SUPPLEMENTARY APPENDIX

Bone marrow mesenchymal stromal cells induce nitric oxide synthase-dependent differentiation of CD11b$^+$ cells that expedite hematopoietic recovery

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 doi:10.3324/haematol. 2016.155390

Received: September 5, 2016.

Accepted: February 1, 2017.

Pre-published: February 9, 2017.

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Supplementary materials

Methods

Mice

C57Bl/6 mice were purchased from Harlan, Bicester UK. CD45.1 C57Bl/6 mice were bred in-house in the Biological Service Unit of the James Black Centre, King's College London. *Nos2*$^{-/-}$ donors, in the C57Bl/6 background, were bred in the animal facility of IRCCS Istituto Oncologico Veneto (Veneto Oncology Institute - IOV). Mice were used at 8-12 week of age. All procedures were conducted in accordance with the Home Office Animals Act of 1986.

Cell cultures and media

For murine BM derived MSC, femurs and tibias of 8 to 12 weeks old WT C57Bl/6 or *Nos2*$^{-/-}$ mice were directly crushed and plated in Mesencult medium with MSC stimulatory supplement (StemCell Technologies, Canada). After 72 hours non-adherent cells were removed and fresh medium was added to the cultures. The medium was changed every 3 to 4 days for 8 to 10 weeks and a homogeneous cell population was obtained. The identity of MSC was confirmed by immunophenotypic criteria based on the expression of PDGFRα and Sca-1, and the absence of hematopoietic marker CD45 (**Supplementary Figure S1**), the ability to differentiate into the three mesenchymal lineages and to suppress CD3-CD28-induced T cell proliferation.

All human samples were collected after informed consent according to institutionally approved protocols. Human MSC were isolated from washouts of discarded BM collection bags of healthy donors. Briefly, BM derived mononuclear cells (MNC) were seeded at a density of 2 x 10^5 cells/cm^2 in growth medium, composed by Dulbecco's modified Eagle's medium (DMEM)-low glucose (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (Biosera, Nuaille, France), 100U/ml penicillin-streptomycin (Invitrogen) and 100U/ml L-glutamine (Invitrogen). After 48 h, non-adherent cells were removed and fresh medium was added. When cultures reached 70% of confluence, adherent cells were trypsinized, re-plated at 2 x 10^3 cells/cm^2 and maintained in culture for up to 8 passages.

Generation of MSC-induced myeloid cells

For co-cultures experiments with murine BM MNC, murine BM-derived MSC were plated at $2x10^5$ cells/well in 24 well plates (Costar), and kept at 37°C, 5% CO_2 and 95% humidity in a cell incubator. When the MSC were attached to the plastic, 10^6 BM MNC were added. After 4 days, cells were collected by adding PBS containing 2mM ethylene-diamine-tetraacetic acid (EDTA). Where described, $CD11b^+$ were sorted at the end of the co-cultures by collecting the cells with PBS containing 2mM EDTA and purified with $CD11b^+$ Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purity of isolation was assessed by flow cytometry (**Supplementary Figure S2**).

For co-culture experiments with human BM MNC, human MSC were plated in complete RPMI 1640 medium (Euroclone, Pero, IT) at 10^5 cells/well in 24 well plates and kept in the incubator overnight to allow cell adhesion. Human BM MNC were obtained by Ficoll-Paque Plus (GE Healthcare Europe, Freiburg, Germany) separation from fresh BM of healthy donors, and added to the MSC layer (10^6 cells/well). After 7 days, cells were collected by trypsinization.

Flow cytometry

For the analysis of MSC purity, monoclonal antibodies PDGFRα (clone APA5, eBioscience), CD45 (clone 30-F11, eBioscience) and Sca-1 (clone D7, eBiosience) were used (**Supplementary Figure S1**).

For the analysis of myeloid populations within murine co-cultures, monoclonal antibodies CD11b (clone M1/70, Biolegend) and Gr-1 (clone RB6-8C5, Biolegend), CD115 (AFS98, eBioscience), IL4Rα (CD124, clone mIL4R-M1), CD169 (clone 3D6.112, AbDSerotec), CD206 (clone MR5D3, AbDSerotec), CD68 (clone FA-11, Biolegend), F4/80 (clone CI:A31, AbDSerotec), Ly6C (clone AL21, BD Biosciences) and Ly6G (clone 1A8, BD Biosciences) were used.

For sorting primitive HSC and myeloid progenitors, BM MNC were firstly incubated with whole mouse IgG to block unspecific binding sites, and then stained for lineage antibody cocktail (CD3e, CD11b, CD45R/B220, TER-119, and Ly-6G and Ly-6C –

BD Pharmingen), c-Kit (CD117 – clone 2B8, eBioscience), IL-7Rα (CD127 – clone A7R34, eBioscience), CD34 (clone RAM34), CD16/32 (FcγR – clone 93, eBioscience), Sca-1 (Ly6A-E, clone D7, eBioscience) and Flk2 (CD135, clone A2F10.1, BD Bioscience) antibodies. Samples were acquired with a BD FACSAria (BD Biosciences). IL-7Rα positive cells were excluded, as well as all lineage positive cells. Following the isolation method proposed by Weissman group [25], c-kit$^+$ and Sca-1$^-$ cells were subdivided in CD16/32^{low}CD34$^+$, CD16/32^{low}CD34$^-$, and CD16/32^{high}CD34$^+$ populations (MEP, CMP, GMP respectively). HSC were instead defined as c-kit$^+$Sca-1$^+$Flk-2$^-$ cells. Analysis of the sorted populations is shown in **Supplementary Figure S3A** and **Supplementary Figure S3B**.

