lyz2Cre;Maffl/fl* and *Maffl/fl* littermate controls. (F) UMAP plot depicting the transcriptional identity of lung myeloid cells from *Lyz2Cre;Maffl/fl* mice and littermate controls (n = 5 pooled mice per group). (G) Histogram showing frequency of each cluster in *Lyz2Cre;Maffl/fl* mice and littermate controls. (H) Volcano plot depicting the DE genes between IM from *Lyz2Cre;Maffl/fl* mice and littermate controls. Transcripts significantly upregulated in IM from control and *Lyz2Cre;Maffl/fl* mice are colored in red and blue, respectively (log2 fold-change +/− 0.5 and adjusted *P* value < 0.05). (I) Surface expression levels of MerTK and CD206 in lung AM and IM, quantified by flow cytometry in *Lyz2Cre;Maffl/fl* andlittermate controls. (C, E, I) Data show mean +/− SEM and individual values and are pooled from (C) 3 independent experiments (n=9/group) or (E, I) 2 independent experiments (n=4-5/group). *P* values were calculated using (C) a one-way ANOVA with Tukey’s post-hoc test (for bulk IM) or a two-tailed Student’s *t* test (for IM subsets), or (E, I) a two- way ANOVA with Tukey’s post-hoc test. \*, *P* < 0.05; \*\*\*\*, *P* < 0.0001; ns, not significant.

**STAR Methods text**

**RESOURCE AVAILABILITY**

**Lead contact**

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](mailto:t.marichal@uliege.be)).

**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 codes have been deposited at GitHub and are available for reviewers with the following credentials: link: <https://github.com/BlanQwall/Lung_IM_differentiation>, ID: FictiveReviewer; password: ImmunoPhysiology. DOIs are listed in the key resources table [Qiang, do we have DOIs at this point?].

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODELS AND SUBJECT DETAILS**

**Mice**

The following mice of the C57BL/6 background were used in this study: CD45.2 C57BL/6 wild-type (WT) (The Jackson Laboratory), CD45.1 C57BL/6J WT (Shen et al., 1985) (The Jackson Laboratory, Strain #002014), *Cx3cr1Gfp/+* (Jung et al., 2000) (The Jackson Laboratory, Strain #005582), *Tmem119Cre* (see below), *Cx3cr1LSL-DTR/+* (Diehl et al., 2013) (The Jackson Laboratory, Strain #025629), *Nr4a1-/-* (Lee et al., 1995) (The Jackson Laboratory, Strain #006187), *Maffl/fl* (Wende et al., 2012) (kindly provided by Dr. Fabienne Andris), *Mafbfl/fl* (generated by Dimitri Pirottin and the GIGA Mouse facility and Transgenics Platform, Liège University, Belgium), *Lyz2Cre* (Clausen et al., 1999) (The Jackson Laboratory, Strain #004781) and *Ms4a3Cre* (Liu et al., 2019) (kindly provided by Dr. Florent Ginhoux). Myeloid-restriced *Maf* or *Mafb* depletion was achieved by crossing *Maffl/fl* or *Mafbfl/fl* mice with *Lyz2Cre* or *Ms4a3Cre* mice.

C57BL/6 *Tmem119Cre* knock-in mice 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 back-crossed to C57BL/6J WT mice for at least for four generations. *Tmem119Cre* mice were genotyped by PCR using the following primers: PCR Primers 1 fur mutant allele (Annealing Temperature 60.0 ºC): Forward primer: 5’- TCCGTAACCTGGATAGTGAAACAG -3’, Reverse primer: 5’- ATATGTCCTTCCGAGTGAGAGAC -3’, Product size: 270 bp (Mutant). PCR Primers 2 for WT allele (Annealing Temperature 60.0 ºC): Forward primer: 5’- ACCGAGGACAGAAATGAATAAGATG -3’; Reverse primer: 5’- AGGGAACGAGGATGGGTAGTAG -3’; Product size: 643 bp (WT).

