Supporting Information:

1) **Materials and Methods**
2) **Legend for Dataset S1**
3) **Supplementary Figures**
4) **Supplementary Tables**

SUPPLEMENTARY MATERIALS AND METHODS

Animals

The C57BL/6J (CD45.1 and CD45.2), *Cx3cr1*$^{-/-}$, *Csf1r*^{CreER} and *Rosa26*$^{LSL\text{-}YFP}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). *Flt3*^{Cre} transgenic mice were kindly provided by Dr. Kory Levin (Washington University School of Medicine, St. Louis, MO, USA). The *Ccr2*$^{-/-}$ mice were originally kindly provided by Dr. Israel Charo (1), which were backcrossed nine times with the C57BL/6J mice as previously described (2). Because the *Flt3*^{Cre} transgene located on the Y chromosome, male *Flt3*^{Cre} mice were crossed with female *Rosa26*$^{LSL\text{-}YFP}$ mice to generate *Flt3*^{Cre}-*Rosa26*$^{LSL\text{-}YFP}$ mice. Similarly, male *Flt3*^{Cre}-*Rosa26*$^{LSL\text{-}YFP}$ mice were crossed with female *Ccr2*$^{-/-}$ mice to obtain *Flt3*^{Cre}-*Rosa26*$^{LSL\text{-}YFP}$-*Ccr2*$^{+/-}$ mice, which were further crossed with female *Ccr2*$^{-/-}$ mice to generate *Flt3*^{Cre}-*Rosa26*$^{LSL\text{-}YFP}$-*Ccr2*$^{-/-}$ mice. The *Csf1r*^{CreER}-*Rosa26*$^{LSL\text{-}YFP}$ mice were generated by crossbreeding *Csf1r*^{CreER} mice with *Rosa26*$^{LSL\text{-}YFP}$ mice. Our study protocols were approved by the Institutional Animal Care and Use Committee at the Boston University Medical Center (Boston, MA, USA) and at the University of Texas Southwestern Medical Center (Dallas, TX, USA).

Labeling yolk sac primitive macrophages and tracing their progeny.

Csf1r^{CreER} mice were crossed with *Rosa26*$^{LSL\text{-}YFP}$ mice to obtain *Csf1r*^{CreER}-*Rosa26*$^{LSL\text{-}YFP}$ mice. To date the age of embryos, limited-time cross was setup by putting male and female mice together after 10:00 pm, and vaginal plug was checked the next day before 8:00 am. Females

with the presence of the plug was designated as day 0 post-conception before 12:00 pm and as day 0.5 after 12:00 pm.

4-Hydroxytamoxifen (4-OH) (H-7904, Sigma Aldrich, St Louise, MO, USA) induction of Cre expression, Cre/lox recombination, and YFP expression by the CSF1R-expressing yolk sac primitive MPs, was performed as described by Nakamura E. et al. (3). Briefly, 4-OH was first dissolved at a concentration of 100 mg/ml in 100% Ethanol and then diluted to a final concentration of 10 mg/ml into sterile sunflower oil (S-5007, Sigma Aldrich). The solution was then injected intraperitoneally at a dose of 2 mg/mouse at day 8.5 of embryo (E 8.5). To counteract the mixed estrogen agonist effect of 4-OH, which can result in late fetal abortions, progesterone (P-3972, Sigma Aldrich) dissolved in sunflower oil was injected simultaneously at a dose of 1 mg/mouse. Four weeks after birth, the pups were genotyped for Cre, and tissues were collected for MP analysis following perfusion.

Bone marrow transplantation

Bone marrow cells were collected from the tibias and femurs of the donor mice (CD45.2 background). After removing red blood cells using RBC lysis buffer (Biolegend, San Diego, CA, USA), bone marrow cells were re-suspended in sterile PBS at a concentration of 2×10^7 cells/ml. Recipient mice (CD45.1 background, 6-7 weeks of age) were sub-lethally irradiated at a dose of 800 rad. Three hours after irradiation, donor bone marrow cells were intravenously injected into recipient mice at a dose of 2×10^6 cells/mouse.

In vivo intravascular staining of CD45$^+$ cells and perfusion

Mice were anesthetized by intraperitoneal injection of Ketamine/Xylazine solution provided by the Animal Science Center of Boston University. Fluorescence-labeled anti-CD45 antibody was then injected intravenously at a dose of 10 μg/injection. Five minutes after injection, blood was sampled and mice were subsequently perfused with PBS for 10 min using a peristaltic pump.

Preparation of single-cell suspension, flow cytometry analysis (FACS) and cell sorting

Peritoneal cells were collected by washing with PBS. Cells were then pelleted by centrifuging at 300 g for 10 minutes and re-suspended in FACS staining buffer (PBS containing 2% normal mouse serum (Invitrogen, Frederick, MA, USA) and 2% BSA (Sigma-Aldrich)).

For all other tissues, single-cell suspension was prepared by collagenase/dispase digestion (4-6). Briefly, each tissue was minced in 2.5 ml digestion solution (1 U/ml collagenase B and 1 U/ml dispase II (Roche Diagnostics, Indianapolis, IN, USA) in PBS) and incubated at 37ºC for 1 hour. The reaction was terminated by adding 10 ml PBS containing 10% fetal bovine serum (FBS). The mixture was then filtered through a 70-μm cell strainer and centrifuged at 250 g for 5 minutes. The pellet was collected and the supernatant was centrifuged again at 250 g for 5 minutes. The pellet was combined with the pellet from the first centrifugation, washed with PBS, and centrifuged at 670 g for 10 minutes. The pellet was re-suspended in 3 ml PBS and filtered through a 40-μm cell strainer. For brain, cell suspension was layered on equal volume of 50% isotonic Percoll (Sigma-Aldrich) and centrifuged at 600 g for 25 minutes. Supernatant was then

discarded and cell pellet was re-suspended in FACS staining buffer. For skeletal muscle, heart and lung, cell suspension was layered on equal volume of the Lympholyte-M solution (Cedarlane, Burlington, NC, USA) and centrifuged at 2,095 g for 45 minutes. Cells at the interface were collected, centrifuged at 670 g for 10 minutes, and re-suspended in FACS staining buffer.

The antibodies used for flow cytometry were listed in the Supplementary Table 3. We used fluorescence-labelled corresponding normal IgG isotypes as negative controls for gating. All flows were done using LSR II (BD Bioscience, San Jose, CA, USA), and data were analyzed using Flowjo software (Tree Star, Inc., Ashland, OR, USA). Cell sorting was performed by the Flow Cytometry Core of University of Texas Southwestern Medical Center.