For the analysis of myeloid populations after *in vivo* adoptive transfer of WT or Nos2$^{-/-}$ MSC, neutrophils were identified as Gr-1$^+$CD115$^-$ (gate I), whereas macrophages and eosinophils were distinguished on the basis of forward and side scatter characteristics within the Gr-1^{neg}CD115^{int}F4/80$^+$ (gate II and III) (**Supplementary Figure S4A**). Monocytes were characterized as CD11b$^+$Ly6G$^-$ Ly6C$^+$ (**Supplementary Figure S4B**).

Donor hematopoietic engraftment after *in vivo* adoptive transfer of CD11b$^+$ cells was evaluated by quantitating analysis of CD45.1 and CD45.2 percentage (CD45.1, clone A20, eBioscience; CD45.2, clone 104, eBioscience); neutrophils were identified as Gr-1^{high} (Gr-1, RB6-8C5, Biolegend) CD115$^+$ (AFS98, eBioscience), monocytes as Gr-1^{neg}CD115$^+$, B cells as CD19$^+$ (CD19, clone 1D3, BD Biosciences), T cells as CD3$^+$ (CD3, clone 145-2C11, eBioscience).

The analysis of human co-cultures was performed using antibodies against cellular markers CD45 (clone HI30, Life Technologies), CD14 (clone 61D3, eBioscience), CD16 (clone NKP15, BD Biosciences) and HLA-DR (clone L243, BD Biosciences).

Supplementary figures

SUPPLEMENTARY FIGURE S1. Phenotypic analysis of bone marrow derived MSC. BM-derived MSC, generated as described in Supplementary Methods section, were evaluated at phenotypic analysis. The proportion of $CD45^-$ cells is represented in the live gate. The percentages of $PDGFR\alpha^+$ $Sca\text{-}1^+$ cells are represented in the $CD45^-$ gate using a dot plot. A representative example out of 3 independent preparations of MSC is shown.

SUPPLEMENTARY FIGURE S2. Purification of $CD11b^+$ cells from the cultures. BM MNC were cultured alone or with MSC for 4 days, and cells were collected to isolate the $CD11b^+$ population. Zebra plots from a representative experiment show the proportion of $CD11b^+$ cells within the live gate before and after the purification step described in Supplementary Methods section.

SUPPLEMENTARY FIGURE S3. Sorting of myeloid progenitors in mouse bone marrow. BM MNC were fractionated for MEP, CMP, GMP and HSC as described in Supplementary Methods section. **A.** Contour plots represent the gating strategy within the live gate. The $IL\text{-}7R\alpha^-Lin^-$ $Sca\text{-}1^-c\text{-}Kit^+$ fraction of BM cells was subdivided into $CD16/32^{low}$ $CD34^+$, $CD16/32^{low}$ $CD34^-$, and $CD16/32^{high}$ $CD34^+$ populations (MEP, CMP, GMP respectively), whereas the $IL\text{-}7R\alpha^-Lin^-Sca\text{-}1^+c\text{-}Kit^+$ was subdivided into $Flk2^-Sca\text{-}1^+$ (HSC). **B.** Contour plots from a representative experiment show the sorted $CD16/32^{low}$ $CD34^+$ (MEP), $CD16/32^{low}$ $CD34^-$ (CMP), $CD16/32^{high}$ $CD34^+$ (GMP) and $Flk2^-Sca\text{-}1^+$ (HSC) populations.