C57BL/6 *Mafbflox* mice were generated using recombinerring technologies, Briefly, genomic segment covering *Mafb* single exon was retrieved to PL253 vector using BAC recombineering. The loxP-EM7-Neo-loxP cassette was cloned by PCR from PL452 plasmid and ligated to the 5’ of Mafb segment (PL253/Mafb/Neo 5’) and then the cassette was “pop out” by electroporating to SW106 cells expressing Cre and 5’ loxP left in the construct. The FRT-Neo-FRT-loxP cassette was cloned from PL451 plasmid and ligated to the 3’ of Mafb segment. The purified plasmid was electroporated into mouse ES cells and the cells were selected under G418 treatment for 1 week. The bona fide clones with successful homologous recombination were screened by Southern blot. Successfully recombined clones were injected into blastocysts to make *Mafbflox-Neo* mice. These mice were crossed to a *FLP*-expressing line to remove the Pgk-Neo cassette and generate *Mafbflox* mice.

*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 homozygous CD45.1 WT mice.

A mix of male and female mice between 6 and 10 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.

**METHOD DETAILS**

***In vivo* treatments with chemicals**

For DT-induced depletion of IM, IM-DTR mice were injected intraperitoneally (i.p.) with a single dose of 50 ng of DT (List Biological Labs, Cat#150), unless otherwise stated. Control mice were either IM-DTR mice injected with PBS, or *Tmem119Cre/+* littermate control mice injected with DT. For EdU incorporation experiments, IM-DTR mice were injected i.p. with 1mg of EdU (Santa Cruz Biotechnology, Cat#sc-284628) in 200 µL PBS 16 h before sacrifice. For experiments with CSF1-R inhibitors, 100 mg/kg of pexidartinib (PLX3397) (MedChemExpress, Cat#HY-16749) was given by i.p. injection 24 and 48 h post-DT injection.

**Generation of BM (competitive) chimeras**

Eighteen-week-old CD45.2 or CD45.1/2 IM-DTR mice were anesthetized by i.p. injection of 200 µL of PBS containing Ketamine (75 mg/kg, Dechra, Cat#804132) and Xylazine (10 mg/kg, Bayer, Cat#0076901). The thoracic cavity was 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 anesthesia, mice were reconstituted by intravenous (i.v.) administration of 107 BM cells from congenic CD45.1 WT mice. For mixed BM chimeras, mice were injected i.v. with 107 BM cells consisting of a 1:1 mix of 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.

The refiling rate of a cell type in bone-marrow chimera mice shown in Figure 3D was defined as chimerism (CD45.1+ frequency) of the cell type normalized to chimerism of blood monocytes (CD11b+ SSClo CD115+) in the same sample.

**Adoptive transfer of BM monocytes**

BM monocytes were isolated from congenic CD45.1 WT mice using the Monocyte Isolation Kit (Miltenyi Biotec, Cat#130-100-629). 2.106 BM monocytes were administered i.v. into CD45.1/2 IM-DTR mice that were injected i.p. with 50 ng DT 24 h before monocyte transfer to deplete endogenous IM.

**Blood and tissue leukocyte isolation**

Blood was collected by retro-orbital plexus bleeding of terminally-anesthetized mice. Mice were then euthanized by cervical dislocation. Peritoneal lavage was obtained by injecting 10 mL HBSS (Lonza, Cat#BE10-508F) into the peritoneal cavity and collecting the washout. Mice were then perfused with 10 mL 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 (ThermoFisher, Cat#10270098), 1 mg/mL collagenase A (Sigma, Cat#14190094) and 0.05 mg/mL DNase I (Sigma, Cat#11284932001). After 45 min of digestion, the suspension was flushed using a 18 G needle to dissociate aggregates. Ice-cold PBS (ThermoFisher, Cat#) containing 10 mM of EDTA (Merck Millipore, Cat#1084181000) was added to stop the digestion process and cell suspensions were filtered using a cell strainer (70 µM, Corning, Cat#352350). Mononuclear leukocytes from lungs, brain and liver were enriched using a Percoll density gradient (GE Healthcare, Cat#17089101) and harvesting cells from the 1.080:1.038 g/mL interface.

For the isolation of leukocytes from the small intestines and colons, small intestines and colons were dissected from the pylorus and the rectum, were separated from the mesenteric tissue from Peyer’s patches and from fat and were placed in ice-cold HBSS with 2% FBS. Intestinal content was removed with PBS, and the small intestines and colons were opened by a longitudinal cut and washed 3 times in ice-cold HBSS with 2% FBS. To remove mucus and epithelial cells, small intestines and colons were incubated with HBSS with 2% FBS and 1 mM 1,4 dithiothreitol (DTT, Sigma, 10197777001) for 20 min with constant shaking followed by an incubation with HBSS containing 2% FBS and 1.3mM EDTA for 40 min. Tissue pieces were then cut into small pieces and incubated for 1 h at 37 ˚C with RPMI containing 2% FBS, 2 mg/mL collagenase IV (ThermoFisher, Cat#17104019) and 40 U/mL DNase I. At the end of incubation, the suspension was homogenized with a 19G syringe and filtered through a 70 µM strainer.