CD68 immunostaining

After perfusion with PBS for 10 minutes to remove intravascular circulating cells, muscles were collected with epimysium included and frozen in liquid nitrogen-chilled isopentane, sectioned at 8 μm, stained with anti-CD68 antibody (FA-11, Bio-Rad, Hercules, CA, USA) as described previously (7).

Giemsa Staining

Slides containing a single layer of sorted macrophages were prepared by cytospin. The Giemsa Stain reagent (Sigma Aldrich) was then used for staining following the manufacturer's instruction.

Single cell-based mRNA sequencing

FACS-sorted MPs from quadriceps, diaphragm, peritoneum and lung of mice at 10 weeks of age were collected by centrifugation and re-suspended in FACS buffer at a final concentration of 1,000 cells/ml. To minimize the individual variability, cells collected from 10 mice were pooled together for the analysis. Single-cell encapsulation with beads using 10× Genomics Chromium Single Cell 3' v2 kit (PN 120235 and PN 120234 Module 1) was performed following the manufacturer instructions (User Guide Rev A, 10× Genomics, Pleasanton, CA, USA). Briefly, 4 wells of a 10× microfluidic chip (PN 120236) were loaded each with an individual sample to target 5,000 cells per sample. Single-cell gel beads in emulsion (GEMs) were generated and reverse-transcription was performed in the emulsion prior to 12 cycles of cDNA amplification. Quality control and quantification of the amplified cDNA were assessed by Bioanalyzer. Libraries were constructed according to the manufacturer instructions (PN 120234 Module 2). Each library was tagged with a different index for multiplexing (PN 120262) during sequencing. Library quality controls were assessed by Bioanalyzer and quantified by Qubit and quantitative PCR (KAPA Biosystems Quantification for Illumina platforms). Sequencing was performed on a HiSeq 2500 (Illumina, Inc., San Diego, CA, USA) using the following read length: 26 bp Read1 (cell barcode + UMI), 8 bp i7 index (sample index), and 98 bp Read2 (insert).

Single cell-based mRNA sequencing analysis

Cell Ranger version 3.0.0 (10x Genomics) was used to process the raw sequencing data. Raw BCL files were converted to FASTQ files and aligned to mouse mm10 reference transcriptome. Transcript counts of each cell were quantified using barcoded UMI and 10xBC sequences. The gene x cell expression matrices were loaded to the R package Seurat version 3.0.0 for downstream analyses. Cells with low quality were filtered out based on at least 200 genes being detected per 1,000 UMIs and mitochondrial gene content. Only those genes found in more than 3 cells were retained. "LogNormalize", the Seurat default global-scaling normalization method, was used. In this method UMI counts are first scaled by the total sequencing depth ('size factors') followed by pseudocount addition and log-transformation.

With the above filters in place we obtained 13,611 genes from 3,239 cells from the quadriceps muscle macrophage sample, 13,231 genes from 2,730 cells from the diaphragm muscle macrophages, 14,003 genes from 3,542 cells from the peritoneal macrophage sample and 14,759 genes 4,919 cells from the lung alveolar macrophages. The highly variable features (genes) for this data were then calculated with "FindVariableFeatures" in Seurat which uses a mean variability plot. Here average expression and dispersion per feature was calculated and features were divided into bins to get z-scores for dispersion per bin.

After regressing out the number of UMI and percentage of mitochondrial gene content, the resultant data were scaled and dimensional reduction was performed with principle component

analysis and visualization using tSNE plots. The number of Principal Components (n=5~7) to use in downstream analysis was calculated based on a Jackstraw and elbow plot of the same. For each sample, a Shared Nearest Neighbor (SNN) Graph was constructed with "FindNeighbors" in Seurat by determining the k-nearest neighbors of each cell. The clusters were then identified by optimizing this SNN modularity using the "FindClusters" function. This allowed for a sensitive detection of rare cell types. We obtained 5-7 clusters for each sample with a resolution of 0.3. There were small clusters identified as non-MPs and therefore were removed from the following analysis. Four clusters for each muscle sample were presented and further analyzed.

The differential expression testing was carried out using "negbinom" test in Seurat with a Likelihood ratio test which assumes an underlying negative binomial distribution suitable for UMI datasets like the 10X single cell transcriptome. This was carried out to get the top markers for each cluster. The genes identified as relatively overexpressed in a cluster as compared to all other cells in a sample were termed markers. A heatmap visualization of the top 15 markers per cluster has been shown for each sample and along with the functional enrichment analysis used to identify and name the clusters.

Specific marker distribution in clusters were represented as featureplots, violinplots and heatmaps using the Seurat tool.

To compare the clusters and cell types in all the samples, they were combined using the method described by Stuart T. et al. (8), where Canonical Correlation Analysis is applied to identify

correspondences between samples and create a common reference. A t-SNE visualization was plotted to highlight the commonalties between samples Diaphragm and Quadriceps macrophages as compared to Peritoneal and Lung tissue macrophages.

Functional Enrichment Analysis

The top 65-70 markers [adj. *p*-value < 0.05] per cluster were then used to identify the functional enrichment categories using gprofiler (9, 10). We used pathway gene sets from biological processes of Gene Ontology (http://www.geneontology.org/) and molecular pathways of Reactome (http://www.reactome.org/), CORUM (11) and KEGG (12). Results from different databases were compared to ensure the reliability of the analysis. Results presented were from KEGG.

Statistical analysis

Data were analyzed with GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) and individual values were plotted as Interleaved Scattered Plot with mean value indicated. The Mann-Whitney test was performed to compare between two groups; the Kruskal-Wallis test followed by Dunn's test was performed to compare multiple (>=3) groups. A *p* value of <0.05 was considered statistically significant.