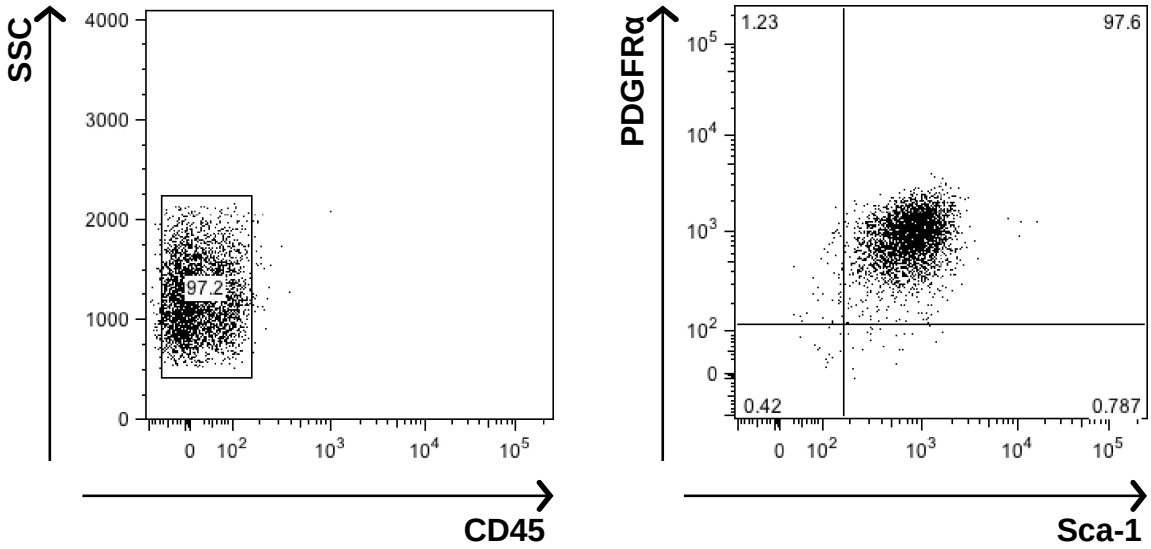
SUPPLEMENTARY FIGURE S4. Characterization of MSC-educated donor macrophage engraftment. Sublethally irradiated (800cGy) CD45.2 WT recipients were injected with $2x10^6$ CD45.1 WT BM cells, either alone or in combination with $0.2x10^6$ CD45.2 WT MSC or *Nos2*$^{-/-}$ MSC. 13 days after the transplant, BM and spleen were analyzed by FACS. **A.** Gating strategy of BM mononuclear phagocytes. Within $CD45.1^+$ donor cells, the $Gr\text{-}1^+CD115^-$ population was individuated as neutrophils (I). The $Gr\text{-}1^{low}$ fraction was further subdivided into an $F4/80^+CD115^-$ population, which can be subdivided into SSC^{hi} eosinophils (III) and SSC^{low} macrophages (II). CD11b expression within the SSC^{low} subset is represented in histogram plot (open histogram) against unstained control (filled histogram) **B.** Gating strategy for $Ly6G^-Ly6C^+$ monocytes. Within $CD45.1^+$ donor cells, the $CD11b^+$ population was subdivided in different populations of $Ly6G^+Ly6C^+$ cells, $Ly6G^-Ly6C^-$

and $Ly6G^{-}Ly6C^{+}$ monocytes. Frequency (**C**) and absolute number (**D**) of myeloid populations within the donor hematopoiesis in the BM (BM alone – white bars; BM+ WT MSC– black bars; BM+*Nos2*$^{-/-}$ MSC – grey bars). Mean of 3 independent experiments ±SEM, * $p<0.05$, *** $p<0.001$ Unpaired t test. **E.** Absolute number of $Ly6G^{-}Ly6C^{+}$ monocytes within donor hematopoiesis (BM alone – white bars; BM+ WT MSC – black bars; BM+*Nos2*$^{-/-}$ MSC – grey bars). Mean of 3 independent experiments ±SEM.

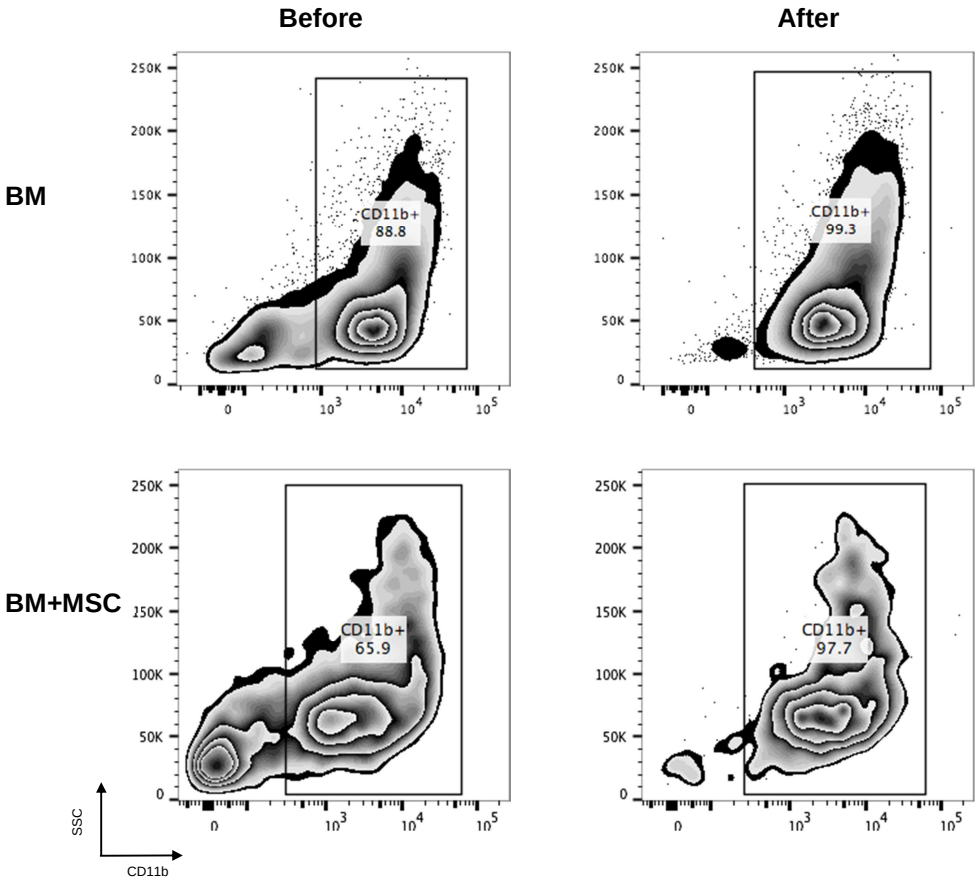
SUPPLEMENTARY FIGURE S5. Characterization of $CD11b^{+}$ cell induced engraftment. Sublethally irradiated (800cGy) CD45.2 WT recipients were injected with $5x10^{4}$ CD45.1 WT BM cells, either alone (BM) or in combination with $2x10^{6}$ MSC-induced $CD11b^{+}$ cells (BM+$CD11b^{+}$). Peripheral blood samples were taken 2, 4, 6 and 8 weeks after the transplant, and analyzed by FACS. Absolute number of T cells ($CD3^{+}$), B cells ($CD19^{+}$), monocytes (Gr-$1^{neg}CD115^{+}$) and neutrophils (Gr-$1^{high}CD115^{+}$) in the whole hematopoiesis at 2 weeks (**A**), and at 2, 4, 6 and 8 weeks after the transplant (**B**). Mean of 4 independent experiments ±SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Unpaired t test.

