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Supplemental Information

Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis

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INVENTORY OF SUPPLEMENTAL ITEMS

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of *Cx3cr1^{cre}* and *Cx3cr1^{creER}* mice. For the generation of ***Cx3cr1^{cre}*** mice, a *Cre* recombinase cassette gene with a “floxed” *neomycin* resistant gene was introduced into the Bacterial Artificial Chromosome (BAC) using the RedE/T recombineering system replacing the *Cx3cr1* coding exon; pKP-Select DK was cloned downstream of LH to select against random integration. For the generation of the *Cx3cr1^{creER}* mice the *Cre*ERT2 cassette gene, a kind gift of D. Metzger (Indra et al., 1999) and *loxP* flanked neomycin resistance gene were introduced replacing the first 390 bp of the *Cx3cr1* gene, a HSV-tk gene was cloned downstream of the LH to select against random integration (Jung et al., 2000). ES-cells were manipulated as described previously (Jung et al., 2000). Chimeric mice were generated following blastocyst aggregation of targeted ES-cells with eight-cell stage embryos. Germline transmitting chimeric males were crossed to founder lines, which were backcrossed for >8 generations onto the C57BL/6 background (for the *Cx3cr1^{creER}*, mice). *Cx3cr1^{cre}* mice were generated on C57BL/6 background. *Neomycin* cassettes were removed by crossing the respective *Cx3cr1^{creER}* and *Cx3cr1^{cre}* animals with the global *Pgk-cre* deleter mouse (Lallemand et al., 1998). Both *Cx3cr1^{cre}* and *Cx3cr1^{creER}* mice are typed

by PCR using the forward primer 5'-GGTTCTTGCGAACCTCATCAC-3' and the reverse primer 5'-GCAGGACAAGCATAGCAGTC-3' that amplify a 2.3 kb fragment from the transgenic *Cx3cr1* locus.

Tamoxifen treatment. To induce gene recombination in *Cx3cr1*^{creER} mice, tamoxifen was administered either orally via gavage/Tamoxifen-containing food (Kiermayer et al., 2007) or dissolved in warm corn oil and administered s.c. twice 48 hrs apart. Mice were examined seven days thereafter, or as described in the text.

Antibodies. CD3 (clone: 145-2C11), CD4 (clone: GK1.5), CD11c (clone: N417), CD19 (clone: 6D5), CD36 (clone: HM36), CD45R (clone: RA3-6B2), CD135 (clone: A2F10) and GR-1 (clone: RB6-8C5) were purchased from BioLegend. CD8 (clone: 53-6-11), CD11a (clone: M17/4), CD11b (clone: M1/70), CD45 (clone: 30F11), CD115 (clone: AFS98), CD117 (clone: 2B8), Ly6G (clone: 1A8) NK1.1 (clone: PK136) and Sca1 (clone: D7) were purchased from ebioscience. CD62L (clone: MEL14) and EMR1 (also know as F4/80) (clone: CI:A3-1) were from AbD Serotec. CD64 (clone: 90322) and TREM-1 were purchased from R&D Systems. M-CSFR (clone:M279), anti-CCR2 antibody (clone:MC21) (Bruhl et al., 2007).

Isolation of tissue samples. For leukocyte analysis peripheral blood was collected by tail bleeds or cardiac puncture under anaesthesia, 15% Xylazin (20mg/ml, Vitamed) 85% Ketamine (100 mg/ml, Fort Dodge Animal Health) (Yona et al., 2010). For analysis of granulocytes, erythrocytes were lysed from whole blood using FACS Lysing Solution (BD Biosciences), in accordance with the manufacturer's instructions. BM cells were harvested as previously described (Yona et al., 2010). Briefly, cells were harvested from the femura and tibiae; mononuclear cells were enriched by Ficoll density gradient centrifugation (1000 x *g*. 15 min at 20ºC with low acceleration and no brake). Small and large intestines were washed of fecal content and opened longitudinally, cut into 0.5 cm pieces and washed in HBSS supplemented with 5% FCS, 2 mM EDTA 0.15mg/ml DTT (300 rpm 37°C). Intestinal tissue was subsequently digested in collagenase VIII (1.5 mg/ml; Sigma, 300 rpm 37°C) and filtered through 80 μM wire mesh. For microglia analysis mice were perfused with 30 ml PBS via the left ventricle. Brain sections were harvested from individual mice and tissues homogenized. Homogenized sections were filtered through 80 μM wire mesh and resuspended in 40% Percoll, prior to density centrifugation (1000 x *g*. 15 min at 20ºC with low acceleration and no brake). For the analysis of liver mononuclear phagocytes mice were perfused with 30 ml of PBS via the left ventricle. The liver was excised, digested with 1 mg/ml collagenase D (Roche, 1 h