References:

1. Landin Boring JG, Stephen W. Chensue, Steven L. Kunkel, Robert V. Farese, Jr.,Hal E. Broxmeyer, and Israel F. Charo (1997) Impaired Monocyte Migration and Reduced Type 1 (Th1) Cytokine Responses in C-C Chemokine Receptor 2 Knockout Mice. *The Journal of Clinical Investigation* 100(10):2552-2561.
2. Peters W, Dupuis M, & Charo IF (2000) A mechanism for the impaired IFN-gamma production in C-C chemokine receptor 2 (CCR2) knockout mice: role of CCR2 in linking the innate and adaptive immune responses. *J Immunol* 165(12):7072-7077.
3. Nakamura E, Nguyen MT, & Mackem S (2006) Kinetics of tamoxifen-regulated Cre activity in mice using a cartilage-specific CreER(T) to assay temporal activity windows along the proximodistal limb skeleton. *Dev Dyn* 235(9):2603-2612.
4. Wang X, Zhao W, Ransohoff RM, & Zhou L (2016) Identification and Function of Fibrocytes in Skeletal Muscle Injury Repair and Muscular Dystrophy. *J Immunol* 197(12):4750-4761.
5. Zhao W, Lu H, Wang X, Ransohoff RM, & Zhou L (2016) CX3CR1 deficiency delays acute skeletal muscle injury repair by impairing macrophage functions. *FASEB J* 30(1):380-393.
6. Zhao W, Wang X, Ransohoff RM, & Zhou L (2016) CCR2 deficiency does not provide sustained improvement of muscular dystrophy in mdx5cv mice. *FASEB J*.
7. Hill NR, Cook HT, Pusey CD, & Tarzi RM (2018) RIPK3-deficient mice were not protected from nephrotoxic nephritis. *BMC Nephrol* 19(1):61.
8. Stuart T*, et al.* (2019) Comprehensive Integration of Single-Cell Data. *Cell* 177(7):1888-1902 e1821.

9. Reimand J, Kull M, Peterson H, Hansen J, & Vilo J (2007) g:Profiler--a web-based toolset for functional profiling of gene lists from large-scale experiments. *Nucleic Acids Res* 35(Web Server issue):W193-200.

10. Raudvere U*, et al.* (2019) g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Res* 47(W1):W191-W198.

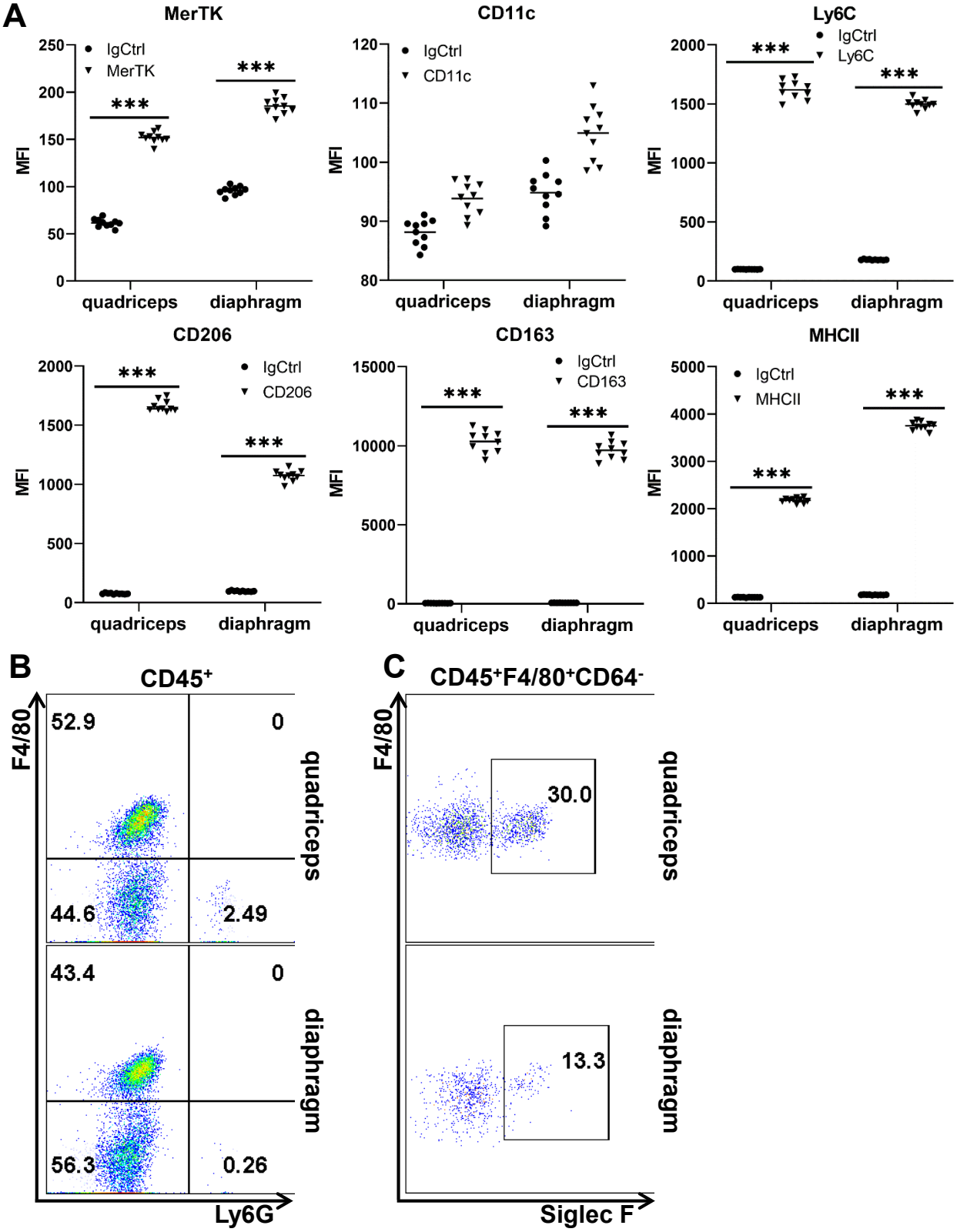
11. Giurgiu M*, et al.* (2019) CORUM: the comprehensive resource of mammalian protein complexes-2019. *Nucleic Acids Res* 47(D1):D559-D563.

12. Kanehisa M, Furumichi M, Tanabe M, Sato Y, & Morishima K (2017) KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res* 45(D1):D353-D361.

