RBP-J regulates homeostasis and function of circulating Ly6C^{lo} monocytes

Tiantian Kou[1,2,3,6], Lan Kang[1,3,6], Bin Zhang[1,3], Jiaqi Li[1], Baohong Zhao[4,5], Wenwen Zeng[1,2,3] and Xiaoyu Hu[1,2,3,7,*]

Affiliations:

[1]Institute for Immunology and School of Medicine, Tsinghua University, Beijing, China.

[2]Tsinghua-Peking Center for Life Sciences, Tsinghua University, Beijing, China.

[3]Beijing Key Laboratory for Immunological Research on Chronic Diseases, Beijing, China.

[4]Arthritis and Tissue Degeneration Program and the David Z. Rosensweig Genomics Research Center, Hospital for Special Surgery, New York, NY.

[5]Department of Medicine, Weill Cornell Medicine, New York, NY.

[6]These authors contributed equally.

[7]Lead Contact

[*]Correspondence: xiaoyuhu@tsinghua.edu.cn.

Summary

Notch-RBP-J signaling plays an essential role in maintenance of myeloid homeostasis. However, its role in monocyte cell fate decisions is not fully understood. Here we showed that conditional deletion of transcription factor RBP-J in myeloid cells resulted in marked accumulation of blood Ly6C^{lo} monocytes that highly expressed chemokine receptor CCR2. Bone marrow transplantation and parabiosis experiments revealed a cell intrinsic requirement of RBP-J for controlling blood Ly6C^{lo}CCR2^{hi} monocytes. RBP-J-deficient Ly6C^{lo} monocytes exhibited enhanced capacity competing with wildtype counterparts in blood circulation. In accordance with alterations of circulating monocytes, RBP-J deficiency led to markedly increased population of lung tissues with Ly6C^{lo} monocytes and CD16.2^{+} interstitial macrophages. Furthermore, RBP-J deficiency-associated phenotypes could be genetically corrected by further deleting *Ccr2* in myeloid cells. These results demonstrate that RBP-J functions as a crucial regulator of blood Ly6C^{lo} monocytes and thus derived lung-resident myeloid populations, at least in part through regulation of CCR2.

Key words:

RBP-J; Ly6C^{lo} monocyte; CCR2; Lung interstitial macrophage

45 **Introduction**

Monocytes are integral components of the mononuclear phagocyte system (MPS) that develop from monocyte precursors in the bone marrow[1]. Several functionally and phenotypically distinct subsets of blood monocytes have been defined on the basis of expression of surface markers[2-5]. In mice, Ly6C^{hi} monocytes (also called inflammatory
50 or classical monocytes) characterized by Ly6C^{hi}CCR2^{hi}CX3CR1^{lo} exhibit a short half-life, and are recruited to tissue and differentiate into macrophages and dendritic cells[6]. Ly6C^{hi} monocytes are analogous to CD14^{+} human monocytes based on gene expression profiling[7]. A second subtype of monocytes is called Ly6C^{lo} monocytes (also termed patrolling monocytes or non-classical monocytes), which express high level of
55 CX3CR1 and low level of CCR2, equivalent to CD14^{lo}CD16^{+} human monocytes. Ly6C^{lo} monocytes are regarded as blood-resident macrophages that patrol blood vessels and scavenge microparticles attached to the endothelium under physiological conditions, and exhibit a long half-life in the steady-state[8-10]. Ly6C^{lo} monocytes may exert a protective effect by suppressing atherosclerosis in mice, while high levels of
60 nonclassical monocytes have been associated with more advanced vascular dysfunction and oxidative stress in patients with coronary artery disease[11-13]. In the inflammatory settings, Ly6C^{lo} monocytes often show anti-inflammatory properties, yet also exhibit a proinflammatory role under certain circumstances[5,9,14-18]. In addition, Ly6C^{lo} monocytes have demonstrated protective functions in tumorigenesis, such as engulfing
65 tumor materials to prevent cancer metastasis, activating and recruiting NK cells to the lungs[19,20]. While the functions of Ly6C^{lo} monocytes are complex and sometimes contradictory depending on the animal models and experimental approaches, it is clear that they play a crucial role in both health and disease.

70 The generation and maintenance of Ly6C^{lo} monocytes are regulated by several transcription factors, including Nr4a1 and CEBPβ[21,22]. The colony-stimulating factor 1 receptor (CSF1R) signaling pathway is important for the generation and survival of Ly6C^{lo} monocytes, and neutralizing antibodies against CSF1R can reduce their numbers[23]. Interestingly, recent studies have suggested that different subsets of

monocytes may be supported by distinct cellular sources of CSF-1 within bone marrow niches, in which targeted deletion of *Csf1* from sinusoidal endothelial cells selectively reduces Ly6C^{lo} monocytes but not Ly6C^{hi} monocytes[24]. LAIR1 has been shown to be activated by stromal protein Colec12, and LAIR1 deficiency leads to aberrant proliferation and apoptosis in bone marrow non-classical monocytes[25]. These findings further underscore the complexity of the mechanisms that regulate Ly6C^{lo} monocyte, and suggest that multiple factors are involved in this process.

Recombination signal-binding protein for immunoglobulin kappa J region (RBP-J; also named CSL in humans) is commonly known as the master nuclear mediator of canonical Notch signaling[26]. In the absence of Notch intracellular domain, RBP-J associates with co-repressor proteins to repress transcription of downstream target genes. In the immune system, the best studied functions of Notch signaling are its roles in regulating the development of lymphocytes, such as T cells and marginal zone B cells[26]. Notch signaling also has been reported to regulate the differentiation and function of myeloid cells including granulocyte/monocyte progenitors, osteoclasts and dendritic cells [27-30]. In osteoclasts, RBP-J represses osteoclastogenesis, particularly under inflammatory conditions[30]. In dendritic cells, Notch-RBP-J signaling controls the maintenance of CD8^{-} DC[28]. In addition, Notch2-RBP-J pathway is essential for development of CD8^{-}ESAM^{+} DC in the spleen and CD103^{+}CD11b^{+} DC in the lamina propria of the intestine[29]. However, the role of Notch-RBP-J signaling pathway in regulating monocyte subsets remains elusive. Here, we demonstrated that RBP-J controlled homeostasis of blood Ly6C^{lo} monocytes in a cell intrinsic manner. RBP-J deficiency led to a drastic increase in blood Ly6C^{lo} monocytes, lung Ly6C^{lo} monocytes and CD16.2^{+} IM. Our results suggest that RBP-J plays a critical role in regulating the population and characteristics of Ly6C^{lo} monocytes in the blood and lung.

Results

RBP-J is essential for maintenance of blood Ly6C^{lo} monocytes

To investigate the role of RBP-J in monocyte subsets, we utilized mice with RBP-J

105 specific deletion in the myeloid cells (*Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ mice). Efficient deletion of *Rbpj* in blood monocytes was confirmed by quantitative real-time PCR (qPCR) (Figure 1 - figure supplement 1A). Flow cytometric analysis revealed that RBP-J deficient mice had a significant increase in the proportion of Ly6C^{lo} monocytes, but not Ly6C^{hi} monocytes, in blood, compared to age-matched control mice with the genotype
110 *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ (Figure 1A). In contrast to circulating monocytes, RBP-J deficient mice exhibited minimal alterations in the percentages of monocyte subsets in bone marrow (BM) and spleen (Figure 1B; Figure 1 - figure supplement 1C). Additionally, RBP-J deficiency in myeloid cells did not appear to have an effect on neutrophils (Figure 1- figure supplement 1B). Therefore, among circulating myeloid populations,
115 RBP-J selectively controlled the subset of Ly6C^{lo} monocytes.

BM progenitors that give rise to circulating monocytes are monocyte-dendritic cell progenitors (MDPs) and common monocyte progenitors (cMoPs)[21,31-33]. Next, we analyzed BM progenitors, and found that the percentages of MDPs and cMoPs were
120 equivalent in control and RBP-J deficient mice (Figure 1C). These results in conjunction with the observations of normal BM monocyte populations suggest that alterations of peripheral blood Ly6C^{lo} monocytes may not originate from BM. Next, we examined whether RBP-J may influence the egress of Ly6C^{lo} monocytes from BM, and measured BM exit rate of Ly6C^{lo} monocytes using *in vivo* labeling of sinusoidal cells
125 as previously described (Figure 1D)[34]. The results showed that RBP-J deficiency did not affect egress of Ly6C^{lo} monocytes from BM (Figure 1E). Taken together, these data revealed RBP-J as a critical regulator controlling homeostasis of peripheral blood Ly6C^{lo} monocytes.

130 **RBP-J deficiency does not affect Ly6C^{hi} monocyte conversion or Ly6C^{lo} monocyte survival and proliferation**

Next, we aimed to determine whether the increase of blood Ly6C^{lo} monocytes in RBP-J deficient mice was due to decreased cell death or enhanced proliferation. We first stained monocytes with Annexin V and 7-AAD to identify apoptotic cells and observed

comparable percentages of apoptotic blood Ly6C^{lo} monocytes in control and RBP-J deficient mice (Figure 2A). Given the crucial role of Nr4a1 in the survival of Ly6C^{lo} monocytes[21], we detected the expression of Nr4a1, which was similar in Ly6C^{lo} monocytes from control and RBP-J deficient mice (Figure 2- figure supplement 1A). We then assessed the proliferative capacity by analyzing Ki-67 expression as well as *in vivo* EdU incorporation. Ki-67 levels in blood monocytes displayed no differences between control and RBP-J deficient mice (Figure 2B). The percentage of EdU^{+} monocytes did not significantly differ between control and RBP-J deficient mice at the indicated time points, implying that the turnover of Ly6C^{lo} monocytes was normal in RBP-J deficient mice (Figure 2C). Fluorescent latex beads as particulate tracers could be phagocytosed by monocytes after intravenous injection, and stably label Ly6C^{lo} monocytes[35]. Thus, we intravenously injected fluorescent latex beads into control and RBP-J deficient mice to track circulating monocytes (Figure 2D). By day 7 post injection, only Ly6C^{lo} monocytes were latex^{+} as previously reported[35], whereas control and RBP-J deficient mice groups presented a similar frequency of latex^{+} monocytes (Figure 2E). Together, these data indicated that RBP-J did not influence monocyte survival and proliferation.

Previous studies have shown that Ly6C^{lo} monocytes are observed in recipient mice following the adoptive transfer of Ly6C^{hi} monocytes, indicating that Ly6C^{hi} monocytes can convert into Ly6C^{lo} monocytes[10,31]. We next wished to evaluate whether conversion of Ly6C^{hi} monocytes was regulated by RBP-J by isolating bone marrow GFP^{+}Ly6C^{hi} monocytes from *Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ control or *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ mice and adoptively transferring them into *Ccr2*$^{RFP/RFP}$ recipients (Figure 2F). Sixty hours after transfer, a subset of cells from donors were converted into Ly6C^{lo} monocytes (Figure 2 - figure supplement 1B), and equal percentages of Ly6C^{lo} monocytes were derived from control and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ donors (Figure 2G). Thus, the conversion of Ly6C^{hi} monocytes into Ly6C^{lo} monocytes was not affected by RBP-J deficiency.

RBP-J regulates blood Ly6C^{lo} monocytes in a cell intrinsic manner

We next wondered whether the increase of Ly6C^{lo} monocytes in RBP-J deficient mice was bone marrow-derived and cell intrinsic. We performed bone marrow transplantation by engrafting lethally irradiated mice with a 1:4 mixture of *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ control and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ BM cells (CD45.2) and *Cx3cr1*$^{gfp/+}$ BM cells (CD45.1) (Figure 3A). Eight weeks after transplantation, we analyzed the frequencies of CD45.2^{+} donor cells in the bone marrow and blood of recipient mice and found that the frequencies of CD45.2^{+} cells within total cells were similar to the mixture ratio of bone marrow cells for Ly6C^{hi} monocytes and neutrophils in both BM and blood (Figure 3B; Figure 3 - figure supplement 1A-B). Specifically, more blood Ly6C^{lo} monocytes were derived from RBP-J deficient donors than control donors (Figure 3B), reflecting that the contribution of RBP-J deficient cells in blood Ly6C^{lo} cells was significantly higher than that of control cells. These results implied that RBP-J deficient BM cells were highly efficient in generating blood Ly6C^{lo} monocytes.