at 37°C), and filtered through 80 μM wire mesh. The pellet was resuspended in 40% Percoll layered over 80% Percoll prior to density centrifugation (1000 x *g*. 15 min at 20ºC with low acceleration and no brake); enriched cells were isolated from the interphase and erythrocytes removed by ACK lysis. Cells were resuspended and stained. Spleens were excised and digested with 1 mg/ml collagenase D (Roche, 1 h at 37°C), filtered through 80 μM wire mesh, erythrocytes removed by ACK lysis. For peritoneal cell isolation, peritoneal cavities were washed with PBS supplemented with 3mM EDTA. Following gentle massage, the cavity was opened by abdominal incision and lavage fluid collected. For Langerhans' cells, ears were split into two layers and incubated in trypsin-EDTA (1 h at 37°C), the epidermis was then peeled from the dermis and filtered through 80 μM wire mesh. Fetal livers were excised and mechanically dissociated by passing cells through 80 μM wire mesh, erythrocytes were removed by ACK lysis.

Isolation of monocytes for adoptive transfers. BM cells were harvested from the femora, tibiae, coxal and humerus; mononuclear cells were enriched by Ficoll density gradient and filtered through 70 μM wire mesh. Splenocytes were filtered through an 80 μM wire mesh, erythrocytes were removed by ACK lysis. Splenic monocytes were isolated by MACS enrichment using biotinylated anti-CD115 antibody and streptavidin-coupled magnetic beads (Miltenyi Biotec). Both BM and splenic cells were resuspended and stained with CD11b CD115 and GR-1 antibodies. Ly6C^{hi} monocytes were identified as CD11b^{+} CD115^{+} and Gr-1^{+} and in certain cases GFP^{int}, cells were purified by high-speed cell sorting using a FACS Aria (Becton-Dickson). Sorted cells with a purity of >95% were resuspended to 7.5 x10^{6} monocytes/ml in sterile PBS and 200 μl injected i.v. into congenic CD45.1 WT mice.

BrdU pulsing. Mice were treated with 3 injections of 2 mg BrdU (5-bromo-2-deoxyuridine; BD Pharmingen) i.p. 3 hours apart or a single 2 mg i.p injection. To assess BrdU incorporation, bood, bone marrow or splenic monocytes were stained for CD11b, CD115 and Gr-1 (Ly6C/G), fixed and permeabilized using Cytofix/Cytoperm and Perm/Wash buffer (BD Pharmingen) according to the manufacturer's instructions. Cells were incubated at 37°C for 60 min in 30 μg of DNase, followed by staining with anti-BrdU-APC for 30 min washed and analyzed by flow cytometry. To calculate the half-life of monocytes, an exponential trendline using the $N(t) = N(0) * e^{-\lambda t}$ equation was fitted to the decay curves, the decay constant λ was calculated from the trendline. Following, the half life $T_{½}$ was calculated by dividing ln(2) (0.693) by the decay constant λ.

Antibody and cytokine treatment: Mice were treated with 150 µl of CCR2 mAb (clone: MC21) conditioned media (29 µg ab/ml), i.p. every 48 hours to ablate blood $Ly6C^+$ monocytes. To block CD115, mice were treated with 200 µg of the CD115 mAb (clone: M279, MacDonald et al. 2010) i.p. twice 72 hrs apart. Recombinant M-CSF (PeproTech) (25µg/mouse) was given i.p.

Multiplex assay C57Bl/6 mice were administered MC21 hybridoma supernatant containing 6 µg of anti-CCR2 antibody i.v. control media, or a CL positive control. 100 µl of peripheral blood was collected prior the injection (0 h), and 12 and 24 hours after. Blood was analyzed by flow cytometry to confirm depletion of $Ly6C^+$ monocytes. Serum was analyzed for CSF-1, G-SCF, IL-6 and VEGF-A using a custom Procarta Cytokine assay (Affymetrix) according to the manufacturer's instructions on a Luminex 200 instrument (Luminex Corp.).