**Flow cytometry**

Cells (0.5–5 x 106) were pre-incubated with Mouse BD Fc Block™ (BD biosciences, Cat#553142) to avoid unspecific binding to Fc receptors and stained with appropriate antibodies at 4°C in the dark for 30 min. For Ki-67 stainings, extracellular-stained cells were permeabilized and stained using FITC Mouse Anti-Ki-67 Set (BD Biosciences, Cat#556026). For EdU staining, extracellular-stained cells were permeabilized and stained using Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit (Thermo Fisher, Cat#10632), according to manufacturer instructions. Cell viability was assessed using LIVE/DEAD Fixable Near-IR (775) stain (Thermo Fisher, Cat#L34976) and the cell suspensions were analyzed with a LSRFortessa (BD Biosciences). Results were analyzed using FlowJo software (Tree Star Inc.). For scRNA-seq and bulk RNA-seq, lung myeloid cells were sorted using an FACSAriaIII (BD Biosciences). The full list of antibodies used can be found in the Key Resources Table.

**MCP-1/CCL2 quantification**

IM-DTR and littermate control mice were sacrificed at indicated time points after DT administration. Blood was collected and lungs were perfused through the right ventricle with 10 mL PBS and isolated. Blood samples were left undisturbed for 30–45 min at room temperature (RT) to allow clot formation. The serum was separated from the blood clot by centrifugation for 10 min at 2000 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, Cat#11697498001)) using a tissue homogenizer (IKA) with the addition of 1% NP-40 (Sigma, Cat#74385) 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.

**Bulk RNA-seq: sample preparation and analysis**

Native IM subsets, Ly6C+ cMo and AM were isolated from uninjected IM-DTR mice, while repopulated IM subsets were isolated from IM-DTR mice that had been treated i.p. with 50 ng DT 14 days earlier. Cell populations were FACS-sorted using the gating strategy shown in Figure S2A into TRIzol reagent (ThermoFisher, Cat#10296010). Total RNA was extracted with the 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, Cat#R11490). One hundred nano-grams of RNA was used to generate the libraries using the Truseq Stranded mRNA kit (Illumina, Cat#20020594). 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).

**scRNA-seq**

To compare lung monocytes and IM from untreated IM-DTR mice (group “No treatment”) with those from IM-DTR mice treated with 50 ng DT i.p. 96 h before (group “DT96h”), 5 mice from each group was sacrificed and lung single-cell-suspensions were obtained after enzymatic digestion. CD11b+ cells were enriched by MACS using CD11b MicroBeads (Miltenyi Biotec, 130-049-601). Lung monocytes and IM were then FACS-sorted and the 10x Genomics platform (Single Cell 3’ Solution) was used for scRNA-seq. Lung monocytes and IM were FACS-sorted separately and IM were then enriched in the final single cell suspension to reach a monocyte/IM ratio of 5:5. For each sample, an aliquot of Trypan blue-treated cells was examined under the microscope for counting, viability and aggregate assessment following FACS sorting. Viability was above 90% for all samples and no aggregate were observed. Cell preparations were centrifuged at 1503 RCF for 4 min and pellets were resuspended in calcium- and magnesium-free PBS containing 0.4mg ml−1 of UltraPure BSA (Thermo Fisher Scientific, Cat#AM2616).

To analyze lung monocytes and IM from IM-DTR mice treated 12 (group “DT12h”), 24 (group “DT24”) and 48 h (group “DT48h”) before with 50 ng DT i.p., from from *Lyz2Cre;Mafbfl/f* (group “Mafb-KO”) , *Lyz2Cre;Maffl/fl* (group “cMAF-KO”), *Lyz2Cre;Mafbfl/fl;Maffl/fl* (group “dKO”) and mice littermate mice without *Lyz2Cre* (group “Control”), the same protocol was applied, but cells from each group were barcoded with different anti-mouse Hashtag antibodies (Biolegend) before being pooled for encapsulation and library construction. In order to have higher resolution in analyzing lung myeloid cells in myeloid-restricted Mafb and c-Maf-deficient mice, the pooled Mafb-KO/cMAF-KO/dKO/Control sample were composed with monocyte/IM ratio of 3:7 instead of 5:5 for other samples.