SUPPLEMENTARY FIGURE S6. Kinetics of $CD11b^{+}$-cell-induced engraftment. Sublethally irradiated (800cGy) CD45.2 WT recipients were injected with $5x10^{4}$ CD45.1 WT BM cells, either alone (BM) or with $2x10^{6}$ MSC-induced $CD11b^{+}$ cells (BM+$CD11b^{+}$). Peripheral blood samples were taken 2, 4, 6 and 8 weeks after the transplant, and analyzed by FACS as described in Supplementary Materials section. **A.** Frequency of donor hematopoiesis in MNC, T cells ($CD3^{+}$), B cells ($CD19^{+}$), monocytes (Gr-$1^{neg}CD115^{+}$) and neutrophils (Gr-$1^{high}CD115^{+}$). **B.** Absolute number of donor hematopoietic cells in MNC, T cells ($CD3^{+}$), B cells ($CD19^{+}$), monocytes (Gr-$1^{neg}CD115^{+}$) and neutrophils (Gr-$1^{high}CD115^{+}$). Mean of 4 independent experiments ±SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Unpaired t test.

Supplementary Figure S1.

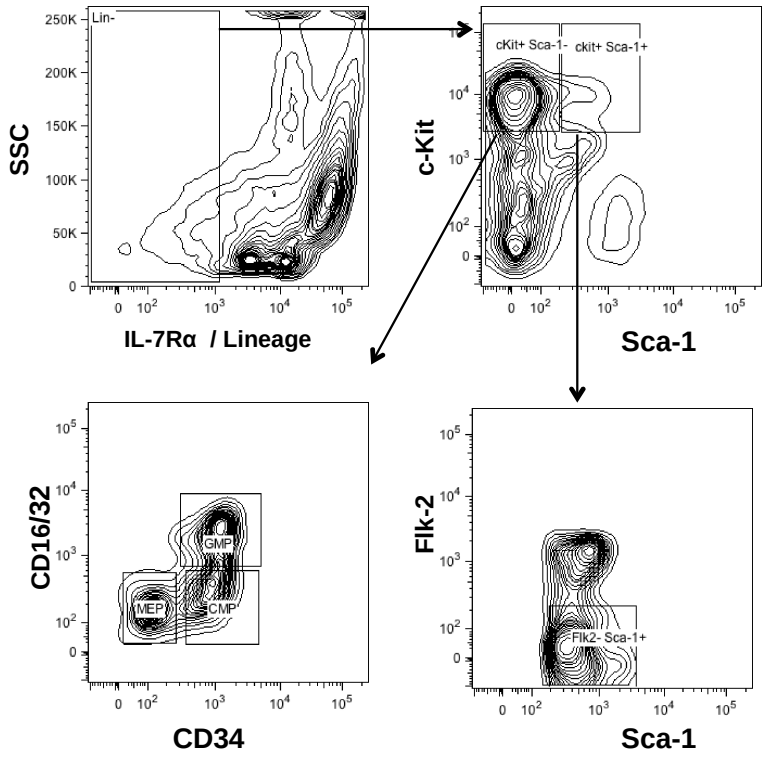


Supplementary Figure S2.

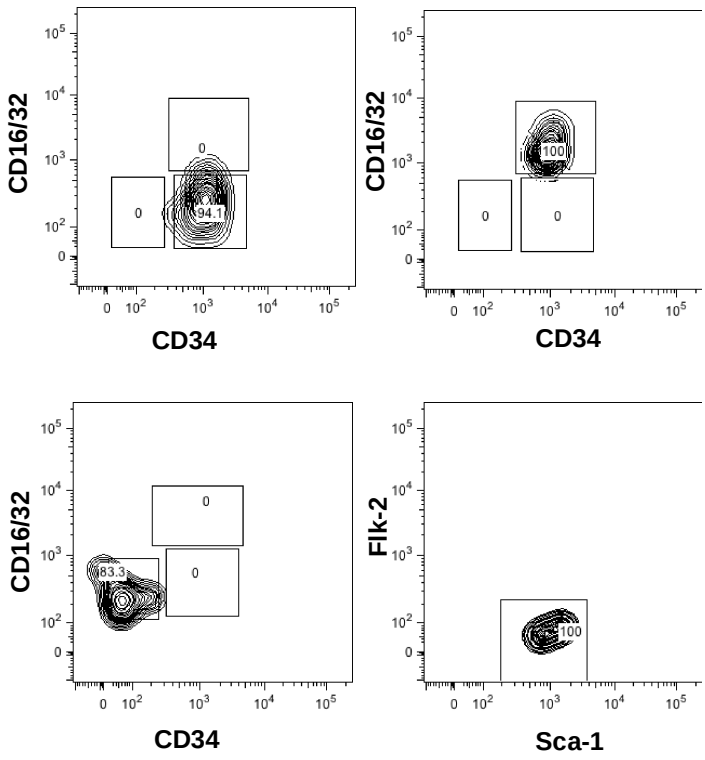


Supplementary Figure S3.