Histology. Mice were anaesthetized and perfused with 30 ml of PBS via the left ventricle, organs were excised, fixed for 18 hours in 4% para-formaldehyde, equilibrated for 36 hours in 30% sucrose in PBS prior to being imbedded in OCT and frozen at -80°C. Cryostatic sections, 10-24 µm thick, sections were post-fixed with cold methanol, blocked with CAS block and stained as described in the text. Slides were finally mounted in fluorescent mounting medium. Analysis by confocal laser scanning microscopy was performed using a Zeiss LSM510 microscope. Image acquisition was processed with Zeiss LSM Image browser software.

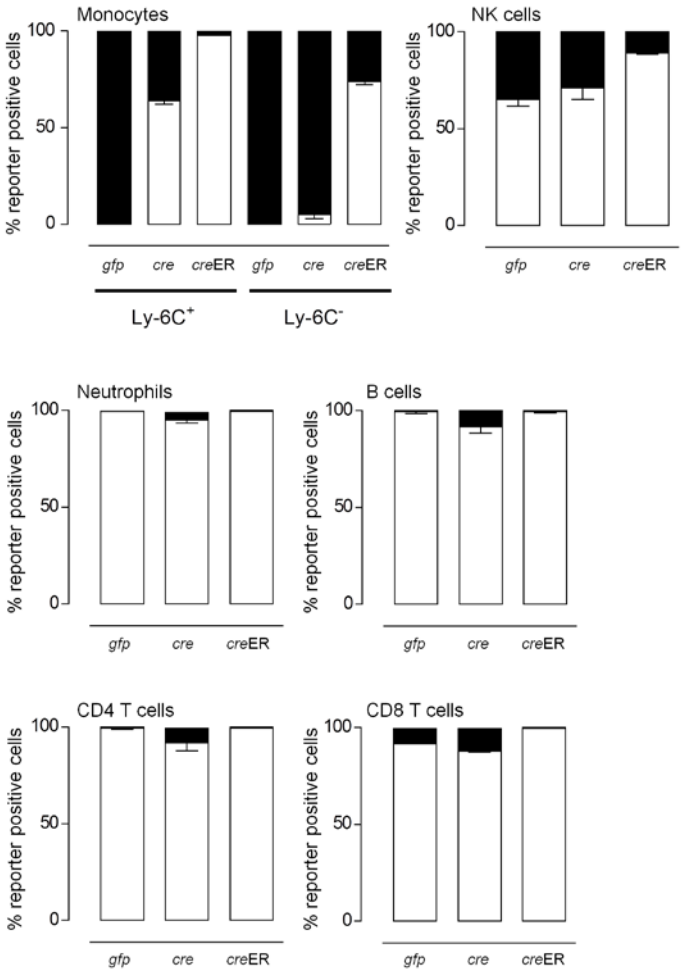

Figure S1. Reporter gene expression pattern in leukocytes of *Cx3cr1^{gfp}*, *Cx3cr1^{cre}:R26-yfp* and *Cx3cr1^{creER}:R26-yfp* mice

Flow cytometric analysis of mononuclear phagocyte populations of *Cx3cr1$^{gfp/+}$*, *Cx3cr1$^{cre/+}$:R26-yfp* and *Cx3cr1$^{creER/+}$:R26-yfp mice*. *Cx3cr1$^{creER/+}$*:R26-*yfp* mice were administered 8 mg of Tamoxifen s.c. 48 h apart twice and were examined 7 days later. Bar graphs summarize mean fluorescence intensity (MFI) of YFP/GFP expression (Black Bar), absence of YFP/GFP fluorescence (White Bar). Reporter gene expression in lymphocytes was low in *Cx3cr1^{cre}* mice and absent from tamoxifen-treated *Cx3cr1^{creER}* mice; neutrophils (6±1.7 % and 0.3+0.3 %, respectively); B cells (8.3+2.8 % and 0.5±0.3 %, respectively); CD4 T cells (6.2±1.2 % and 0.3±0.3 %, respectively); CD8 T cells (11.3±1.2 % and 0.3±0.3 %, respectively). Consistent with CX_3CR1 expression in NK cells, a fraction of these cells underwent recombination in *Cx3cr1^{cre}* mice and tamoxifen-treated *Cx3cr1^{creER}* mice (28±6 % and 11±0.8 %, respectively). Mean ± SEM, n= 4-6 mice per group.