Given that RBP-J deficient mice had more Ly6C^{lo} monocytes in blood than did control mice, we performed parabiosis experiments, a surgical union of two organisms, which allowed parabiotic mice to share their blood circulation. The contribution of circulating cells from one animal in another can be estimated by measuring the percentage of blood cells that originated from each animal[36]. We joined a CD45.1, *Cx3cr1*$^{gfp/+}$ mouse with an age and sex-matched *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ control or *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ mouse (CD45.2) (Figure 3C). Four weeks after the procedure, about 50% of B cells and T cells in parabiotic mice displayed efficient exchange of their circulation (Figure 3 - figure supplement 1C). As expected, RBP-J deficient mice exhibited higher percentages of Ly6C^{lo} monocytes than control animals (Figure 3D). Intriguingly, in the parabiotic *Cx3cr1*$^{gfp/+}$ mice, RBP-J deficient cells still constituted significantly higher proportion of circulating Ly6C^{lo}, but not Ly6C^{hi} monocytes, than RBP-J sufficient counterparts as indicated by the percentages of the GFP-negative population (Figure 3E). These results implicated that the enhanced ability of Ly6C^{lo} monocytes to circulate in the peripheral blood as a result of RBP-J deficiency was cell intrinsic.

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RBP-J regulates phenotypical marker genes in blood Ly6C^{lo} monocytes

To further study the consequences of RBP-J loss of function in blood Ly6C^{lo} monocytes, we performed gene expression profiling by RNA-seq, which revealed that Ly6C^{lo} monocytes in RBP-J deficient mice exhibited low expression of *Itgax* but high
200 expression of *Ccr2*, in comparison to those in control mice (Figure 4A). These differential expression patterns of phenotypic marker genes were also confirmed by qPCR and flow cytometry analysis (Figure 4B). However, Ly6C^{hi} blood monocytes and BM monocytes displayed normal levels of CD11c and CCR2 (Figure 4B; Figure 4 - figure supplement 1A). Moreover, principal component analysis showed distinct
205 expression pattern of monocytes between control and RBP-J deficient mice (Figure 4C). To determine whether the regulation of phenotypic markers by RBP-J in blood Ly6C^{lo} monocytes was cell-intrinsic, we performed BM transplantation as described above (Figure 3A). The findings indicated that Ly6C^{lo} monocytes derived from RBP-J deficient BM cells expressed high levels of CCR2 but low levels of CD11c, in
210 comparison to those derived from control BM cells, whereas no significant changes were observed in blood Ly6C^{hi} and BM monocytes, as expected (Figure 4D; Figure 4 - figure supplement 1B). These results collectively suggested that RBP-J regulated the expression of CCR2/CD11c in a cell-intrinsic manner.

215 **RBP-J-mediated control of blood Ly6C^{lo} monocytes is CCR2 dependent**

Given that RBP-J deficiency led to enhanced CCR2 expression in Ly6C^{lo} monocytes and that CCR2 is essential for monocyte functionality under various inflammatory and non-inflammatory conditions[37], we wished to genetically investigate the role of CCR2 in the RBP-J deficient background. We generated RBP-J/CCR2 double deficient (DKO)
220 mice with the genotype *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/RFP}$ with *Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/+}$ and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/+}$ mice serving as controls. As expected, the expression of CCR2 in Ly6C^{lo} monocytes was reduced in DKO mice compared to RBP-J deficient mice (Figure 5A), confirming the successful deletion of the *Ccr2* gene. DKO mice showed a lower percentage of both Ly6C^{hi} and Ly6C^{lo} monocytes than RBP-J deficient

225 mice (Figure 5B-C). Notably, the percentage of Ly6C^{lo} monocytes in DKO mice was comparable to that observed in the control mice (Figure 5B-C), implicating that deletion of CCR2 corrected RBP-J deficiency-associated phenotype of increased Ly6C^{lo} monocytes. Whereas, both RBP-J deficient and DKO mice exhibited lower expression levels of CD11c in their Ly6C^{lo} monocyte than control mice, suggesting that the
230 regulation of CD11c expression was independent of CCR2 (Figure 5D). These results suggested that RBP-J regulated Ly6C^{lo} monocytes, at least in part, through CCR2.

Lung Ly6C^{lo} monocytes are accumulated in RBP-J deficient mice

In mice, alveolar macrophages (AM) are maintained by local self-renewal, and arise
235 from fetal liver derived precursors, whereas interstitial macrophages (IM) are probably originated from monocytes [38-40]. Joey et al identified 3 subpopulations of IM according to expression of CD206 and CD16.2 and Ly6C^{lo} monocytes are proposed to give rise to CD64^{+}CD16.2^{+} IM[41]. We next compared the populations of lung monocytes and IM between control and RBP-J deficient mice. The conditional deletion of RBP-J resulted
240 in a significant increase in the absolute and relative numbers of Ly6C^{lo} monocytes and CD16.2^{+} IM, while the numbers of Ly6C^{hi} monocytes, CD206^{-} IM, and CD206^{+} IM remained unchanged (Figure 6A-B). To confirm these findings, lung sections from *Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ control and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ mice were stained with an anti-GFP antibody, which revealed a significant increase in the number of GFP^{+} cells
245 in RBP-J deficient mice (Figure 6C). In addition, we evaluated the proliferative capacity of Ly6C^{lo} monocytes and CD16.2^{+} IM, but did not observe enhanced EdU incorporation in Ly6C^{lo} monocytes and CD16.2^{+} IM from RBP-J deficient mice (Figure 6 - figure supplement 1A-B). These findings suggested that the augmented population of these cells might not be resulted from increased *in situ* proliferation. Previous reports have
250 indicated that LPS can increase the population of IM in a CCR2-dependent manner[40]. We thus challenged mice with LPS, and analyzed monocytes and IM at day 0 and day 4. LPS exposure induced an increase in numbers of Ly6C^{lo} monocytes, CD16.2^{+} IM and CD206^{-} IM compared to baseline both in control and RBP-J deficient mice (Figure 6D-E). There was a trend toward higher Ly6C^{hi} monocyte after LPS treatment, although

255 this observation was not statistically significant. Of note, RBP-J deficient mice exhibited robustly elevated numbers of Ly6C^{lo} monocytes and CD16.2^{+} IM compared with control mice after LPS treatment (Figure 6D-E), suggesting that RBP-J deficient Ly6C^{lo} monocytes were recruited to inflamed tissue in large numbers and differentiated into CD16.2^{+} IM.

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To investigate whether the elevated levels of lung monocytes and CD16.2^{+} IM in RBP-J deficient mice were derived from blood Ly6C^{lo} monocytes, we examined monocytes and IM in *Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/+}$ control, *Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/RFP}$, *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/+}$, and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/RFP}$ (DKO) mice. The deletion
265 of CCR2 led to a decrease in Ly6C^{hi} monocytes, and the Ly6C^{lo} monocytes in DKO mice were reduced to a level similar to that of control mice (Figure 7A, B). While the DKO mice had more CD16.2^{+} IM compared to control mice, these cells were severely reduced compared to RBP-J deficient mice (Figure 7A, C). The above data supported the notion that increased lung Ly6C^{lo} monocytes and CD16.2^{+} IM in RBP-J deficient
270 mice were derived from Ly6C^{lo} blood monocytes. In summary, these results suggested that in RBP-J deficient mice, recruitment of blood Ly6C^{lo} monocytes to the lung was markedly facilitated by increased cell number as well as heightened expression of CCR2, leading to the increase of lung Ly6C^{lo} monocytes and CD16.2^{+} IM.

275 **Discussion**

In this study, we identified RBP-J as a pivotal factor in regulating blood Ly6C^{lo} monocyte cell fate. Our results showed that mice with conditional deletion of RBP-J in myeloid cells exhibited a robust increase in blood Ly6C^{lo} monocytes, and subsequently accumulated lung Ly6C^{lo} monocytes and CD16.2^{+} IM under steady-state conditions.
280 Bone marrow transplantation experiments in which RBP-J deficient cells were transplanted into recipient mice as well as the parabiosis experiment showed similar increase in blood Ly6C^{lo} monocytes, demonstrating that RBP-J was an intrinsic factor required for the regulation of Ly6C^{lo} monocytes. Further analysis revealed that RBP-J regulated the expression of CCR2 and CD11c on the surface of Ly6C^{lo} monocytes.

285 Moreover, the phenotype of elevated blood Ly6C^{lo} monocytes and their progeny was further ameliorated by deleting *Ccr2* in an RBP-J deficient background.

Notch-RBP-J signaling have been shown to play a role in regulating functional polarization and activation of macrophages, and regulated formation of Kupffer cells
290 and macrophage differentiation from Ly6C^{hi} monocytes in ischemia[42-48]. At steady-state, interaction of DLL1 with Notch 2 regulates conversion of Ly6C^{hi} monocytes into Ly6C^{lo} monocytes in special niches of the BM and spleen[49]. Under inflammatory conditions, Notch2 and TLR7 pathways independently and synergistically promote conversion of Ly6C^{hi} monocytes into Ly6C^{lo} monocytes[50]. However, in our study, the
295 percentage of blood Ly6C^{lo} monocytes increased in RBP-J deficient mice. These results somewhat differ from what was observed in mice with conditional deletion of Notch2[49]. Actually, 4 members of the Notch family have been identified in mammals[26]. Thus, Notch receptors seem to be non-redundant in regulating monocyte cell fate, and distinct Notch receptors are likely to act in a concerted manner to coordinate the monocyte
300 differentiation program.

Colony-stimulating factor 1 receptor (CSF1R) signal, CX3CR1, C/EBPβ and Nr4a1 have been suggested to be involved in the generation and survival of Ly6C^{lo} monocytes. The lifespan of Ly6C^{lo} monocytes is acutely shortened after blockade of CSF1R, and
305 the numbers of these cells are reduced, and the percentage of dead cells increased in mice with endothelial cell-specific depletion of Csf1[23,24]. CX3CR1 and C/EBPβ-knockout mice show accelerated death of Ly6C^{lo} monocyte, display the decreased level of Ly6C^{lo} monocytes[22,51]. Nr4a1 knockout mice had significantly fewer monocytes in blood, bone marrow, and spleen, due to differentiation deficiency in MDP and
310 accelerated apoptosis of Ly6C^{lo} monocytes in bone marrow[21]. RBP-J deficient mice had normal MDP and Ly6C^{lo} monocytes, expressed normal level of Nr4a1 and Ki-67, exhibited normal half-life and turnover rate, which suggested that RBP-J may not regulate Ly6C^{lo} monocytes through these factors. Further studies are required to elucidate the precise molecular mechanisms of RBP-J in Ly6C^{lo} monocytes under

315 steady state.

At the steady state, Ly6C^{lo} monocytes patrol endothelium of blood vessel. After R848 induced endothelial injury, Ly6C^{lo} monocytes recruit neutrophils to mediate focal endothelial necrosis and fuel vascular inflammation, and subsequently, the Ly6C^{lo}
320 monocytes remove cellular debris[9,52]. In response to *Listeria monocytogenes* infection, Ly6C^{lo} monocytes can extravasate but do not give rise to macrophages or DCs[8]. Additional evidence shows that Ly6C^{lo} monocytes do not merely act as luminal blood macrophages. In lung, exposure to unmethylated CpG DNA expands CD16.2^{+} IM, which originate from Ly6C^{lo} monocytes and spontaneously produce IL-10, thereby
325 preventing allergic inflammation[41]. A recent study shows that Ly6C^{lo} monocytes give rise to CD9^{+} macrophages, which provide an intracellular replication niche for *Salmonella* Typhimurium in spleen, and Ly6C^{lo}-depleted mice are more resistant to *Salmonella* Typhimurium infection, suggesting that Ly6C^{lo} monocytes exert certain functions in systemic infection[53]. Our data provide evidence for RBP-J in the function
330 of blood Ly6C^{lo} monocytes, as RBP-J deficient Ly6C^{lo} monocytes exhibited enhanced competition in blood circulation, as well as gave rise to increased numbers of lung CD16.2^{+} IM. In summary, we showed that RBP-J acted as a fundamental regulator of the maintenance of blood Ly6C^{lo} monocytes and their descendent, at least in part through regulation of CCR2. These results provide insights into understanding the
335 mechanisms that regulate monocyte homeostasis and function.

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Author contributions:

T. Kou designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. L. Kang designed and performed experiments, analyzed and interpreted data. B. Zhang provided advice on RNA-seq analyses. J. Li performed experiments. B. Zhao provided advice on experiments. W. Zeng provided advice on experiments. X. Hu conceptualized the project, supervised experiments, interpreted data, and wrote the manuscript.

Declaration of interests:

The authors declare no competing interests.