A.

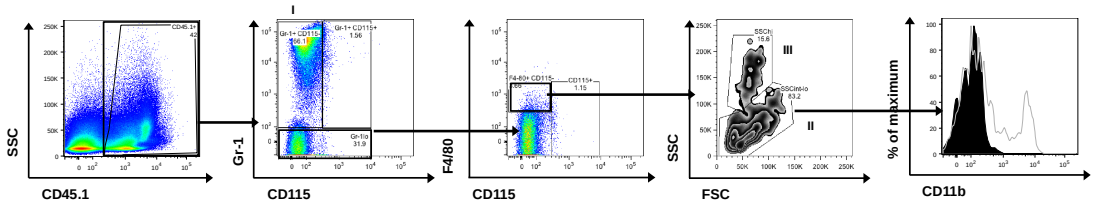


B.



Supplementary Figure S4.

A.



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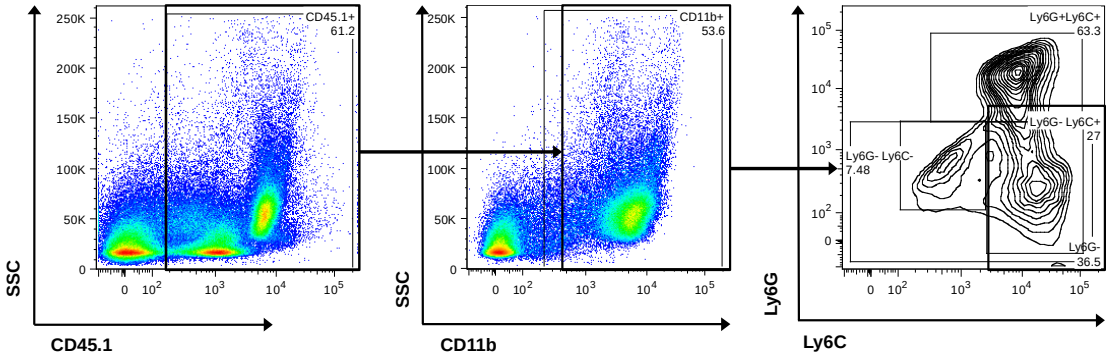
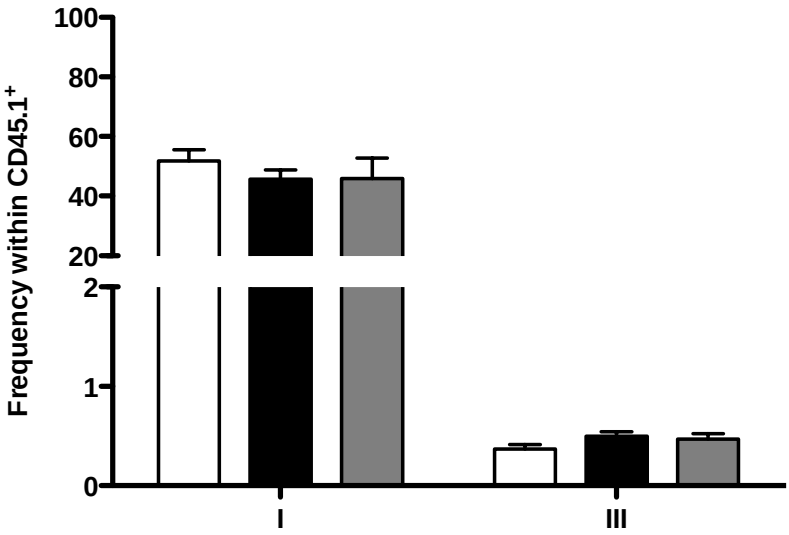
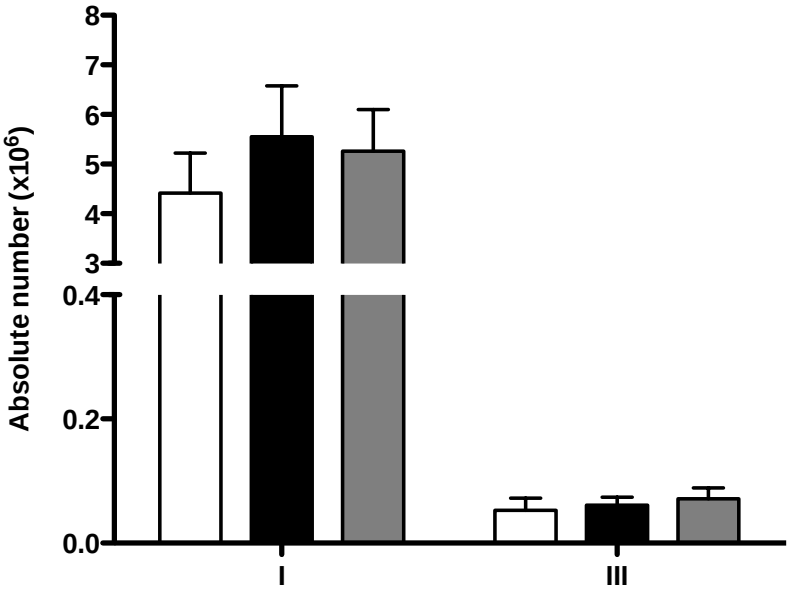