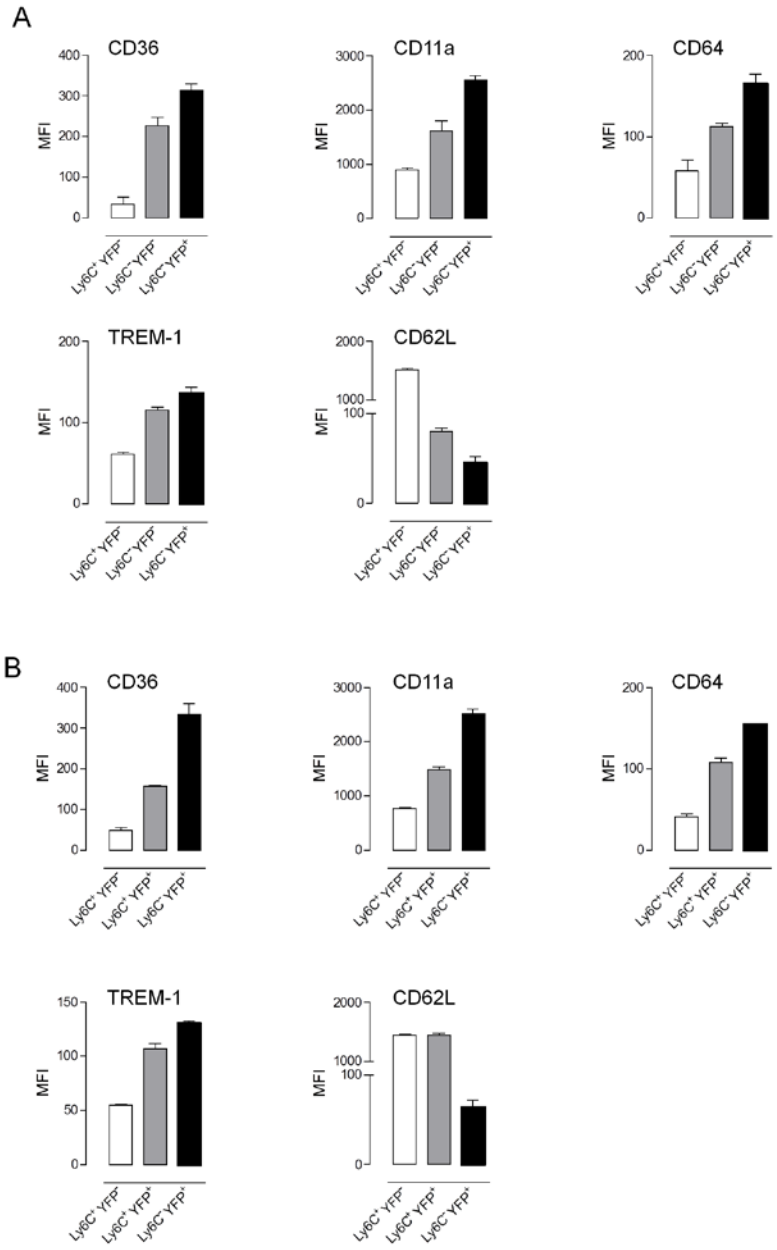

Figure S2. Gradual surface marker acquisition during differentiation from Ly6C$^+$ to Ly6C$^-$ blood monocytes

(A) Flow cytometric analysis of Ly6C$^+$ and Ly6C$^-$ blood monocytes subsets of *Cx3cr1^{cre}:R26-yfp* mice for differentiation markers (n=5-9 mice per group).

(B) Flow cytometric analysis of Ly6C$^+$ and Ly6C$^-$ blood monocytes subsets of *Cx3cr1^{creER}:R26-yfp* mice for differentiation markers. Mean ± SEM (n=5-9 mice per group).