Data availability:

Sequencing data sets are deposited in the Genome Expression Omnibus with assigned accession numbers as follows: RNA-seq (GEO accession no. GSE208772).

Materials and methods

Mice

Cx3cr1$^{gfp/gfp}$ mice (JAX stock 005582; Jung et al., 2000) and *Ccr2*$^{RFP/RFP}$ mice (JAX stock 017586) were purchased from the Jackson Laboratory. Mice with a myeloid-

specific deletion of the *Rbpj* were generated by crossing *Rbpj*$^{fl/fl}$ mice to *Lyz2*-Cre mice as described previously (Hu et al., 2008). *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ were crossed to *Cx3cr1*$^{gfp/gfp}$ mice to obtain *Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ mice. C*x3cr1*$^{gfp/+}$ mice were obtained by crossing *Cx3cr1*$^{gfp/gfp}$ with C57/BL6 CD45.1^{+} mice. *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ mice were crossed with *Ccr2*$^{RFP/RFP}$ mice to obtain *Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/+}$, *Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/RFP}$, *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/+}$ and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/RFP}$ mice. All mice were maintained under specific pathogen-free conditions. All animal experimental protocols were approved by the Institutional Animal Care and Use Committees of Tsinghua University. Gender- and age-matched mice were used at 7–12 weeks old for experiments.

Quantitative RT-PCR

Blood monocytes were sorted by flow cytometry. The total RNA was extracted using total RNA purification kit (GeneMarkbio) and reversely transcribed to cDNA by M-MLV Reverse Transcriptase (Takara). qPCR was performed on a real-time PCR system (StepOnePlus; Applied Biosystems) using FastSYBR mixture (CWBIO). *Gapdh* messenger RNA was used as internal control to normalize the expression of target genes. Primer sequences are provided in supplemental Table 1.

Table 1: Primers sequences for regular qPCR used in this study

Gene	Forward primer	Reverse primer
Gapdh	ATCAAGAAGGTGGTGAAGCA	AGACAACCTGGTCCTCAGTGT
Rbpj	ACCCCTGTGCCTGTCGTAGA A	TCCCGGAATGCAGAAATGTC
Nr4a1	TTGAGCTTGAATACAGGGCA	AGTTGGGGGAGTGTGCTAGA
Ccr2	CCTTGGGAATGAGTAACTGT GTGAT	ATGGAGAGATACCTTCGGAAC TTCT

RNA-seq Analysis

Ly6C^{hi} and Ly6C^{lo} blood monocytes were isolated from *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ mice, and total RNA was extracted using total RNA purification kit (GeneMarkbio). RNA was converted into RNA-seq libraries, which were sequenced

with the pair-end option using an Illumina-HiSeq2500 platform at Beijing Genomics Institute (BGI), China. The significantly down-regulated genes were identified with P value <0.05 and (FPKM + 1) fold changes ≤0.2, and significantly up-regulated genes were identified with P value <0.05 and (FPKM + 1) fold changes ≥5.7. The RNA-seq data are deposited in Gene Expression Omnibus under accession number GSE208772

Annexin V staining

Annexin V and 7-amino-actinomycin D (7-AAD) were used for identification of apoptotic monocytes by flow cytometry. Blood cells were stained with annexin V (eBioscience) and 7-AAD (Biolegend) according to the manufacturers' protocols.

EdU pulsing and latex beads labeling

Mice were injected intravenously with a single 1mg EdU (Thermo Scientific). BM and blood cells were collected and stained with Fluorescence-conjugated mAb against CD45, CD11b, Ly6G, CD115 and Ly6C. Cells were then fixed, permeabilized and stained with reaction cocktail using EdU Assay Kit. Labeled cells were analyzed by flow cytometry.

Mice were injected intravenously with a single 10 μL Latex beads (Polysciences) in 250 μl PBS. Blood cells were harvested at indicated time. Cells were then stained for CD45, CD11b, Ly6G, CD115, Ly6C, and analyzed by flow cytometry.

In vivo labeling of sinusoidal leukocytes

Mice were injected intravenously with 1 μg of PE-conjugated anti-CD45 antibodies. Two minutes after antibody injection, mice were sacrificed, and bone marrow were harvested. BM cells were then stained and analyzed by flow cytometry.

Generation of BM chimera

C57/BL6 CD45.2^{+} mice were lethally irradiated in two doses of 5.5Gy 2 h apart. 0.8 × 10^{5} BM cells from *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ mice (CD45.2^{+}) or *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ mice (CD45.2^{+}) were mixed with 3.2× 10^{5} BM cells from *Cx3cr1*$^{gfp/+}$ mice (CD45.1^{+}), and injected

intravenously into recipient mice. Mice were used for experiments 8 weeks after irradiation.

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Adoptive transfers

BM GFP^{+}Ly6C^{hi} monocytes were sorted from *Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ or *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ mice, and transferred to *Ccr2*$^{RFP/RFP}$ mice. Blood cells were collected 60 hours later for flow cytometry analyses.

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Parabiosis

Cx3cr1$^{gfp/+}$ CD45.1^{+} mice were surgically joined with age-matched female *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ or *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ CD45.2^{+} mice at the age of 6 weeks. Bloods were obtained via cardiac puncture, and cell populations were analyzed by flow cytometry at

660 4 weeks after the surgery.

Intranasal instillations of LPS

Rbpj$^{+/+}$*Lyz2*$^{cre/cre}$ or *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ mice were anesthetized with isoflurane and intranasally instilled with 10 μg LPS in 25 μl of PBS. Lungs were harvested 4 days later

665 for flow cytometry analyses.

Immunofluorescence histology

Lungs from *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ and *Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ mice were fixed in 1% paraformaldehyde and incubated in 30% sucrose separately overnight at 4°C. The

670 samples were then incubated in the mixture of 30% sucrose and OTC compound (Sakura Finetek) overnight at 4°C. The tissues were embedded and frozen in OCT compound and then cut at 10-μm thickness. Tissue sections were dried for 10 min at 50°C and then fixed in 1% paraformaldehyde at room temperature for 10 min, permeabilized in PBS/0.5% Triton X-100/0.3 M glycine for 10 min and blocked in

675 PBS/5% goat serum for 1 h at room temperature. Sections were stained with rabbit anti-mouse GFP antibodies (1:1000; EASYBIO) overnight at 4°C, and washed with PBS/0.1% Tween-20 for 30 min three times at room temperature. Sections were then incubated

with AF488-conjugated goat anti-rabbit antibodies (1:200; proteintech) for 2 h at room temperature, and washed with PBS/0.1% Tween-20 for 30 min three times at room
680 temperature. Sections were stained with DAPI (Solarbio) for 7 min, washed in PBS for 8 min two times at room temperature and mounted with SlowFade Diamond Antifade Mountant (Life Technologies).

Cell isolation and Flow cytometry

685 Peripheral blood was sampled by eyeball extirpating, spleens were mashed through a 70-μm strainer, and bone marrow cells were collected from femurs. Lungs were perfused with 5ml of HBSS (MACGENE) through the right ventricle, excised, and digested in HBSS containing 5% FBS, 1 mg/ml collagenase type I (Sigma-Aldrich) and 0.05 mg/ml DNase I (Sigma-Aldrich) for 1 h at 37 °C. The digested tissues were
690 homogenized by shaking, passed through a 70-μm cell strainer to create a single cell suspension. The suspension was enriched in mononuclear cells and harvested from the 1.080:1.038 g ml-1 interface using a density gradient (Percoll from GE Healthcare). BM, Spleen, PB and lung cells were stained with fluorescently conjugated antibodies. The absolute number of cells was counted by using CountBright™ Absolute Counting
695 Beads (Invitrogen).

Antibodies against CD45 (30-F11), Ly6C (HK1.4), CD64 (X54-5/7.1), CD206 (C068C2), CD16.2 (9E9), CD45.1 (A20), F4/80 (BM8), Biotin, CCR2 (SA203G11) and CD3ε (145-2C11) were purchased from BioLegend. Antibodies against Ly6G
700 (1A8-Ly6g), CD11b (M1/70), CD115(AFS98), CD117(2B8), CD135 (A2F10), CD11c (N418), CD206 (MR6F3), CD45.2 (104), Nur77 (12.14), Ki-67 (SolA15) and lineage maker were purchased from eBioscience. Fluorescence-conjugated mAb against Ly6C (AL-21) were purchased from BD Biosciences. Isotype-matched antibodies (eBioscience) were used for control staining. All antibodies were used in 1:400 dilutions,
705 and surface antigens were stained on ice for 30 min.

For intracellular staining, cells were stained with antibodies to surface antigens, fixed

and permeabilized with the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences), and stained with antibodies or isotype control diluted in permeabilization buffer separately for 30 min at room temperature.

Cells were analyzed on FACSFortessa or FACSAria III flow cytometer (BD Biosciences) using FlowJo software.

Figure Legends:

Figure 1. RBP-J deficient mice display more Ly6C^{lo} blood monocytes.

740 (A) Blood Ly6C^{hi} and Ly6C^{lo} monocytes in *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ control and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ mice were determined by flow cytometry analyses (FACS). Representative FACS plots (left) and cumulative data of cell ratio (right) are shown.

(B and C) Representative FACS plots and cumulative data quantitating percentages of BM monocyte subsets (B) and myeloid progenitor cells (C) (CD45^{+}CD11b^{+}Ly6G^{-}

745 CD115^{+} Ly6C^{hi} monocyte; CD45^{+}CD11b^{+}Ly6G^{-}CD115^{+}Ly6C^{lo} monocyte; MDP, CD45^{+}Lin^{-}CD117^{+}CD115^{+}CD135^{+} Ly6C^{-}; cMoP, CD45^{+}Lin^{-}CD11b^{-}CD117^{+}CD115^{+} CD135^{-}Ly6C^{+}). Lin: CD3, B220, Ter119, and Gr-1, CD11b.

(D) Experimental outline for panel (E).

(E) Cumulative data quantitating percentages of sinusoidal monocytes (CD45^{+}) within

750 total BM Ly6C^{lo} monocytes.

Data are pooled from at least 2 independent experiments; n ≥ 4 in each group. Data are shown as mean ± SEM; n.s., not significant; **, P < 0.01 (two-tailed Student's unpaired t test). Each symbol represents an individual mouse.

755 **Figure 2. Monocyte subsets in RBP-J deficient mice display normal cell death.**

(A) Representative FACS plots of monocyte subsets in blood stained with 7-AAD and Annexin V.

(B) FACS analysis of Ki-67 expression in *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ control and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ blood monocyte subsets. Black lines represent control mice and red lines represent

760 RBP-J deficient mice.

(C) Analysis of time course of EdU incorporation of monocyte subsets in BM and blood after a single 1mg EdU pulsing. The percentages of EdU^{+} cells among the indicated monocyte subsets are shown.

(D) Experimental outline for panel (E).

765 (E) Analysis of time course of latex beads incorporation of monocyte subsets in blood after Latex beads injection. The percentages of latex^{+} cells among the indicated monocyte subsets are shown.

(F) Cartoon depicting the adoptive transfer. BM GFP^{+}Ly6C^{hi} monocytes were sorted from *Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ or *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ mice and transferred into
770 *Ccr2*$^{RFP/RFP}$ recipient mice. Sixty hours after transfer, cell fate was analyzed.

(G) Representative FACS plots are shown in the left panel, and the frequencies of GFP^{+} Ly6C^{lo} monocytes within total Ly6C^{lo} monocytes are shown in the right panel.

Data are pooled from 2 independent experiments (G); n ≥ 2 in each group (C, E and G). Data are shown as mean ± SEM; n.s., not significant; (two-tailed Student's unpaired t
775 test). Each symbol represents an individual mouse (E and G).

Figure 3. The role of RBP-J in blood Ly6C^{lo} monocytes is cell intrinsic.

(A) Cartoon depicting the bone marrow transplantation. BM recipient 6-week-old male C57BL/6 mice (CD45.2) were lethally irradiated. BM cells from donor mice
780 (*Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ or *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ and CD45.1, *Cx3cr1*$^{gfp/+}$) were collected and transferred into recipient mice. Mice were used after 8 weeks of bone marrow reconstitution.

(B) Representative FACS plots (left) and cumulative data (right) quantitating the frequency of *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ donor cells among Ly6C^{hi} and
785 Ly6C^{lo} monocytes in the blood of recipient mice.

(C) Cartoon depicting the generation of *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ control or *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ and *Cx3cr1*$^{gfp/+}$ parabiotic pairs.