LEGEND FOR DATASET S1

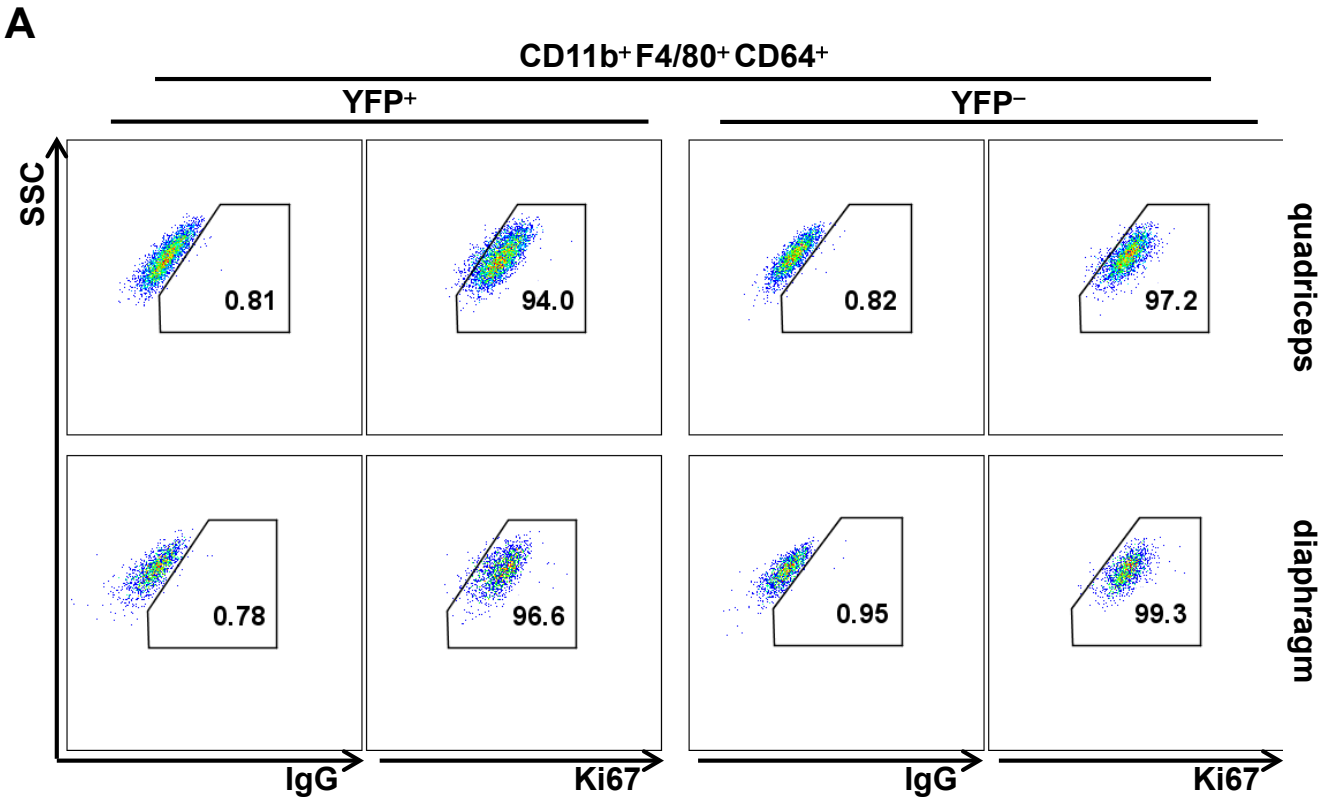
List of genes that are differentially expressed ($Log_2FC \geq 0.5$) by skeletal muscle resident macrophages (SMRMPs) compared to peritoneal macrophages and lung alveolar macrophages.

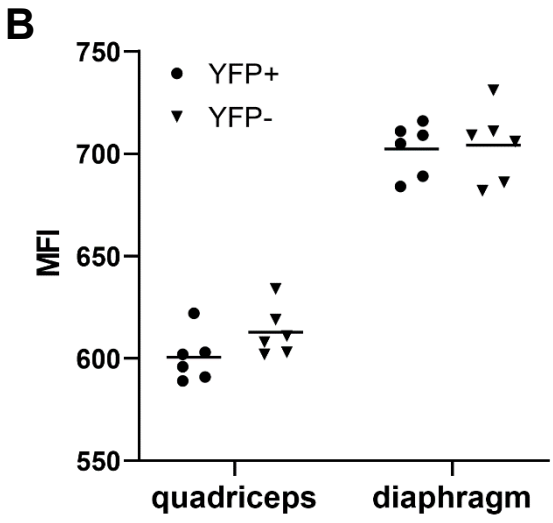
S. Figure 1



Resident MPs are present in adult mouse skeletal muscle in the steady state. **A.** Quantification of expression level gauged by FACS mean fluorescence intensity (MFI) of MerTK, CD11c, Ly6C, CD206, CD163 and MHCII by ivCD45^{-}CD45^{+}CD11b^{+}F4/80^{+}CD64^{+} resident MPs. **B.** FACS analysis of F4/80 and Ly6G expression by CD45^{+} intramuscular cells. **C.** FACS analysis of Siglec F expression by CD45^{+}F4/80^{+}CD64^{-} cells.