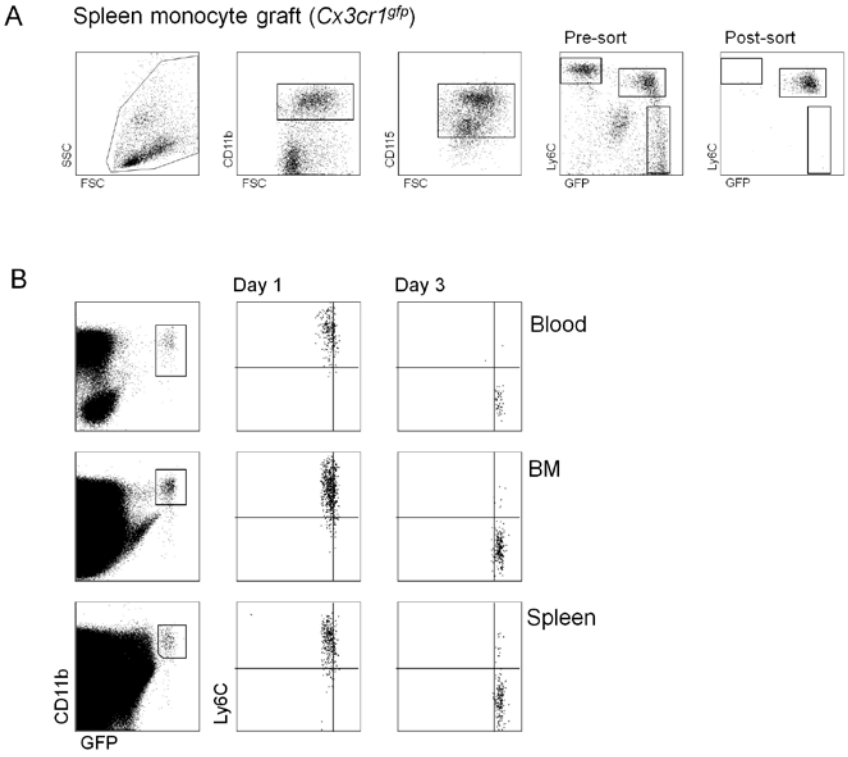

Figure S3. Adoptively transferred Ly6C^{+} BM and spleen monocytes differentiate into Ly6C^{-} monocytes

(A) Gating strategy for monocyte isolation. Monocytes were enriched and stained for CD11b, CD115 and Ly6C before being sorted from *Cx3cr1*$^{gfp/+}$ spleens.
(B) Flow cytometric analysis of congenic WT recipients of Ly6C^{+} spleen monocytes isolated from *Cx3cr1*$^{gfp/+}$ mice 1 and 3 days following engraftment.
Results are representative of 3 analyzed mice.

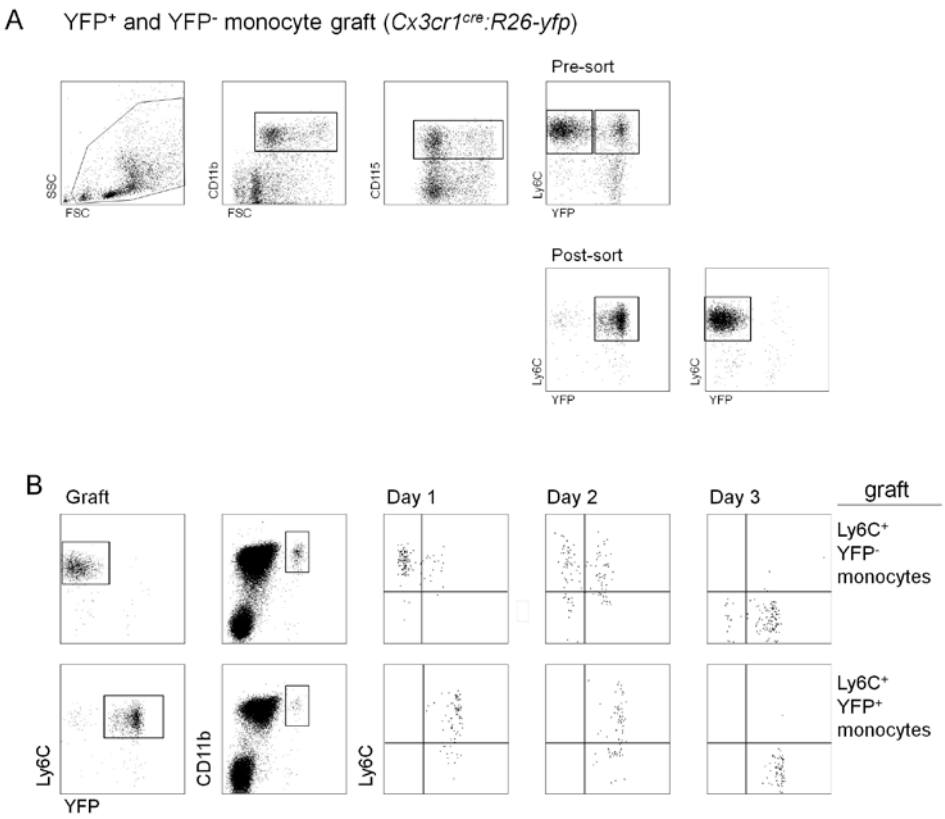

Figure S4. Adoptively transferred Ly6C$^+$ monocytes differentiate into Ly6C$^-$ monocytes

(A) Gating strategy for isolation. Monocytes were enriched and stained for CD11b, CD115 and Ly6C before being sorted from *Cx3cr1^{cre}:R26-yfp* BM monocytes.
(B) Flow cytometric analysis of blood of wt recipients of YFP$^+$ and YFP$^-$ Ly6C$^+$ BM monocytes isolated from *Cx3cr1^{cre}:R26-yfp* mice day 1, 2 and 3 days after engraftment. Results are representative of 3 analyzed mice.