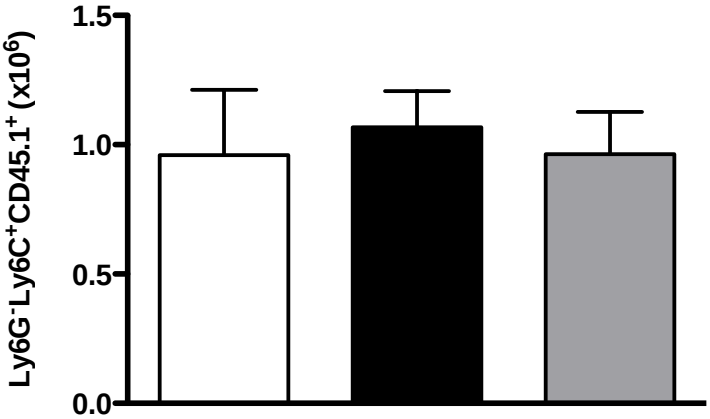
C.



D.

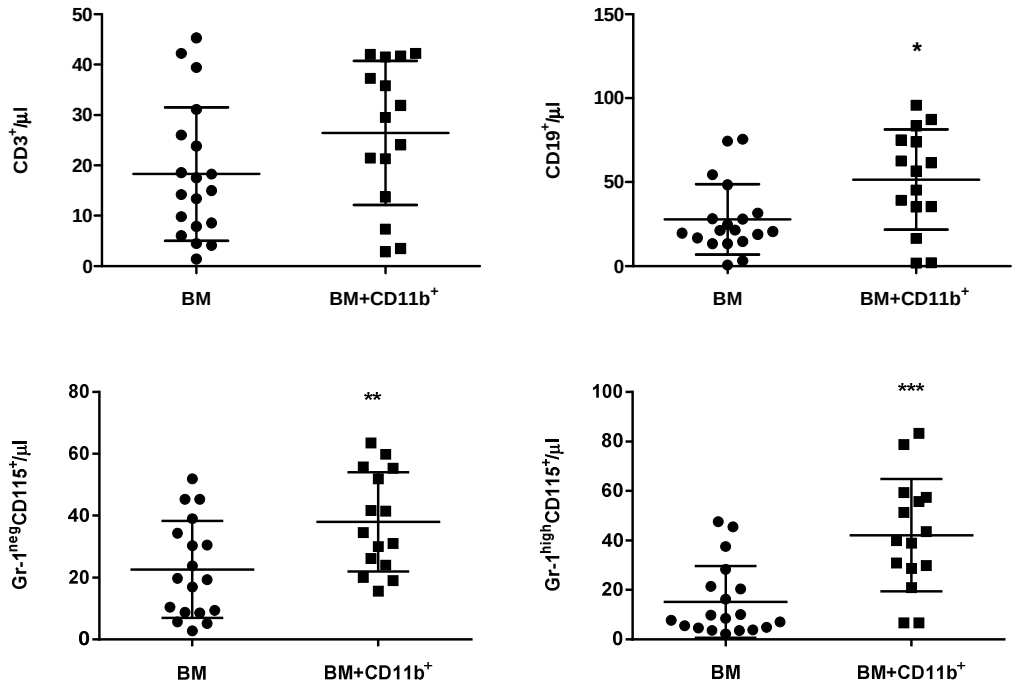

Absolute number (x10^6)
8
7
6
5
4
3
0.4
0.2
0.0
I
III

E.


Ly6G-Ly6C+CD45.1+ (x10^6)
1.5
1.0
0.5
0.0

Supplementary Figure S5.

A.