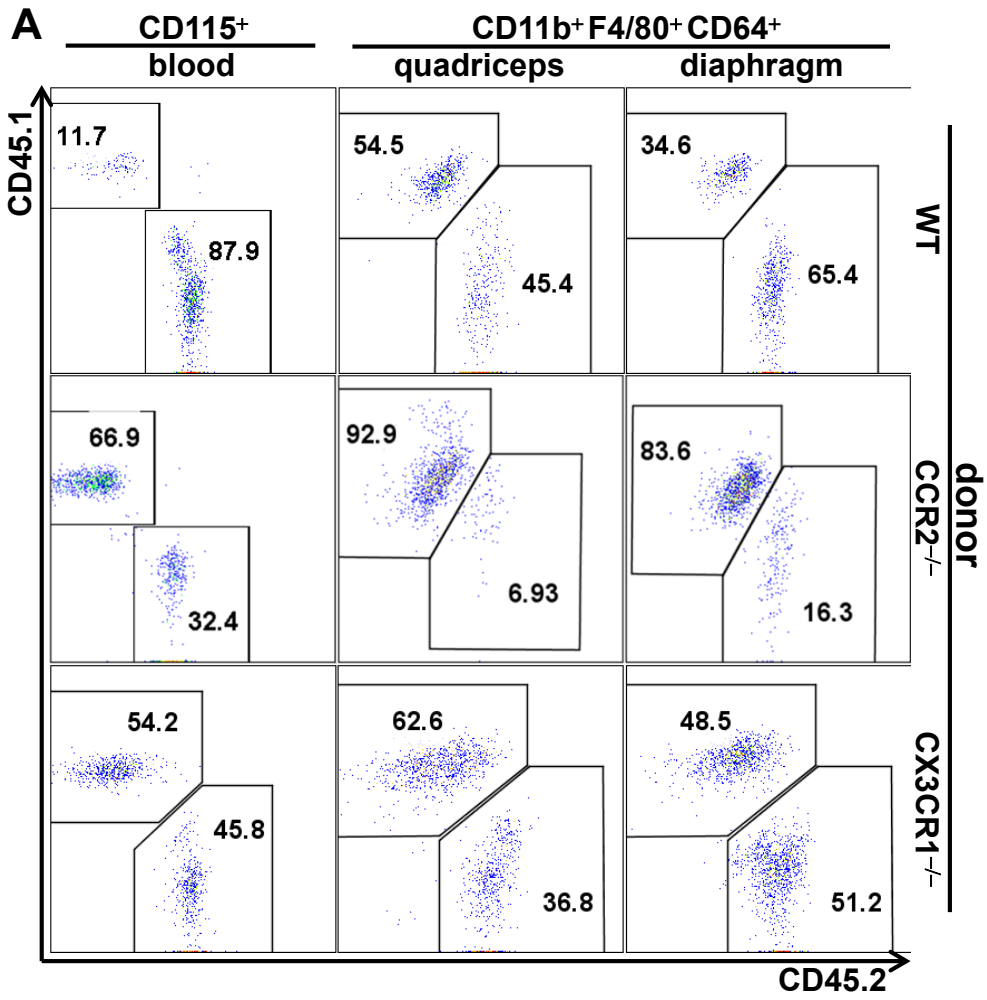
S. Figure 2

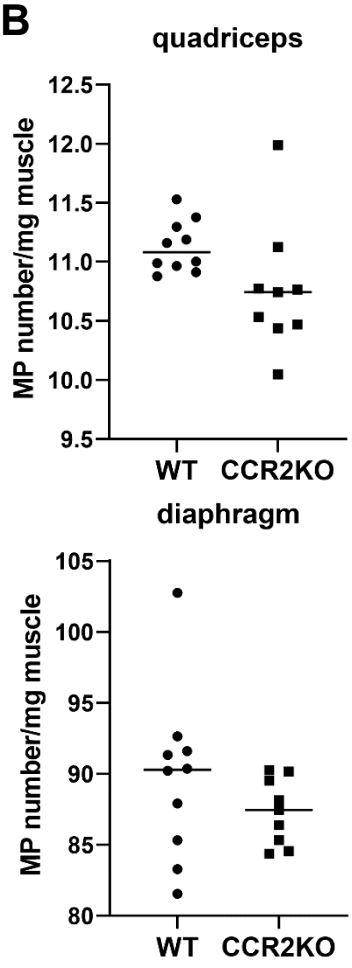


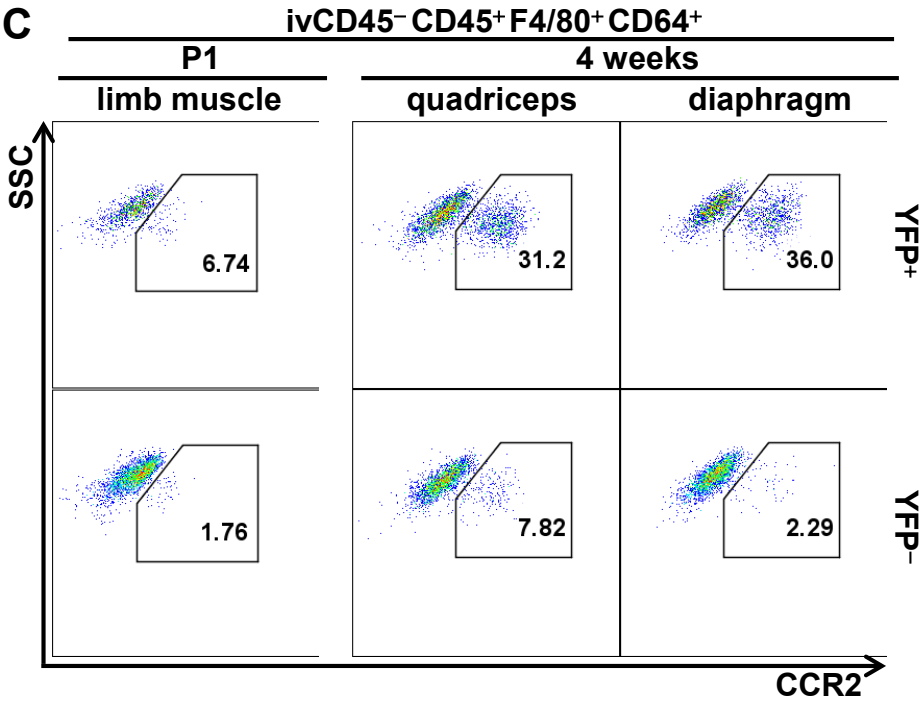


Resident MPs of HSC (YFP^{+}) and non-HSC (YFP^{-}) origins proliferate similarly. FACS analysis of Ki67 expression by skeletal muscle resident MPs from *Flt3^{Cre}-Rosa26$^{LSL\text{-}YFP}$* mice: **A.** dot blot; **B.** quantification of Ki67 expression based on mean fluorescence intensity (MFI). N = 6 mice/each group.