(D) Representative FACS plots (left) and cumulative data (right) quantitating percentages of monocyte subsets derived from control or RBP-J deficient mice in
790 control or RBP-J deficient mice.

(E) Representative FACS plots (left) and cumulative data (right) quantitating percentages of monocyte subsets derived from control or RBP-J deficient mice in *Cx3cr1*$^{gfp/+}$ mice.

Data are pooled from at least 2 independent experiments; n ≥ 6 in each group. Data are
795 shown as mean ± SEM; n.s., not significant; ***, P < 0.001; ****, P < 0.0001 (two-tailed Student's unpaired t test). Each symbol represents an individual mouse. SSC-A, side scatter area.

Figure 4. Phenotypic markers in Ly6C^{lo} monocytes are changed in RBP-J deficient mice.

(A) Heatmap of RNA-seq dataset showing the top 20 downregulated and upregulated genes in blood Ly6C^{lo} monocytes from *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ versus *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ control mice. Blue and red font indicates downregulated and upregulated genes in RBP-J deficient Ly6C^{lo} monocytes respectively.

(B) Representative FACS plots, cumulative MFI, and qPCR analysis of CCR2/CD11c expression in control and RBP-J deficient blood monocyte subsets. Shaded curves represent isotype control, black lines represent control mice and red lines represent RBP-J deficient mice.

(C) PCA of indicated cell types.

(D) Representative FACS plots and cumulative MFI of CCR2/CD11c expression in blood monocyte subsets derived from control or RBP-J deficient mice. Black lines represent control mice, and red lines represent RBP-J deficient mice.

Data are pooled from 2 independent experiments (B and D); n ≥ 6 in each group. Data are shown as mean ± SEM; n.s., not significant; **, P < 0.01; ****, P < 0.0001 (two-tailed Student's unpaired t test). Each symbol represents an individual mouse.

Figure 5. Blood Ly6C^{lo} monocytes are decreased in DKO mice.

(A) Representative FACS plots and cumulative MFI of CCR2 expression in *Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/+}$ control, *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/+}$ and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/RFP}$ (DKO) blood Ly6C^{hi} and Ly6C^{lo} monocytes are shown.

(B and C) Blood monocyte subsets in control, RBP-J deficient and DKO mice were determined by FACS. Representative FACS plots (B) and cumulative data of cell ratio (C) are shown.

(D) Representative FACS plots and cumulative MFI of CD11c expression in control, RBP-J deficient and DKO blood Ly6C^{hi} and Ly6C^{lo} monocytes are shown.

Data are pooled from at least 2 independent experiments; n ≥ 5 in each group. Data are shown as mean ± SEM; n.s., not significant; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 (two-tailed Student's unpaired t test). Each symbol represents an individual

mouse.

830 **Figure 6. RBP-J deficient mice exhibit more lung Ly6C^{lo} monocytes and CD16.2^{+} IM.**

(A and B) Indicated populations in the lungs of *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ mice were determined by FACS. Representative FACS plots (A) and cumulative data of cell ratio and absolute numbers (B) are shown.

835 (C) Immunofluorescence staining for GFP^{+} cells in the lung from *Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Cx3cr1*$^{gfp/+}$ mice (CX3CR1 [green]; DAPI [blue]). Scale bars represent 50 μm.

(D and E) *Rbpj*$^{+/+}$*Lyz2*$^{cre/cre}$ and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$ mice were instilled intranasally with PBS or PBS containing LPS, and lungs were harvested at the indicated time points.

840 Representative FACS plots (D) and cumulative data of cell ratio and absolute numbers (E) are shown.

Data are pooled from at least 2 independent experiments; n ≥ 4 in each group. Data are shown as mean ± SEM; n.s., not significant; *, P < 0.05; **, P < 0.01; ****, P < 0.0001 (two-tailed Student's unpaired t test). Each symbol represents an individual mouse.

845

Figure 7. DKO mice lack lung Ly6C^{lo} monocytes and CD16.2^{+} IM.

(A) Representative FACS plots of lung monocyte and IM subsets in *Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/+}$, *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/+}$, *Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/RFP}$ and *Rbpj*$^{fl/fl}$*Lyz2*$^{cre/cre}$*Ccr2*$^{RFP/RFP}$ mice.

(B and C) Cumulative data of cell ratio and absolute numbers of monocyte (B) and IM

850 (C) subsets.

Data are pooled from 2 independent experiments; n ≥ 4 in each group. Data are shown as mean ± SEM; n.s., not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 (two-tailed Student's unpaired t test). Each symbol represents an individual mouse.

855

Figure 1 - Figure supplement 1. Control and RBP-J deficient mice show similar neutrophils.

(A) qPCR analysis of *Rbpj* expression in sorted monocyte subsets from control and RBP-J deficient mice.

860 (B) Blood and BM $CD45^+CD11b^+Ly6G^+$ neutrophils were determined by FACS. Cumulative data of cell ratio are shown.

(C) Spleen monocyte subsets were determined by FACS. Representative FACS plots (left) and cumulative data of cell ratio and absolute numbers (right) are shown.

Data are pooled from at least 2 independent experiments; n ≥ 2 in each group. Data are

865 shown as mean ± SEM; n.s., not significant; *, P < 0.05 (two-tailed Student's unpaired t test). Each symbol represents an individual mouse.

Figure 2 - Figure supplement 1. The conversion of $Ly6C^{hi}$ monocytes is identical in control and RBP-J deficient mice.

870 (A) Representative FACS plots, cumulative MFI and qPCR analysis of Nr4a1 expression in control and RBP-J deficient blood monocyte subsets. Shaded curves represent isotype control, black lines represent control mice, and red lines represent RBP-J deficient mice.

(B) Representative FACS plots of GFP^+ $Ly6C^{hi}$ monocytes in recipient.

875 Data are pooled from 2 independent experiments (A); n ≥ 4 in each group. Data are shown as mean ± SEM; n.s., not significant (two-tailed Student's unpaired t test). Each symbol represents an individual mouse.

Figure 3 - Figure supplement 1. Cell-intrinsic requirement of RBP-J for $Ly6C^{lo}$

880 **maintenance.**

(A) Representative FACS plots (left) and cumulative data (right) quantitating percentages of blood neutrophils in recipient mice.

(B) BM neutrophils and $Ly6C^{hi}$ monocytes in recipient mice were determined by FACS.

(C) $CD4^+$ T cells and $CD19^+$ B cells were analyzed by FACS in parabiotic mice.

885 Data are pooled from at least 2 independent experiments (A and C); n ≥ 4 in each group. Data are shown as mean ± SEM; n.s., not significant (two-tailed Student's unpaired t test). Each symbol represents an individual mouse.

Figure 4 - Figure supplement 1. Normal expression of CCR2 and CD11c in BM monocytes.

890 (A) Cumulative MFI of CCR2 expression in BM monocyte subsets.

(B) FACS plots of CCR2 and CD11c in BM Ly6C^{hi} and Ly6C^{lo} from control and RBP-J deficient mice.

Data are pooled from 3 independent experiments (A); n ≥ 7 in each group. Data are shown as mean ± SEM; n.s., not significant (two-tailed Student's unpaired t test). Each

895 symbol represents an individual mouse.

Figure 6 - Figure supplement 1. RBP-J is not required for turnover of lung Ly6C^{lo} monocytes and CD16.2^{+} IM.

(A and B) Incorporation of EdU was assessed 24 hours after injection. BM monocyte

900 subsets were used as controls. FACS plots (A) and representative data (B) are shown. Each symbol represents an individual mouse.

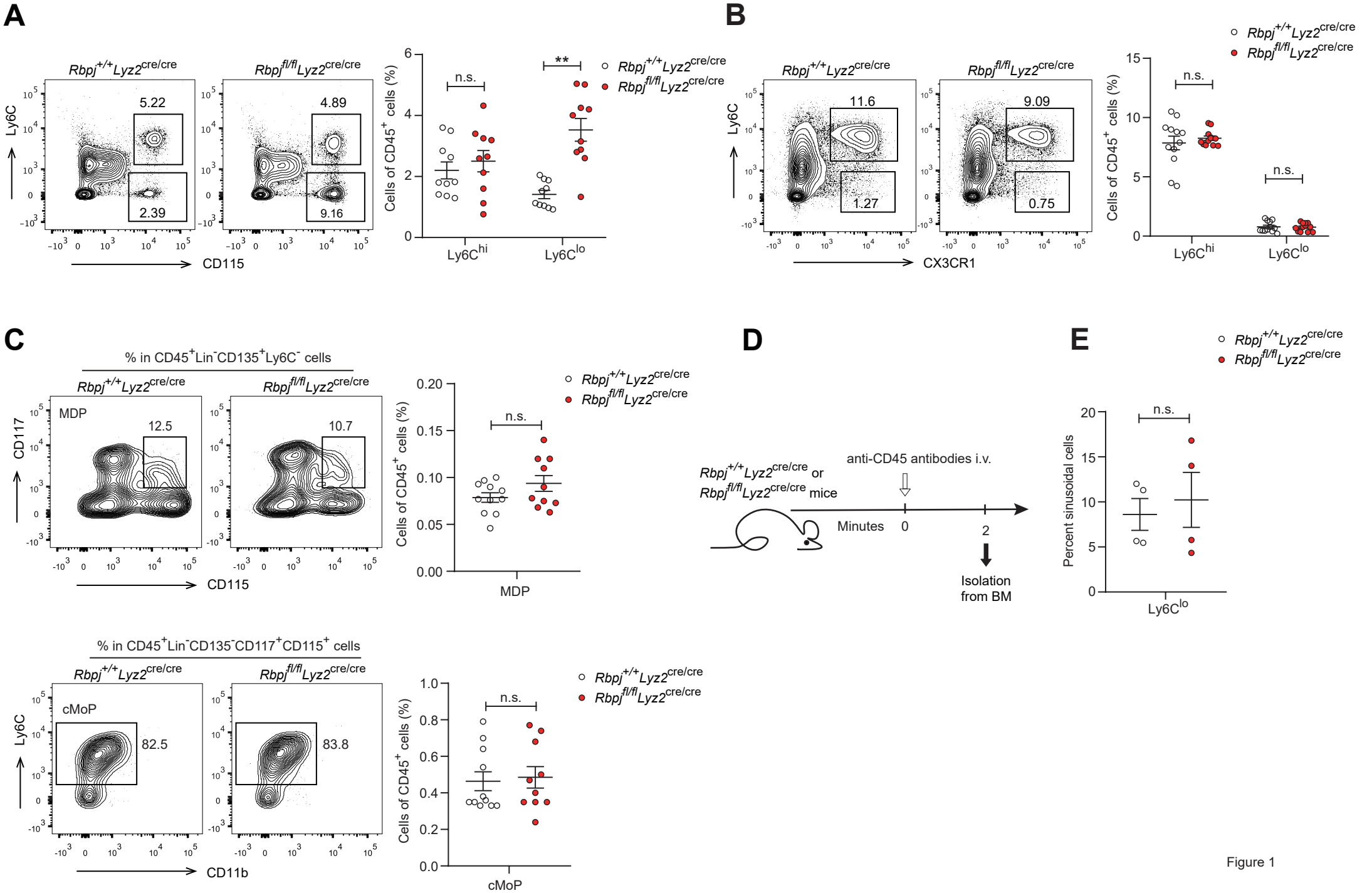

A
Rbpj+/+Lyz2cre/cre
Rbpjfl/flLyz2cre/cre
Ly6C
CD115
5.22
2.39
4.89
9.16
Cells of CD45+ cells (%)
n.s.
**
Ly6Chi
Ly6Clo
B
11.6
1.27
9.09
0.75
CX3CR1
C
% in CD45+Lin-CD135+Ly6C- cells
CD117
MDP
12.5
10.7
% in CD45+Lin-CD135-CD117+CD115+ cells
cMoP
82.5
83.8
CD11b
D
Rbpj+/+Lyz2cre/cre or
Rbpjfl/flLyz2cre/cre mice
anti-CD45 antibodies i.v.
Minutes
0
2
Isolation
from BM
E
Percent sinusoidal cells