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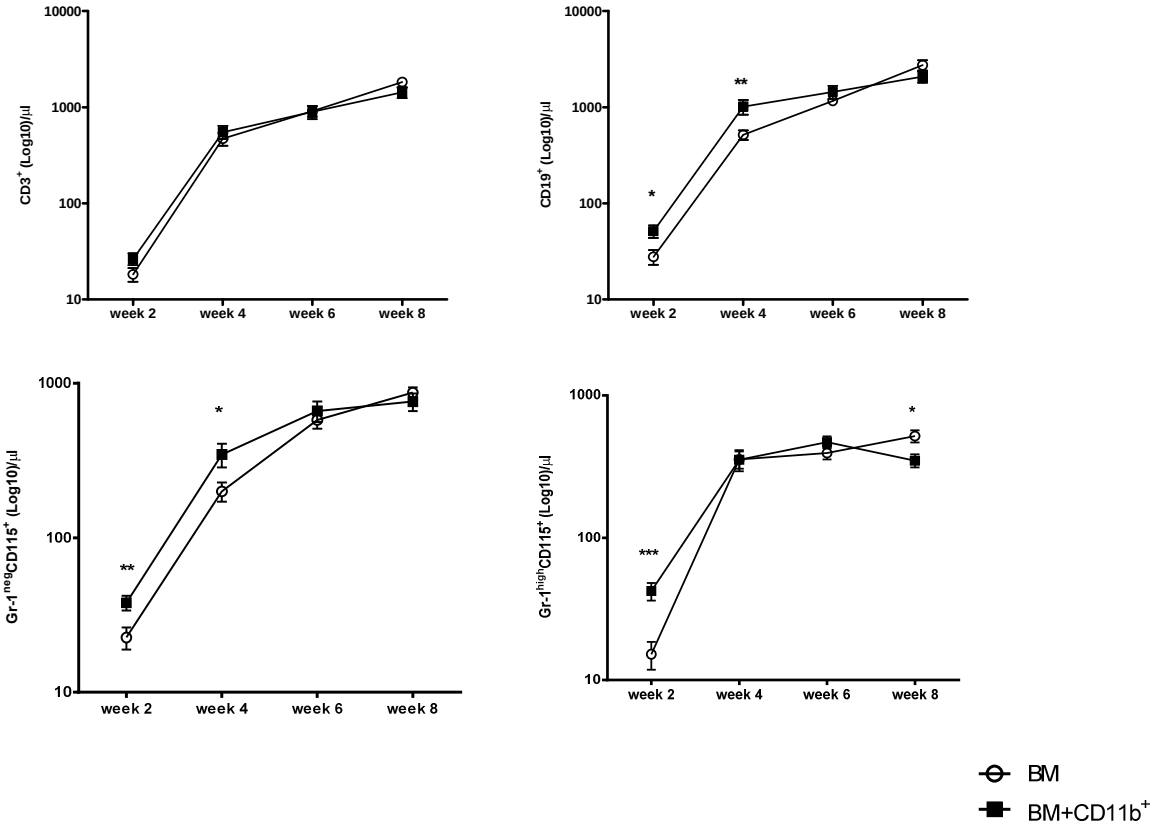
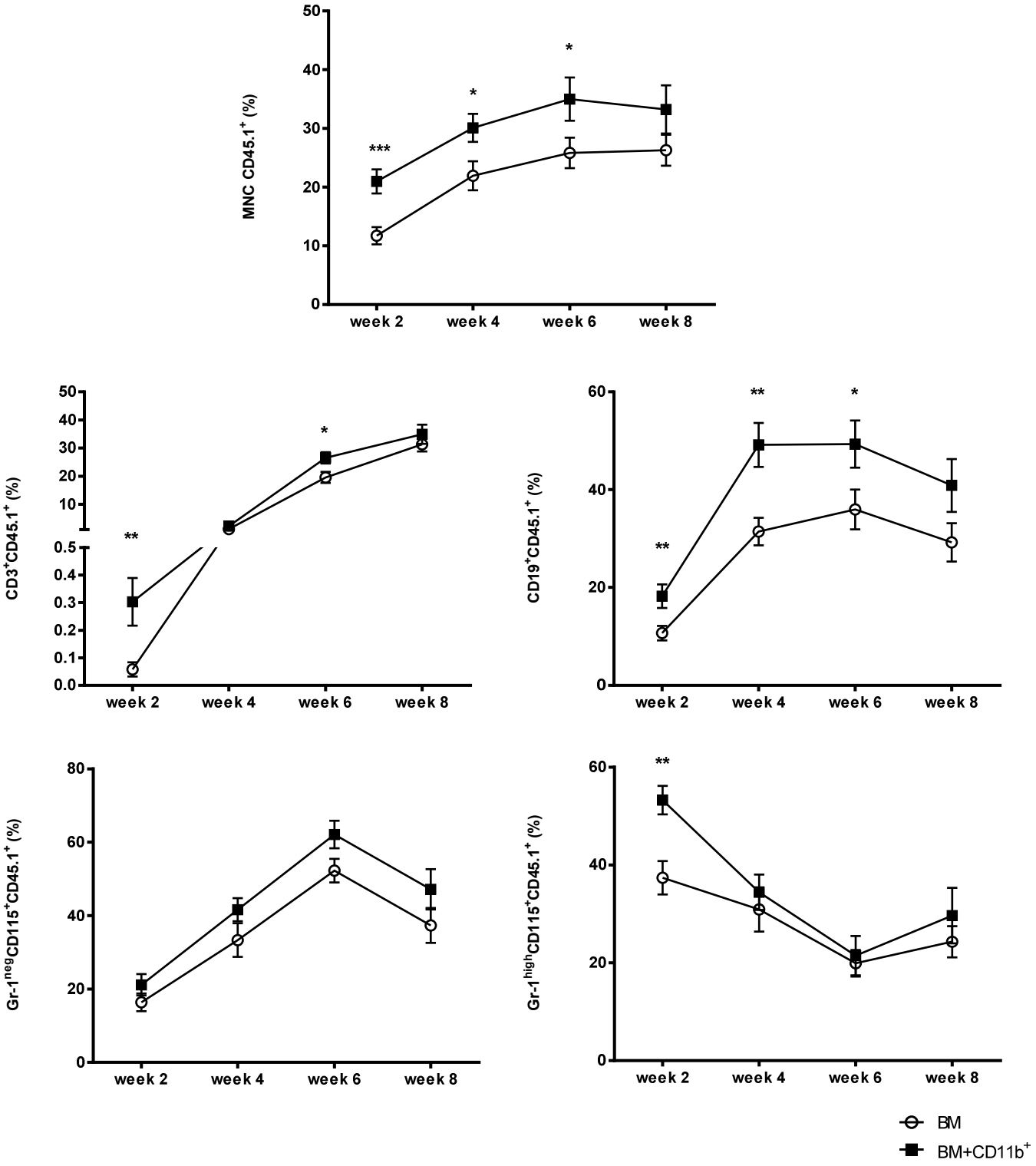
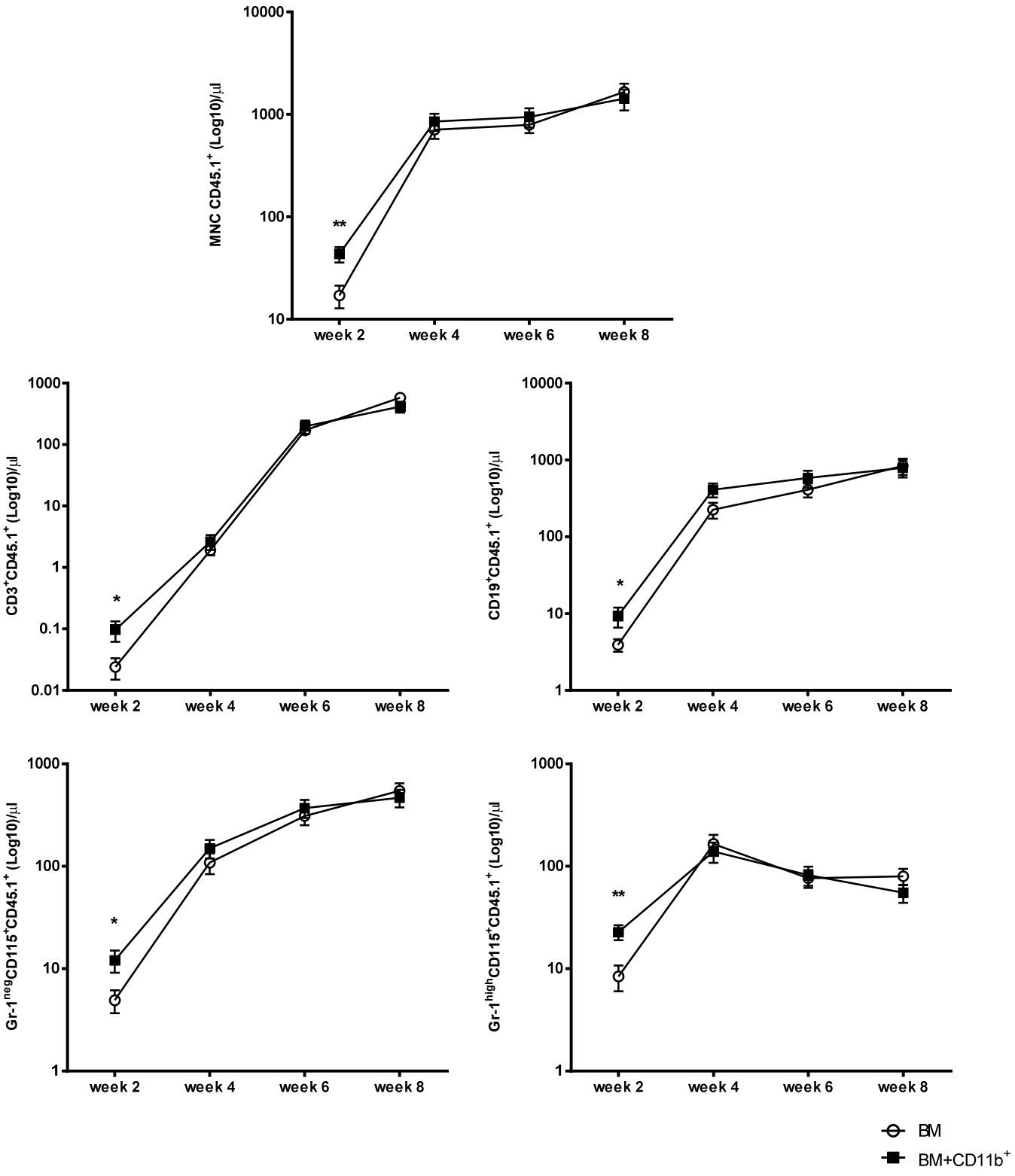

10000
1000
100
10
CD3+ (Log10)/µl
week 2
week 4
week 6
week 8
CD19+ (Log10)/µl
*
**
Gr-1negCD115+ (Log10)/µl
**
*
Gr-1highCD115+ (Log10)/µl
***
*
BM
BM+CD11b+

Supplementary Figure S6.

A.



B.


MNC CD45.1+ (Log10)/µl
10000
1000
100
10
**
week 2
week 4
week 6
week 8
CD3+CD45.1+ (Log10)/µl
1000
100
10
1
0.1
0.01
*
CD19+CD45.1+ (Log10)/µl
Gr-1negCD115+CD45.1+ (Log10)/µl
Gr-1highCD115+CD45.1+ (Log10)/µl
BM
BM+CD11b+