S. Figure 3

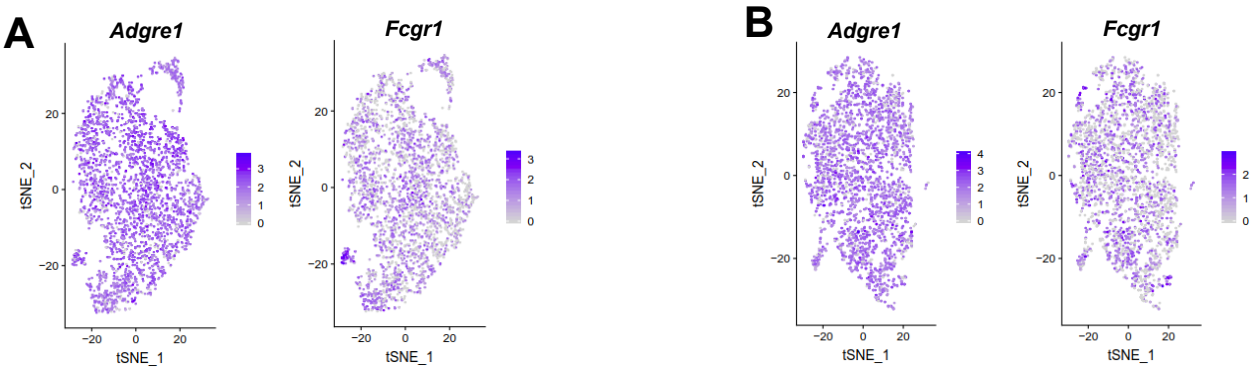


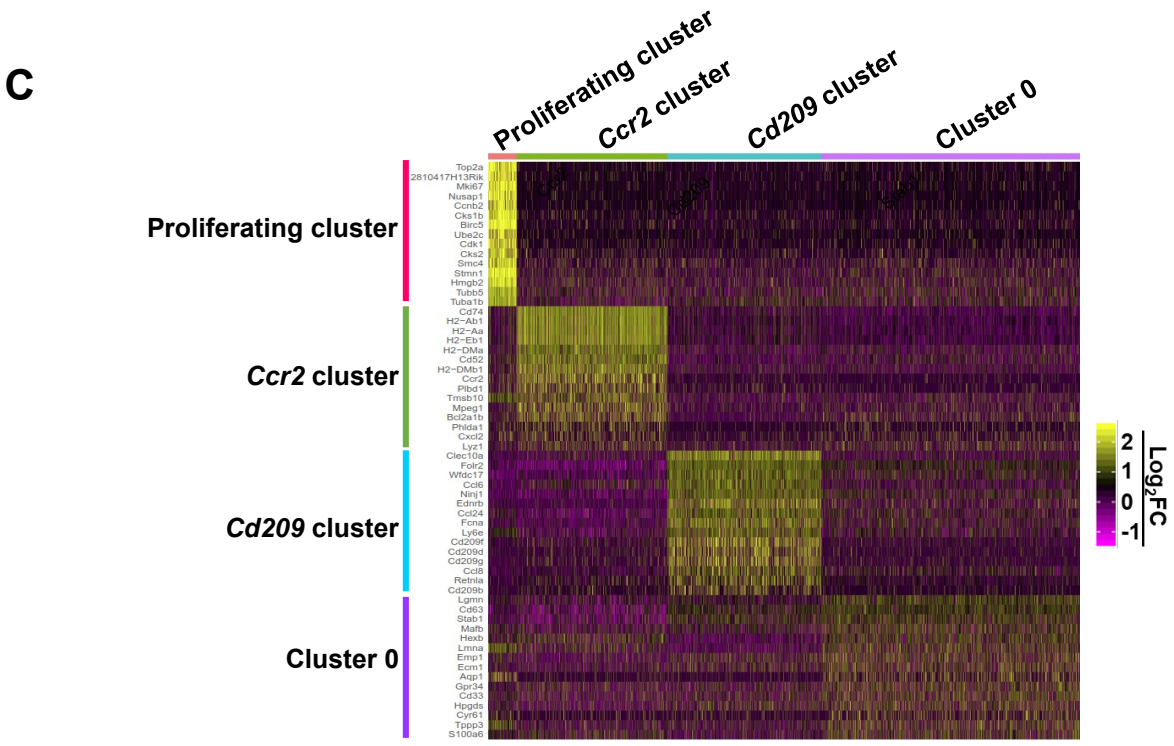




Bone marrow HSC-derived blood MOs contribute to postnatal skeletal muscle resident MPs. A. FACS analysis of CD45.1 and CD45.2 expression by blood MOs and skeletal muscle resident MPs at 4 weeks post bone marrow transplantation. **B.** Comparison of the skeletal muscle resident MP numbers in 4-week-old WT and CCR2KO mice. **C.** FACS analysis of CCR2 expression by skeletal muscle resident MPs in the *Flt3*^{Cre}-*Rosa26*$^{LSL\text{-}YFP}$ mice at indicated ages.

S. Figure 4

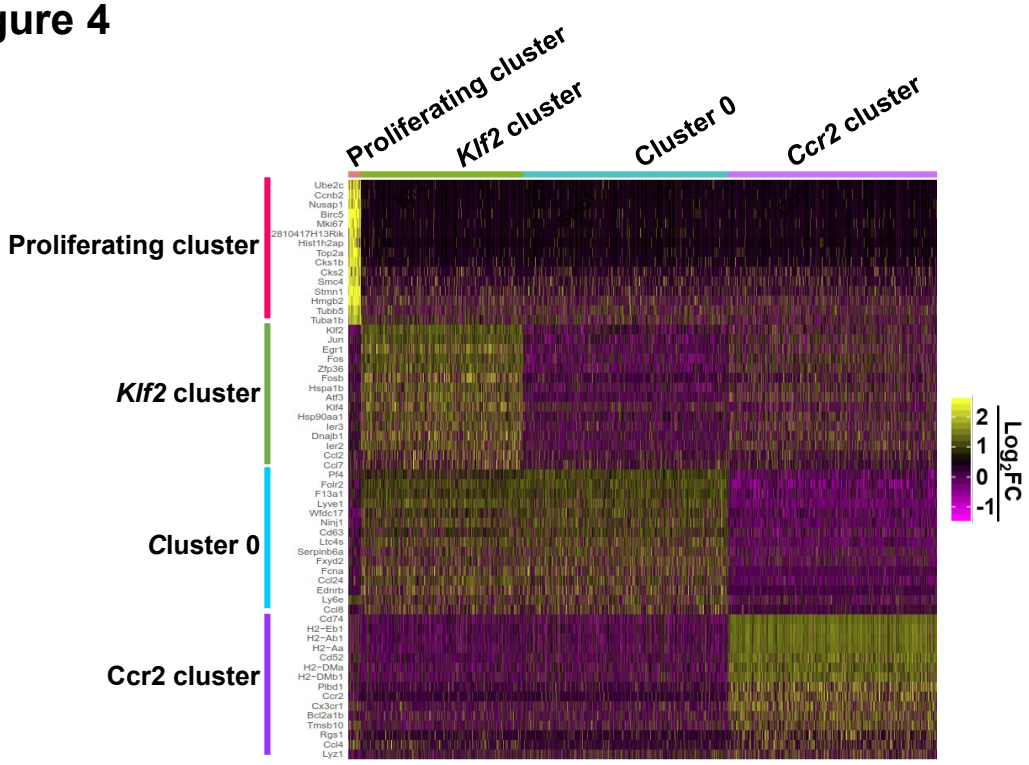




Muscle type	Quadriceps			
Cluster name	Proliferating cluster	Ccr2 cluster	Cd209 cluster	Cluster 0
gene name	Stmn1	Cd74	Cd209f	Aqp1
	Hmgb2	H2-Eb1	Retnla	Tppp3
	Birc5	H2-Aa	Cd209d	Ecm1
	Top2a	H2-Ab1	Cd209g	Cyr61
	Tubb5	Ccr2	Clec10a	Mafb
	Tuba1b	H2-DMb1	Ccl8	S100a6
	2810417H13Rik	Cd52	Fcna	Cd63
	Ube2c	H2-DMa	Ednrb	Emp1
	Mki67	Cxcl2	Ccl6	Stab1
	Nusap1	Tmsb10	Cd209b	Gpr34
	Ccnb2	Phlda1	Wfdc17	Lgmn
	Cks1b	Bcl2a1b	Ccl24	Hexb
	Smc4	Mpeg1	Ly6e	Cd33
	Cdk1	Plbd1	Folr2	Lmna
	Cks2	Lyz1	Ninj1	Hpgds