Figure 1

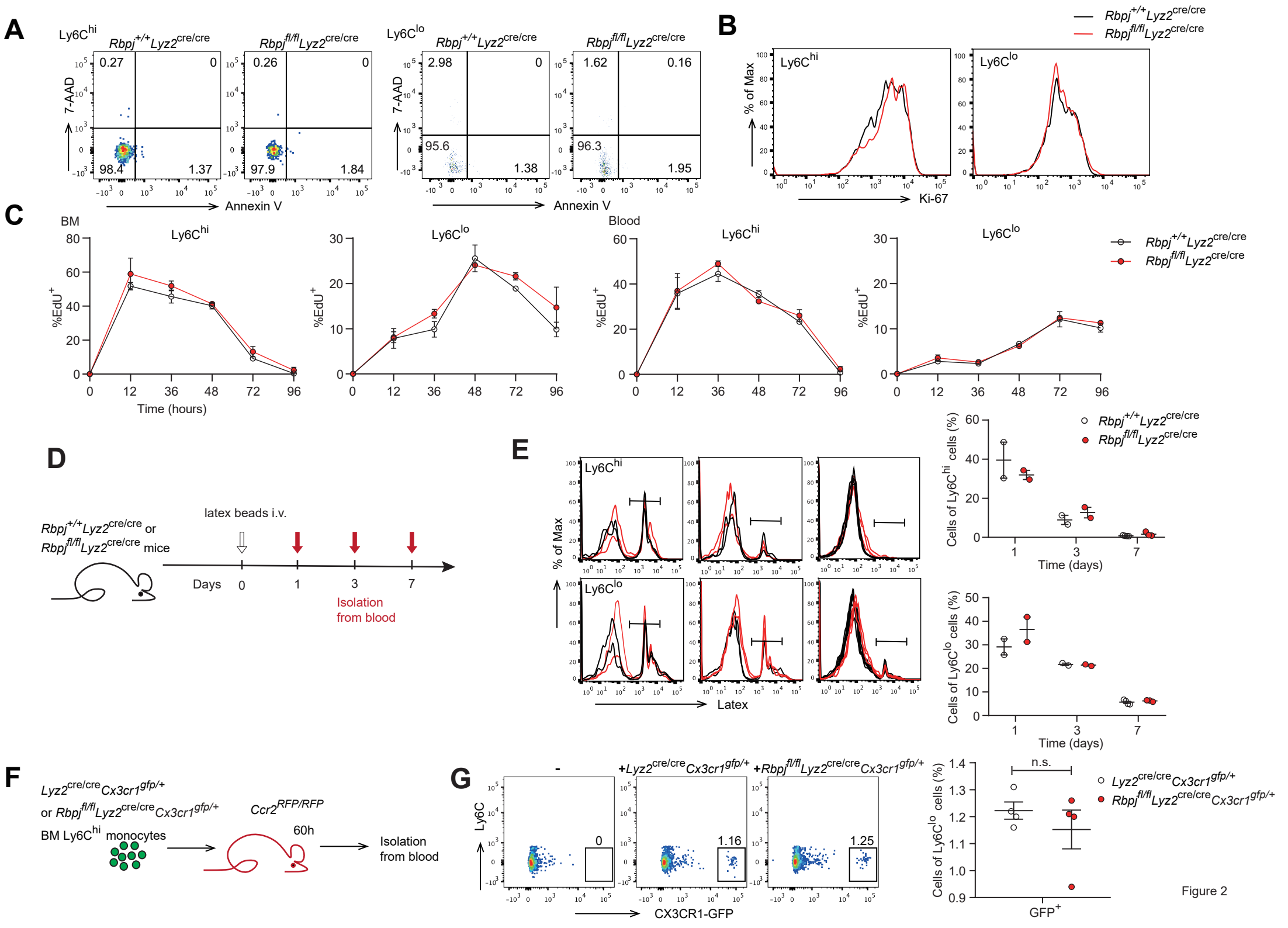

A
Ly6C^hi
Rbpj+/+Lyz2cre/cre
Rbpjfl/flLyz2cre/cre
0.27
0
98.4
1.37
0.26
0
97.9
1.84
Ly6C^lo
2.98
0
95.6
1.38
1.62
0.16
96.3
1.95
7-AAD
Annexin V
B
Rbpj+/+Lyz2cre/cre
Rbpjfl/flLyz2cre/cre
Ly6C^hi
Ly6C^lo
% of Max
Ki-67
C
BM
Blood
Ly6C^hi
Ly6C^lo
%EdU+
Time (hours)
Rbpj+/+Lyz2cre/cre
Rbpjfl/flLyz2cre/cre
D
Rbpj+/+Lyz2cre/cre or
Rbpjfl/flLyz2cre/cre mice
latex beads i.v.
Days 0 1 3 7
Isolation
from blood
E
Ly6C^hi
Ly6C^lo
% of Max
Latex
Cells of Ly6C^hi cells (%)
Cells of Ly6C^lo cells (%)
Time (days)
Rbpj+/+Lyz2cre/cre
Rbpjfl/flLyz2cre/cre
F
Lyz2cre/creCx3cr1gfp/+
or Rbpjfl/flLyz2cre/creCx3cr1gfp/+
BM Ly6C^hi monocytes
Ccr2RFP/RFP
60h
Isolation
from blood
G
-
+Lyz2cre/creCx3cr1gfp/+
+Rbpjfl/flLyz2cre/creCx3cr1gfp/+
Ly6C
0
1.16
1.25
CX3CR1-GFP
n.s.
Cells of Ly6C^lo cells (%)
GFP+
Lyz2cre/creCx3cr1gfp/+
Rbpjfl/flLyz2cre/creCx3cr1gfp/+

Figure 2

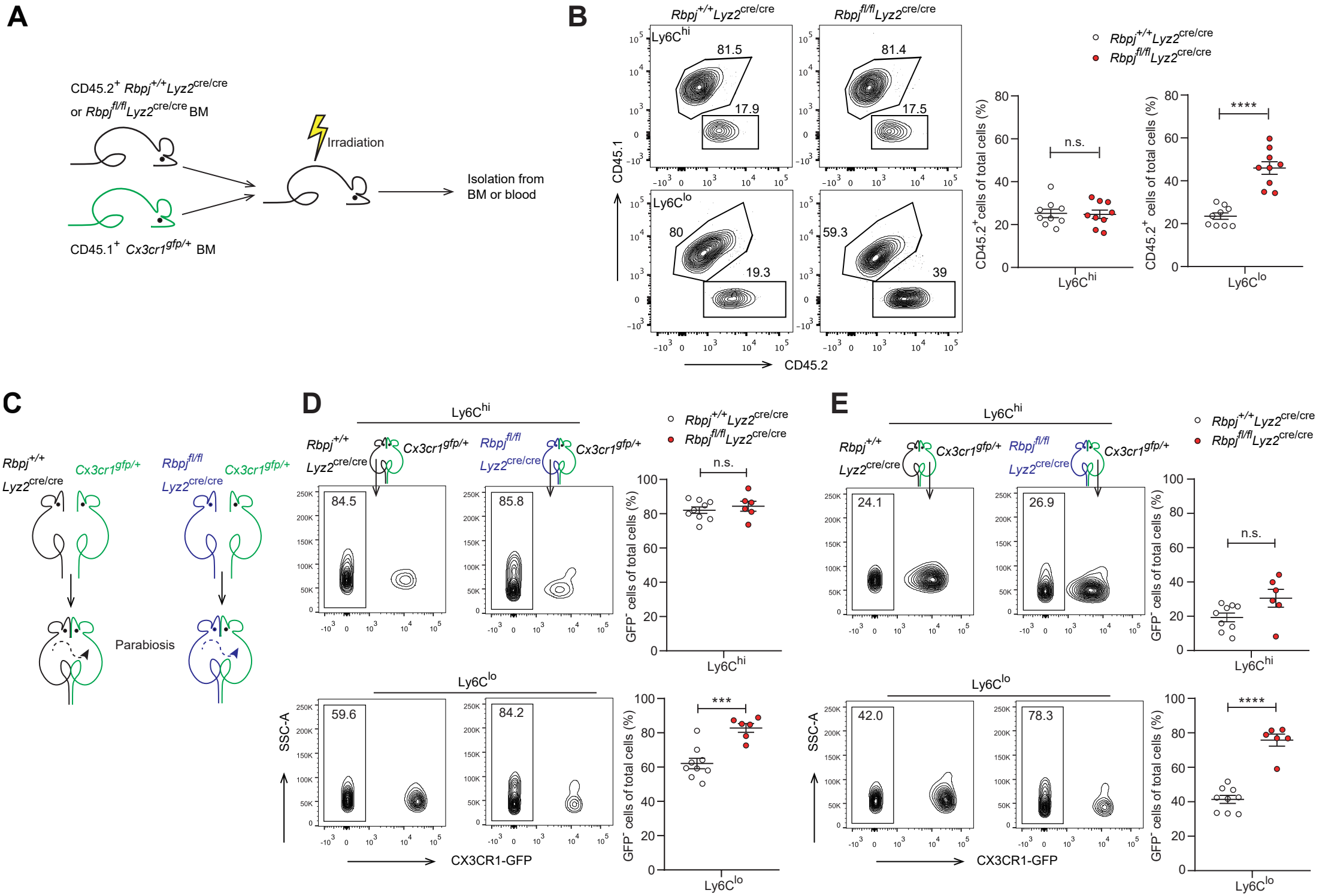

A
CD45.2+ Rbpj+/+Lyz2cre/cre
or Rbpjfl/flLyz2cre/cre BM
Irradiation
Isolation from
BM or blood
CD45.1+ Cx3cr1gfp/+ BM
B
Rbpj+/+Lyz2cre/cre
Rbpjfl/flLyz2cre/cre
Ly6Chi
81.5
17.9
81.4
17.5
Ly6Clo
80
19.3
59.3
39
CD45.1
CD45.2
CD45.2+ cells of total cells (%)
n.s.
****
Ly6Chi
Ly6Clo
C
Rbpj+/+
Lyz2cre/cre
Cx3cr1gfp/+
Rbpjfl/fl
Lyz2cre/cre
Cx3cr1gfp/+
Parabiosis
D
Ly6Chi
84.5
85.8
n.s.
GFP- cells of total cells (%)
Ly6Chi
Ly6Clo
59.6
84.2
***
SSC-A
CX3CR1-GFP
Ly6Clo
E
Ly6Chi
24.1
26.9
n.s.
Ly6Chi
Ly6Clo
42.0
78.3
****
Ly6Clo