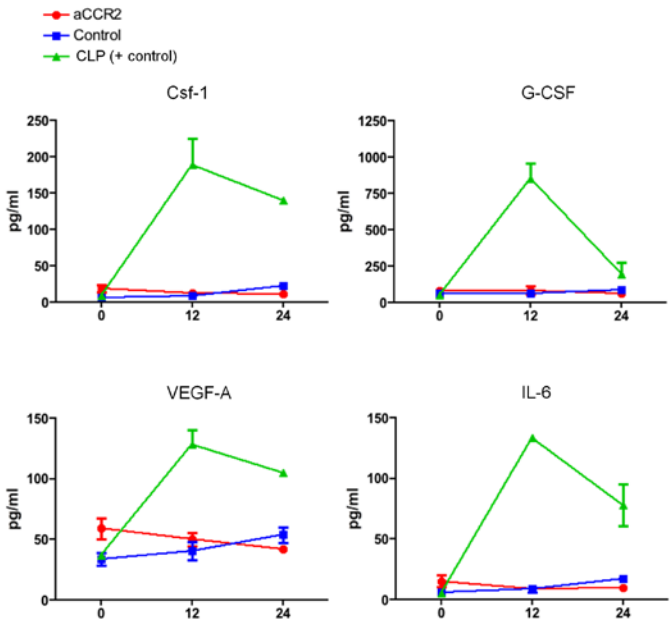

Figure S5. Measurement of serum titers of growth factors and cytokines following administration of the Ly6C$^+$ monocyte-ablating antibody MC21
Serum analysis for CSF-1/ M-CSF, G-SCF, IL-6 and VEGF-A using a custom Procarta Cytokine assay (Affymetrix) and read-out on a Luminex 200 instrument (Luminex Corp.).

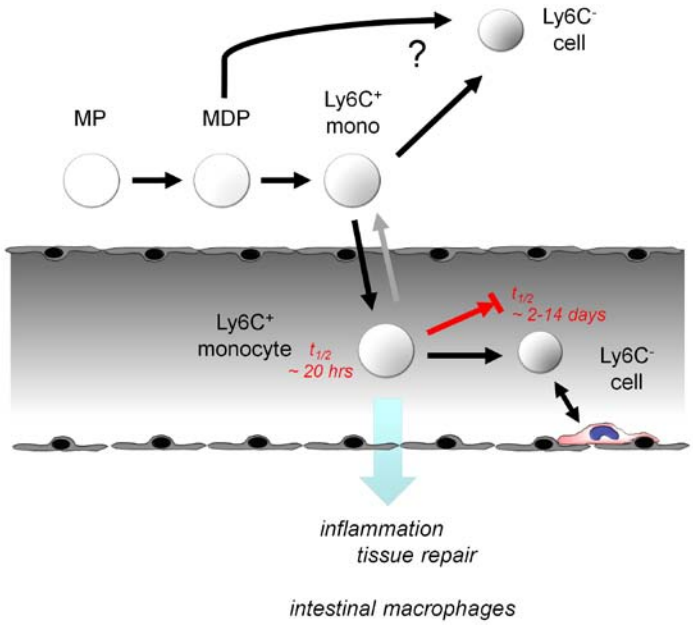

Figure S6. Schematic of murine monocyte development
Ly6C$^+$ BM monocytes arise from MDPs and exit in a CCR2 dependent manner from to the circulation where a fraction of these short-lived cells gives in steady state by default rise to Ly6C$^-$ blood cells. Prevalence of Ly6C$^+$ blood monocytes controls the half-live of Ly6C$^-$ blood cells. The origin of Ly6C$^-$ BM monocytes remains to be determined: either they arise in the BM directly from MDPs or - like their blood counterpart - from a Ly6C$^+$ intermediate.

Supplemental References

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