S. Figure 4

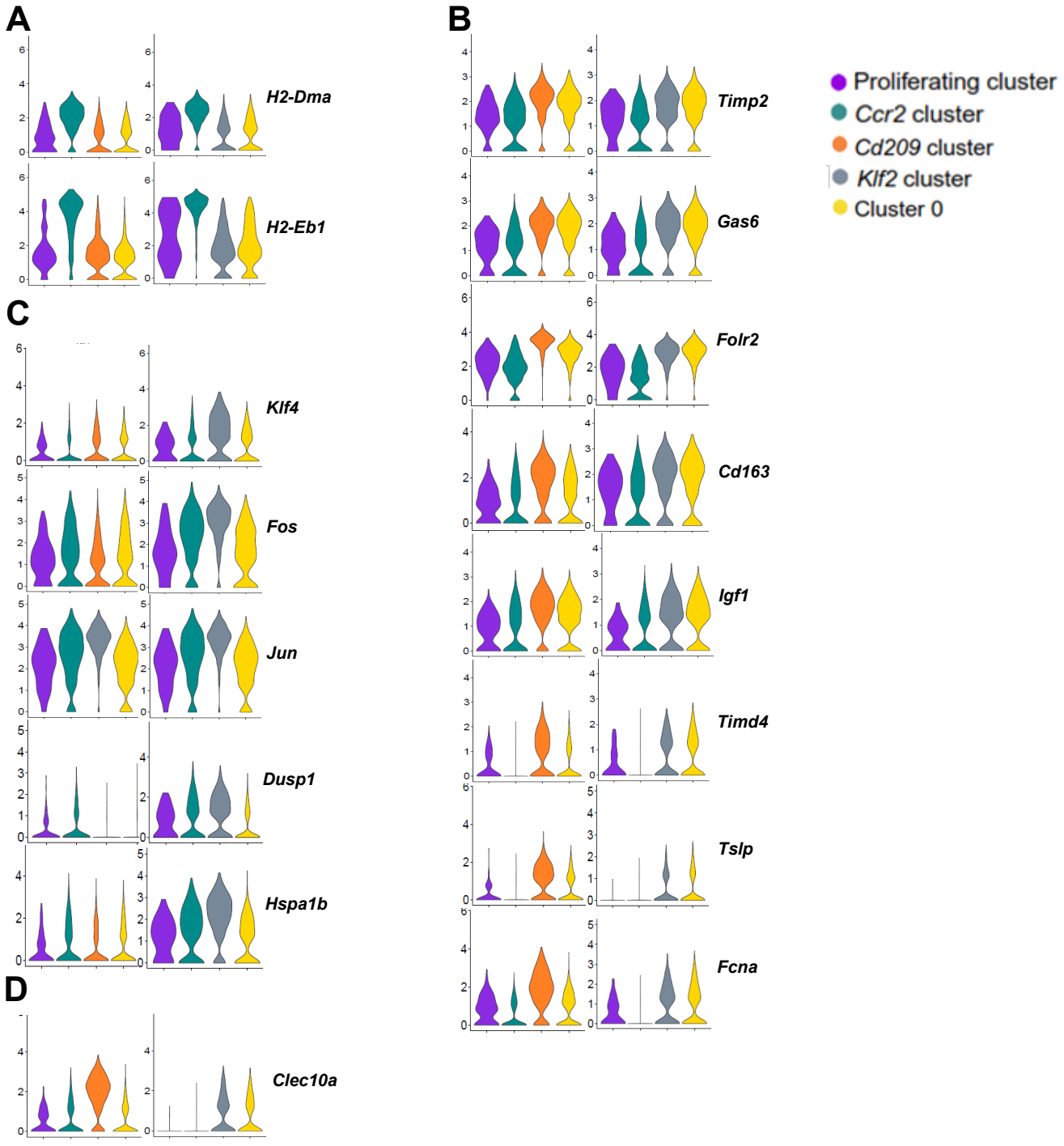
D



Muscle type	Diaphragm			
Cluster name	Proliferating cluster	Ccr2 cluster	Klf2 cluster	Cluster 0
gene name	Stmn1	H2-Eb1	Egr1	Folr2
	Hmgb2	Cd74	Ccl7	Lyve1
	Birc5	H2-Aa	Klf4	Ninj1
	Top2a	H2-Ab1	Dnajb1	Ltc4s
	2810417H13Rik	Cd52	Ccl2	Wfdc17
	Tubb5	Ccr2	Klf2	Pf4
	Tuba1b	H2-DMb1	Fosb	F13a1
	Ube2c	H2-DMa	Atf3	Ccl8
	Mki67	Rgs1	Hspa1b	Fxyd2
	Smc4	Plbd1	Fos	Ly6e
	Ccnb2	Cx3cr1	Zfp36	Fcna
	Hist1h2ap	Tmsb10	Jun	Fcgrt
	Cks1b	Clec4b1	Hsp90aa1	Ccl24
	Nusap1	Ccl4	Rhob	Stab1
	Cks2	Bcl2a1b	Ier3	Serpinb6a

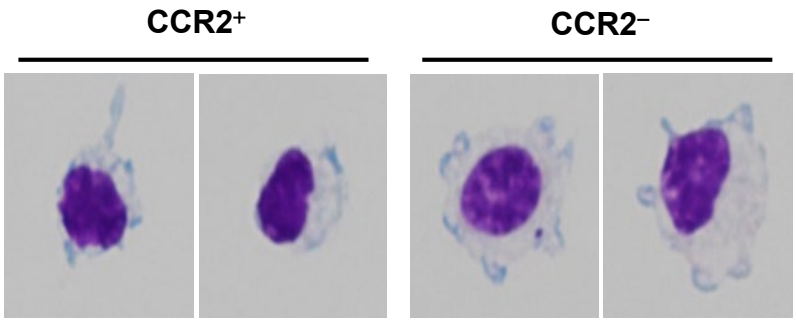
scRNAseq analysis identifies multiple clusters within skeletal muscle resident MPs. scRNAseq data of quadriceps or diaphragm MPs were analyzed individually. **A & B.** Feature plots showing single cell expression of MP marker genes *adgre1* (F4/80) and *fcgr1* (CD64) in cells from quadriceps (**A**) and diaphragm (**B**). **C & D.** Heatmaps of the top 15 most differentially expressed genes of each cluster in MPs isolated from quadriceps (**C**) and diaphragm (**D**).