Figure 3

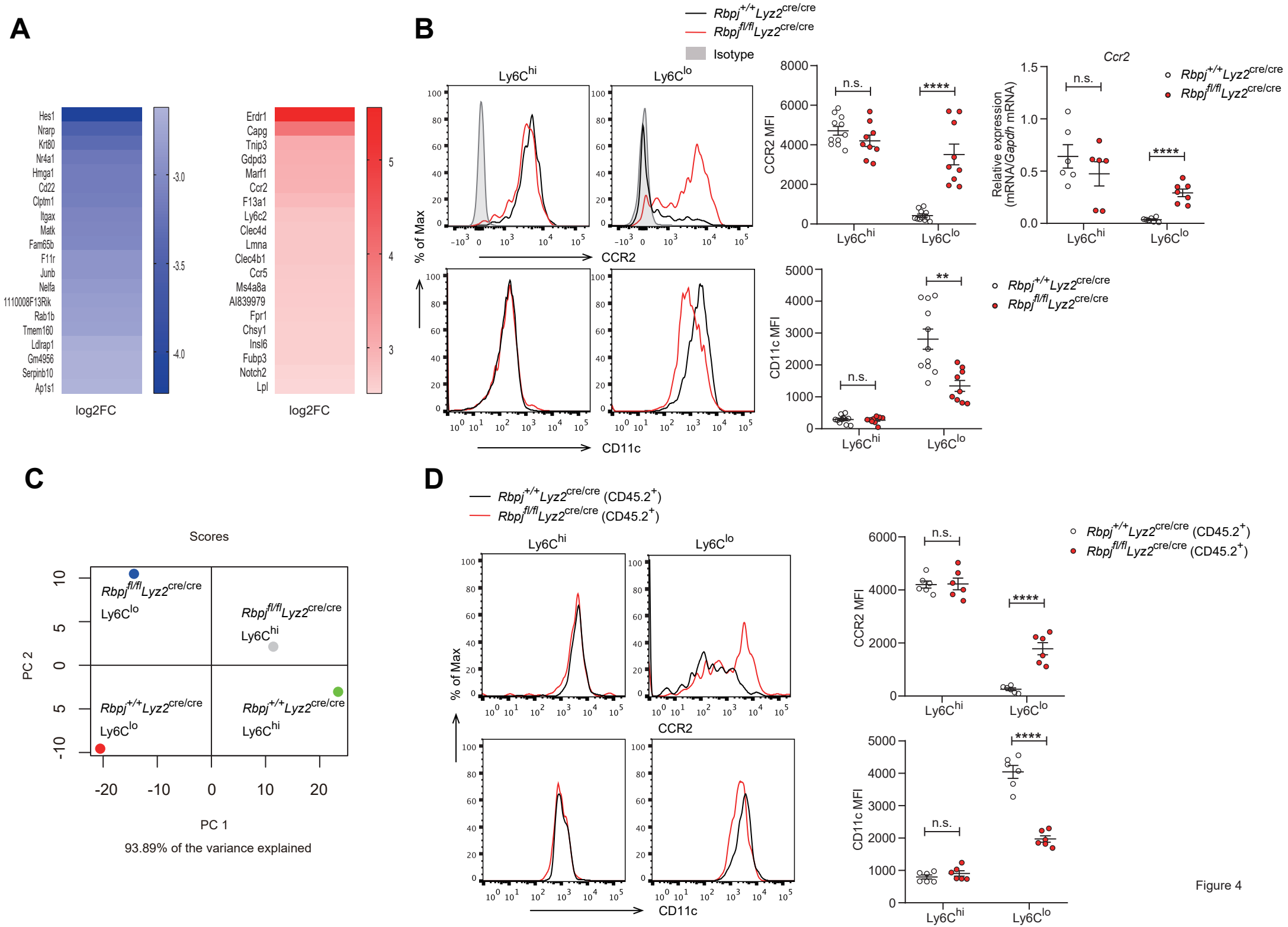

A
Hes1
Nrarp
Krt80
Nr4a1
Hmga1
Cd22
Clptm1
Itgax
Matk
Fam65b
F11r
Junb
Nelfa
1110008F13Rik
Rab1b
Tmem160
Ldlrap1
Gm4956
Serpinb10
Ap1s1
-3.0
-3.5
-4.0
log2FC
Erdr1
Capg
Tnip3
Gdpd3
Marf1
Ccr2
F13a1
Ly6c2
Clec4d
Lmna
Clec4b1
Ccr5
Ms4a8a
AI839979
Fpr1
Chsy1
Insl6
Fubp3
Notch2
Lpl
5
4
3
log2FC
B
Rbpj+/+Lyz2cre/cre
Rbpjfl/flLyz2cre/cre
Isotype
Ly6Chi
Ly6Clo
% of Max
CCR2
CD11c
CCR2 MFI
CD11c MFI
n.s.
****
**
Ccr2
Relative expression (mRNA/Gapdh mRNA)
C
Scores
Rbpjfl/flLyz2cre/cre Ly6Clo
Rbpjfl/flLyz2cre/cre Ly6Chi
Rbpj+/+Lyz2cre/cre Ly6Clo
Rbpj+/+Lyz2cre/cre Ly6Chi
PC 2
PC 1
93.89% of the variance explained
D
Rbpj+/+Lyz2cre/cre (CD45.2+)
Rbpjfl/flLyz2cre/cre (CD45.2+)
Ly6Chi
Ly6Clo
% of Max
CCR2
CD11c
CCR2 MFI
CD11c MFI
n.s.
****

Figure 4

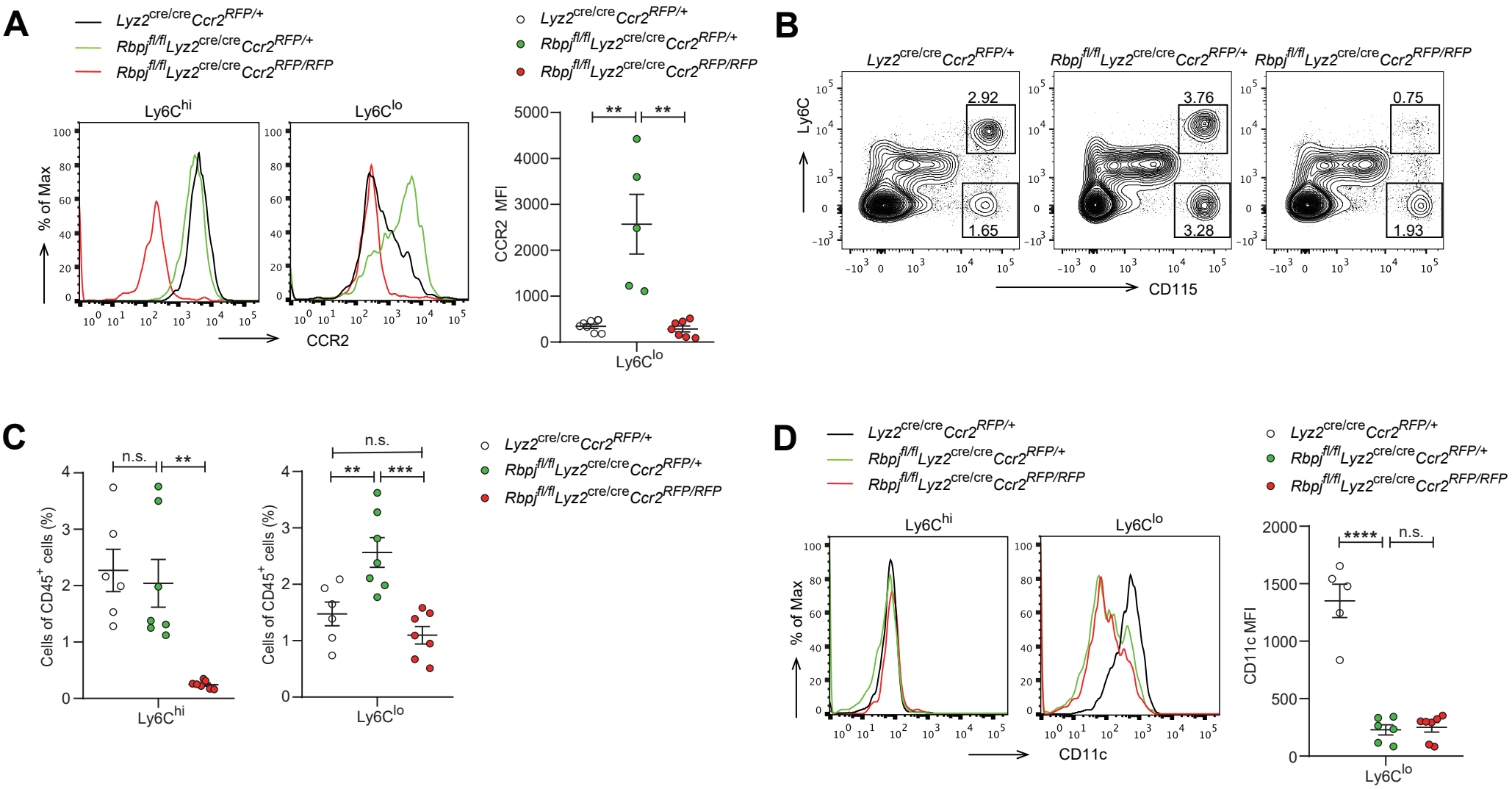

A
Lyz2cre/creCcr2RFP/+
Rbpjfl/flLyz2cre/creCcr2RFP/+
Rbpjfl/flLyz2cre/creCcr2RFP/RFP
Ly6Chi
Ly6Clo
% of Max
CCR2
CCR2 MFI
**
**
B
Lyz2cre/creCcr2RFP/+
Rbpjfl/flLyz2cre/creCcr2RFP/+
Rbpjfl/flLyz2cre/creCcr2RFP/RFP
Ly6C
CD115
2.92
1.65
3.76
3.28
0.75
1.93
C
n.s.
**
n.s.
**
***
Cells of CD45+ cells (%)
Ly6Chi
Ly6Clo
D
Lyz2cre/creCcr2RFP/+
Rbpjfl/flLyz2cre/creCcr2RFP/+
Rbpjfl/flLyz2cre/creCcr2RFP/RFP
Ly6Chi
Ly6Clo
% of Max
CD11c
CD11c MFI
****
n.s.
Ly6Clo

Figure 5

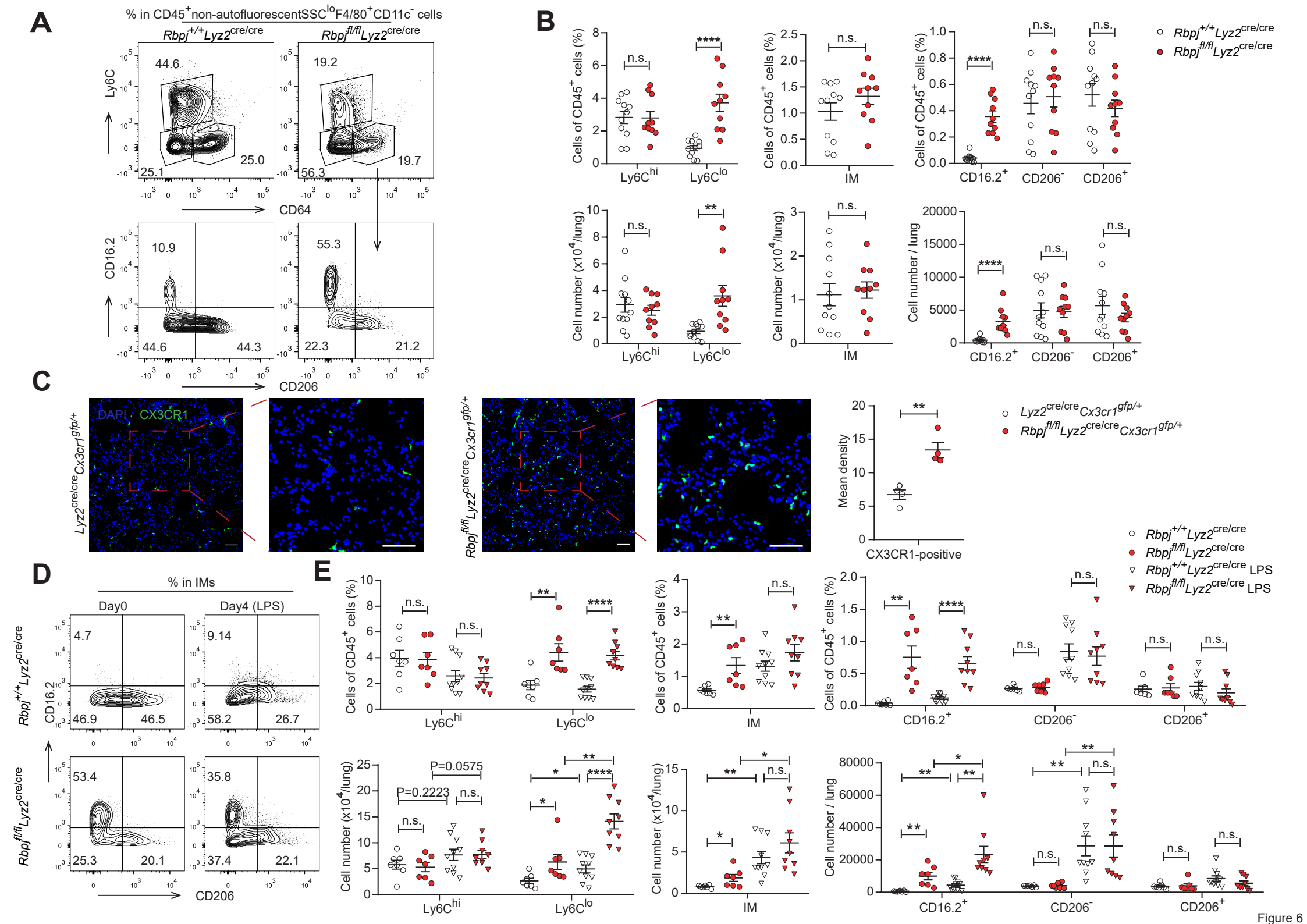

A
% in CD45+non-autofluorescentSSCloF4/80+CD11c- cells
Rbpj+/+Lyz2cre/cre
Rbpj fl/flLyz2cre/cre
Ly6C
CD64
CD16.2
CD206
44.6
25.0
25.1
19.2
19.7
56.3
10.9
44.6
44.3
55.3
22.3
21.2
B
Cells of CD45+ cells (%)
Cell number (x10^4/lung)
Cell number / lung
Ly6Chi
Ly6Clo
IM
CD16.2+
CD206-
CD206+
n.s.
**
****
C
DAPI
CX3CR1
Lyz2cre/creCx3cr1gfp/+
Rbpj fl/flLyz2cre/creCx3cr1gfp/+
Mean density
CX3CR1-positive
D
% in IMs
Day0
Day4 (LPS)
4.7
46.9
46.5
9.14
58.2
26.7
53.4
25.3
20.1
35.8
37.4
22.1
E
P=0.0575
P=0.2223
Rbpj+/+Lyz2cre/cre LPS
Rbpj fl/flLyz2cre/cre LPS