For library preparation, approximately 3,000 cells per sample (for “DT96h” and “No treatment”), or 20,000 cells for pooled hashtag-labeled samples were loaded into the Chromium Controller, in which they were partitioned, their polyA RNAs captured and barcoded using Chromium Single Cell 3’ GEM, Library & Gel Bead Kit v3 (10X Genomics).The cDNAs were amplified and libraries compatible with Illumina sequencers were generated using Chromium Single Cell 3’ GEM, Library & Gel Bead Kit v3 (10X Genomics). For Hash Tag Oligonucleotide (HTO) library, 1 ul HTO Additive primer v2 (0.2 uM stock) were added to the mix at the cDNA amplification step. The libraries were sequenced on an Illumina NovaSeq sequencer on an SP100 cell flow (Read1: 28 cy, read2: 76 cy, index1: 10cy, index2: 10cy) 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 hashtag-labeled samples 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 merging 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 function and the differentially expressed genes were calculated using FindAllMarkers function (Seurat package).

**Single-cell RNA velocity estimation**

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.

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

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. GO 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 regulatory network inference and clustering (SCENIC) analysis**

To predict the potential active transcription factors (TF), Ly6C+ cMo, transit cells, CD206- and CD206+ IM 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 for the 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 represent the SCENIC results, the results of 3.4\_regulonsAUC were added to the metadata of Seurat object so 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 during IM development**

To evaluate trajectory-based DE analysis during IM development in IM-DTR mice, Ly6C+ cMo, transit cells, CD206- and CD206+ IM were subjected to Monocle (Trapnell et al., 2014) analysis. The Monocle CDS object was built with counts and metadata from Seurat object and converted using SeuratWrappers package. Cells were clustered with cluster\_cells function using calculated UMAP coordination and resolution of 0.51E-3. The trajectories along pseudotime were built 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 subjected 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., 2020). The association of average expression of each gene with pseudotime was tested using associationTest and the DE genes between CD206+ and CD206- IM trajectories were calculated with diffEndTest function. The value of the estimated smoother on a grid of pseudotimes was estimated for each of DE gene using predictSmooth. The DE genes with waldStat > 70 and |logFC| > 2 were annotated as “changed genes”, meaning that their expression patterns were different in CD206+ and CD206- IM trajectories, while the rest of DE genes were considered as “unchanged genes”, meaning that the expression patterns were similar in both trajectories. Finally, the scaled estimated smoothers calculated by predictSmooth were used to build heatmap with ComplexHeatmap package (Gu et al., 2016).

**IM and monocyte 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 in the IM and Mafb KO-enriched clusters with VISION package (DeTomaso et al., 2019). The scores were stored in Seurat object and plotted with Seurat package.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analyses were performed with Prism 9 (GraphPad software) and with R Bioconductor (3.5.1) (Huber et al., 2015) and DESeq 2 (Love et al., 2014) or Seurat (3.2.1.) (Stuart et al., 2019) for bulk and sc-RNA-seq data, respectively. Data from independent experiments were pooled for analysis in each data panel unless otherwise indicated. The statistical analyses performed for each experiment are indicated in the respective figure legends. The investigators were blinded during experiments and outcome assessment. We considered a *P* value lower than 0.05 to be significant (\*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001; \*\*\*\*, *P* < 0.0001; ns, not significant).

**ADDITIONAL RESOURCES**

For scRNA-seq data related to IM niche refilling in IM-DTR mice, dimensional reductions, gene expression, DE and Gene Ontology (GO) analyses can be explored online using this platform: <https://gigaimmunophysiology.shinyapps.io/IM-DTR_v2/>.

For scRNA-seq data related to the analysis of lung myeloid cells in myeloid-restricted Mafb and c-Maf-deficient mice, dimensional reductions, gene expression, DE and Gene Ontology (GO) analyses can be explored online using this platform: https://gigaimmunophysiology.shinyapps.io/IM-DTR\_Maf/ .

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