S. Figure 5



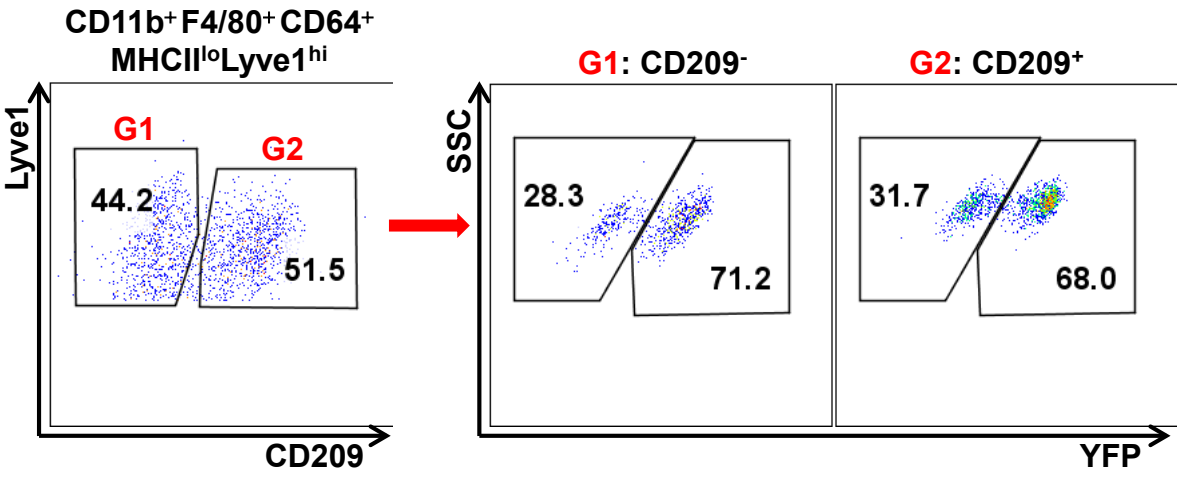
Clusters within SMRMPs are functionally diverse. Violin plots showing expression of: **A.** Genes preferentially expressed by the *Ccr2* cluster; **B.** Genes preferentially expressed by the non-*Ccr2* clusters excluding the Proliferating cluster; **C.** Genes preferentially expressed by the *Klf2* cluster; **D.** Genes preferentially expressed by the *Cd209* cluster.

S. Figure 6



Giemsa staining of CCR2^{+} and CCR2^{-} resident MPs sorted from quadriceps and diaphragm muscles of wild-type C57BL/6J mice at 4-6 weeks of age.

S. Figure 7



Both CD209^{-} and CD209^{+} subsets of MHCII^{lo}Lyve1^{hi} MPs have mixed HSC and non-HSC origins. MPs were collected from the quadriceps of *Flt3^{Cre}-Rosa26$^{LSL\text{-}YFP}$* mice at 10 months of age and subjected to FACS analysis of CD209 and YFP expression.

SUPPLEMENTARY TABLES

Supplementary Table 1

List of enriched pathways by top marker genes (adj. *p*-value < 0.05) of *Ccr2* cluster and non-*Ccr2* clusters in both quadriceps and diaphragm

	***Ccr2* cluster**	**non-*ccr2* clusters**
quadriceps	mRNA translation	Metabolic pathways
	Antigen processing and presentation	C-type lectin receptor signaling pathway
		Endocrine and other factor-regulated calcium reabsorption
		Endocytosis
		Arachidonic acid metabolism
		Lysosome
diaphragm	Antigen processing and presentation	Endocytosis
		chemokine signaling pathway
		Synaptic vesicle cycle
		Endocrine and other factor-regulated calcium reabsorption
		Lysosome
		Metabolic pathways
		MAPK signaling pathway

Supplementary Table 2

List of enriched pathways by top marker genes (adj. p-value < 0.05) of *Cd209* cluster in quadriceps and *Klf2* cluster in diaphragm

Cd209 **cluster**	***Klf2*** **cluster**
Butyrophilin (BTN) family interactions	Regulation of DNA-templated transcription in response to stress
Response to interferon	TNF signaling pathwy
activation of immune response	ameboidal-type cell migration
regulation of multi-organism process	response to topologically incorrect protein
	response to mechanical stimulus
	cellular response to external stimulus
	Fluid shear stress and atherosclerosis
	cell chemotaxis

Supplementary Table 3

Information of antibodies used for flow cytometry analysis.

antigen targeted	flourenscence lable	Company	Cat. No.
CCR2	BV421	BioLegend	150605
CD115	APC	invitrogen	17-1152-82
CD11b	PE	invitrogen	12-0112-82
CD11c	Alexa700	invitrogen	56-0114
CD163	PerCP-eFluor710	invitrogen	46-1631-80
CD206	Alexa647	BD Biosciences	565250
CD209	PE	BioLegend	833003
CD45	PerCP-Cy5.5	BD Biosciences	550994
CD45	PE-Cy7	invitrogen	25-0451-81
CD45.1	FITC	BD Biosciences	553775
CD45.2	PerCP-Cy5.5	invitrogen	45-0454-80
CD64	Alexa647	BD Biosciences	558539
CD64	BV421	BioLegend	139309
F4/80	APC	BioLegend	123116
F4/80	PE	BioLegend	123110
Ki67	PE	invitrogen	12-5698-80
Ki67	PE-Cy7	invitrogen	25-5698-80
Ly6C	PE-Cy7	BioLegend	128018
Ly6C	BV421	BioLegend	128031
Lyve1	PE-Cy7	invitrogen	25-0443-82
MerTK	Alexa700	R&D Systems	FAB5912N
MHC II	PE	BD Biosciences	553552
MHC II	BV421	BD Biosciences	562564
Siglec-F	BB515	BD Biosciences	566211