Figure 6

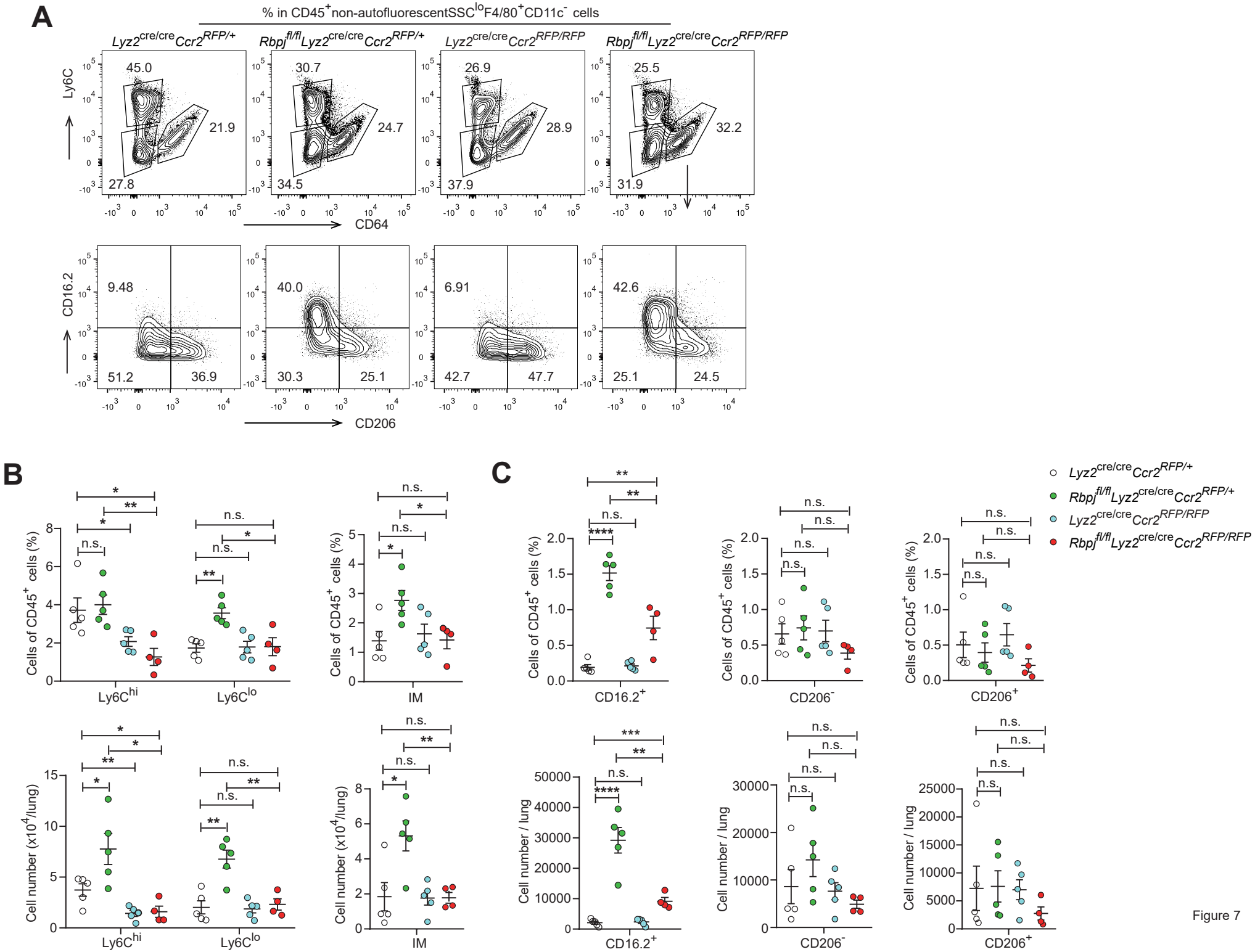

A
% in CD45+non-autofluorescentSSCloF4/80+CD11c- cells
Lyz2cre/creCcr2RFP/+
Rbpjfl/flLyz2cre/creCcr2RFP/+
Lyz2cre/creCcr2RFP/RFP
Rbpjfl/flLyz2cre/creCcr2RFP/RFP
Ly6C
CD64
45.0
21.9
27.8
30.7
24.7
34.5
26.9
28.9
37.9
25.5
32.2
31.9
CD16.2
CD206
9.48
51.2
36.9
40.0
30.3
25.1
6.91
42.7
47.7
42.6
25.1
24.5
B
Cells of CD45+ cells (%)
Ly6Chi
Ly6Clo
IM
Cell number (x10^4/lung)
C
CD16.2+
CD206-
CD206+
Cell number / lung
Lyz2cre/creCcr2RFP/+
Rbpjfl/flLyz2cre/creCcr2RFP/+
Lyz2cre/creCcr2RFP/RFP
Rbpjfl/flLyz2cre/creCcr2RFP/RFP

Figure 7

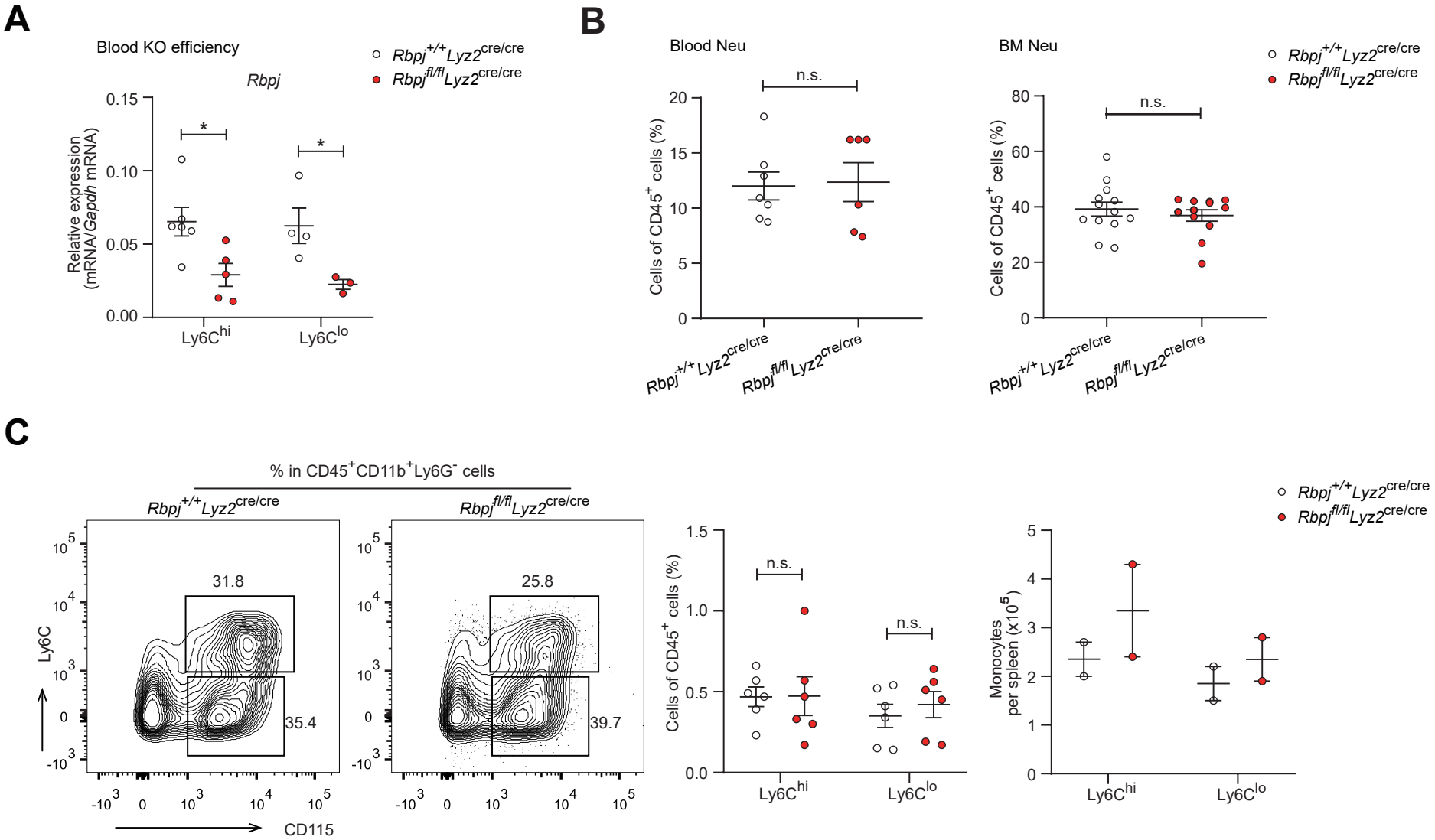


Figure 1- figure supplement 1

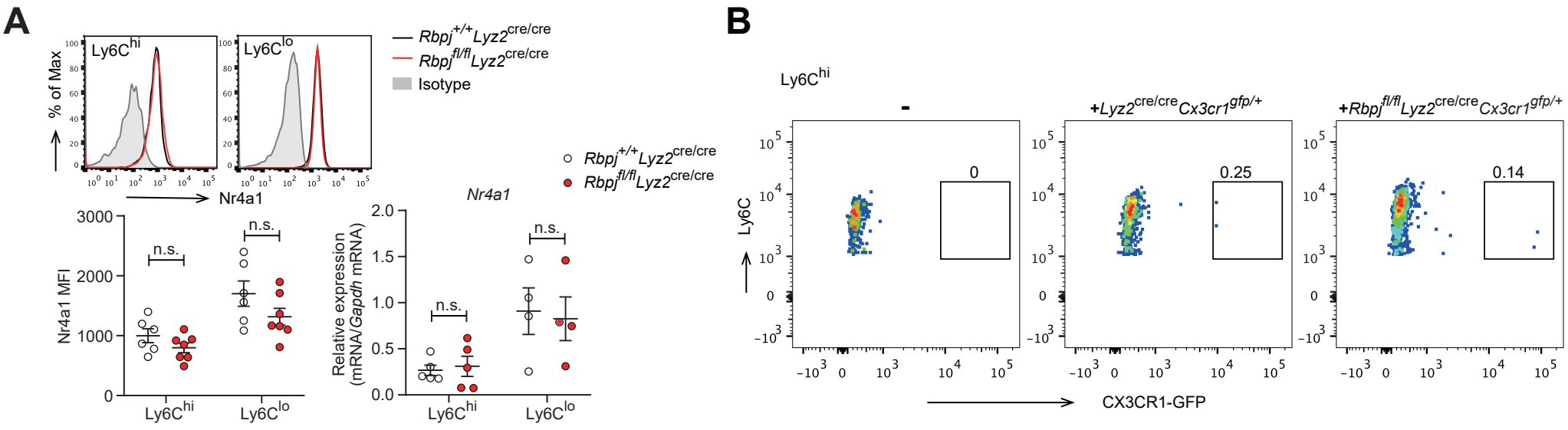


Figure 2- figure supplement 1

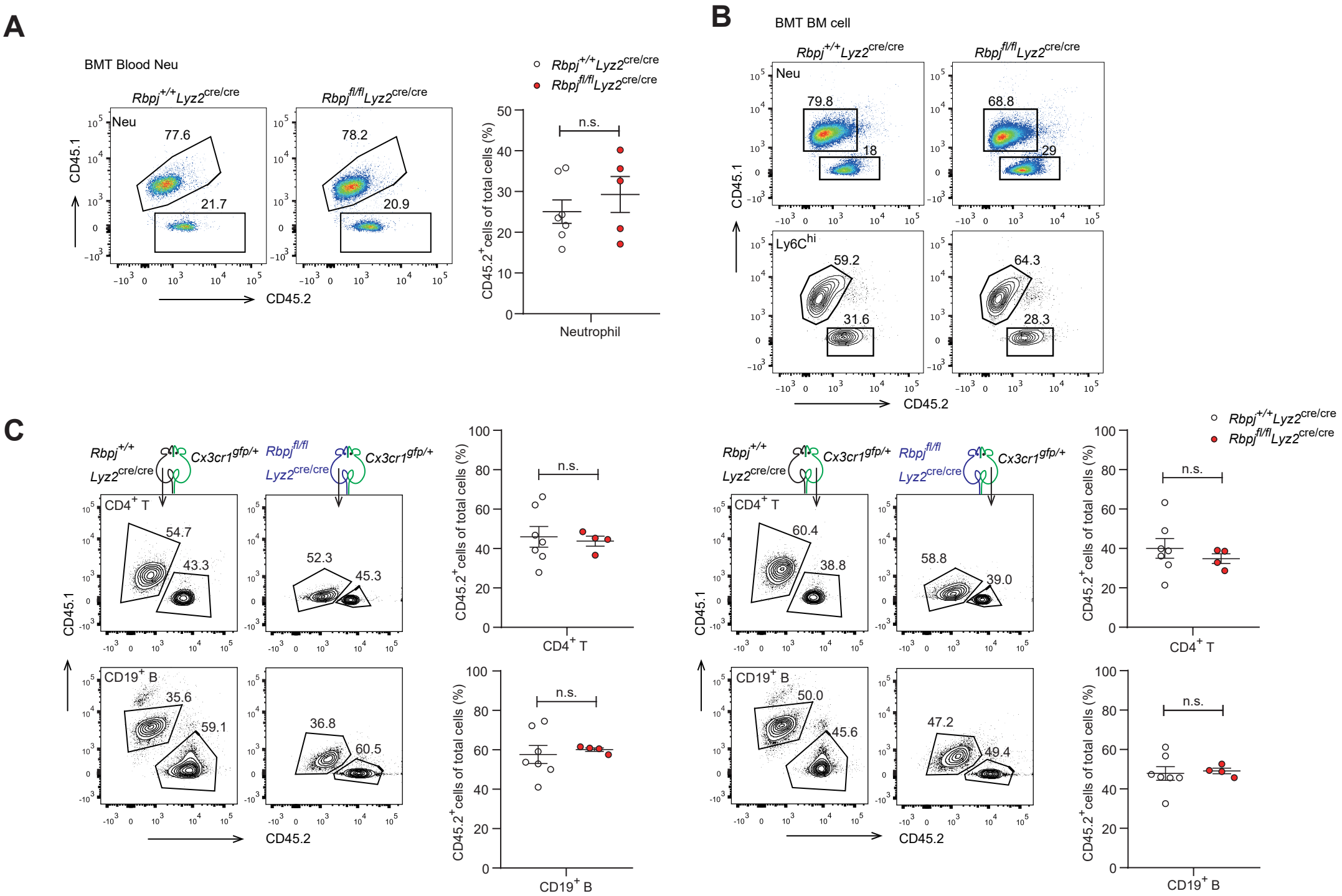


Figure 3- figure supplement 1

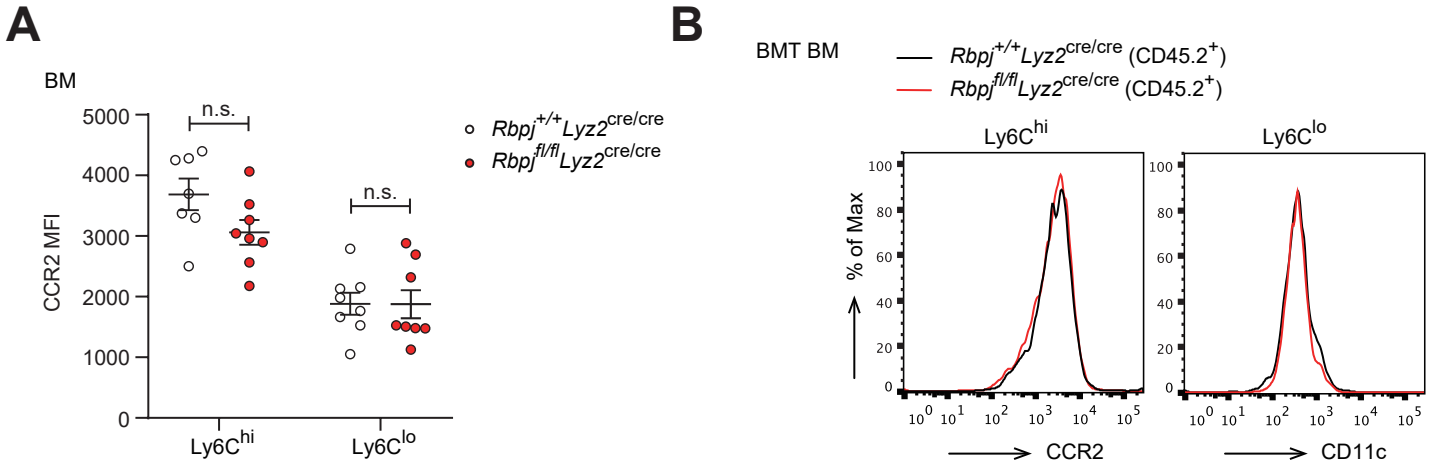


Figure 4- figure supplement 1

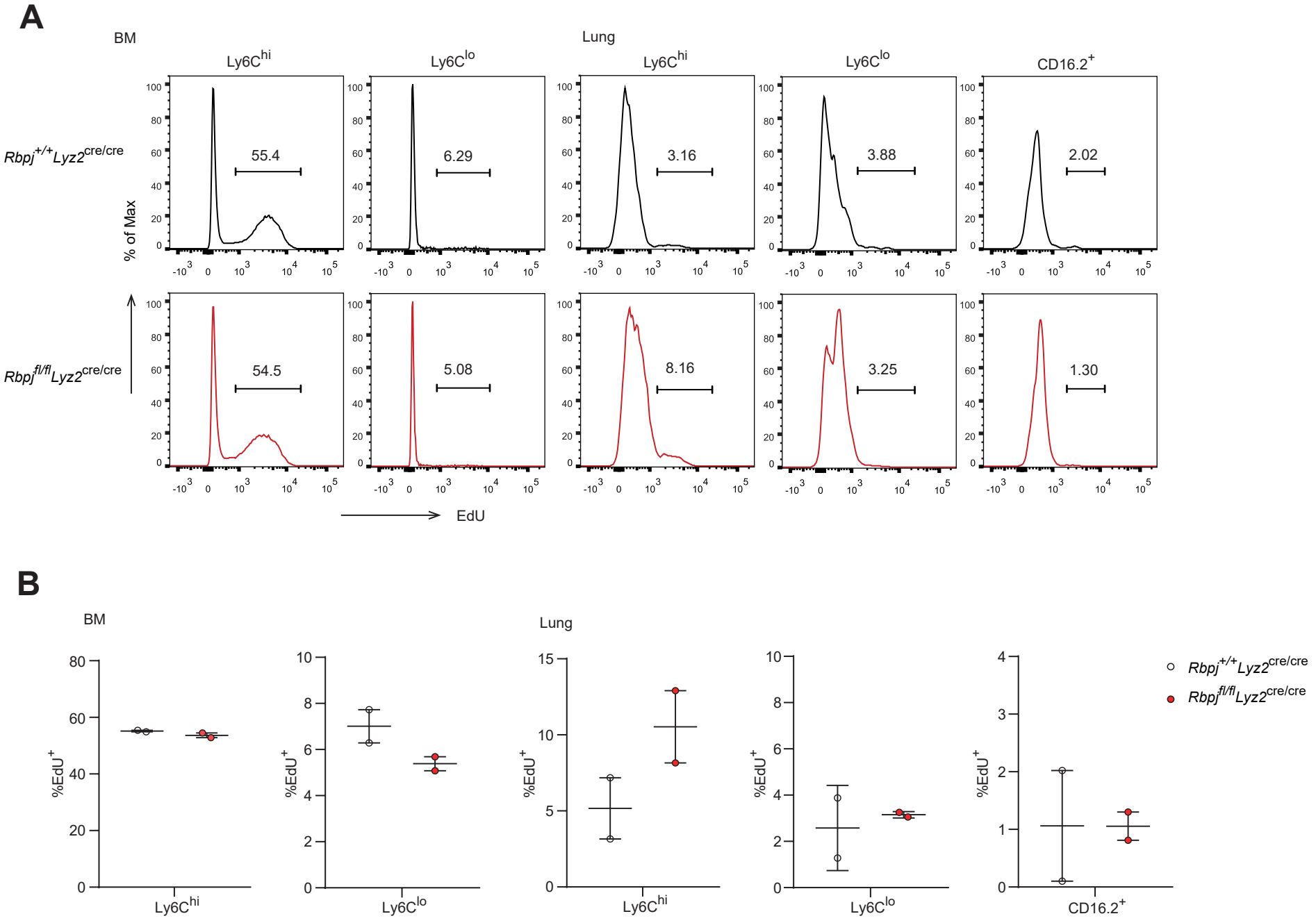
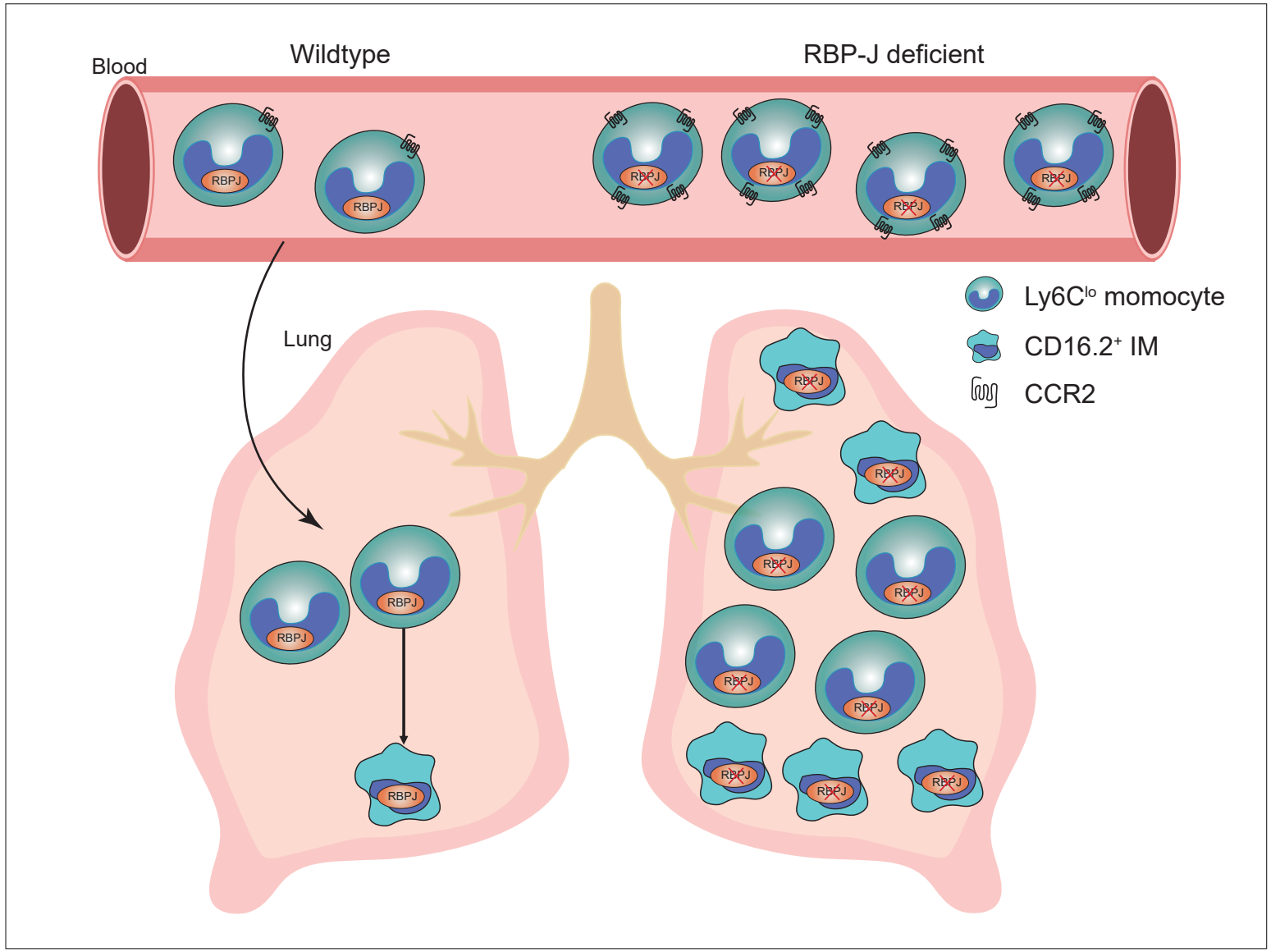


Figure 6- figure supplement 1



Supplementary Figure 7