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Supplemental Information

Large-Scale Hematopoietic Differentiation of Human Induced Pluripotent Stem Cells Provides Granulocytes or Macrophages for Cell Replacement Therapies

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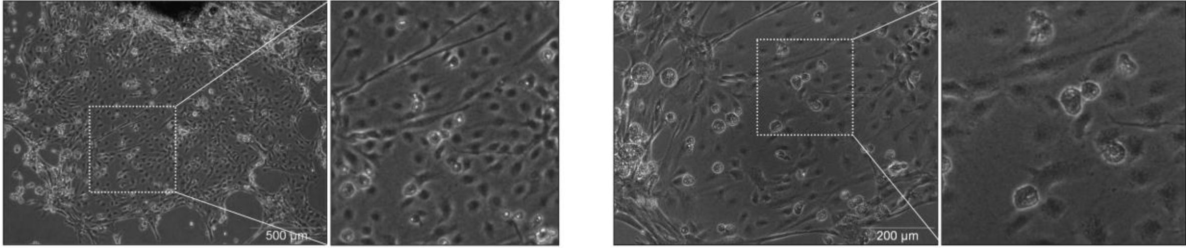
Supplementary Figures

Supp Figure 1

A



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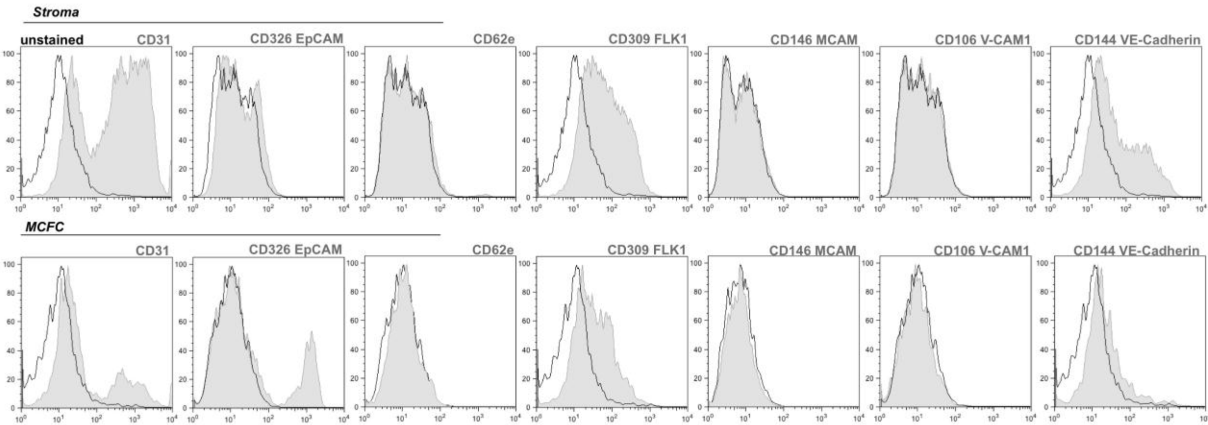
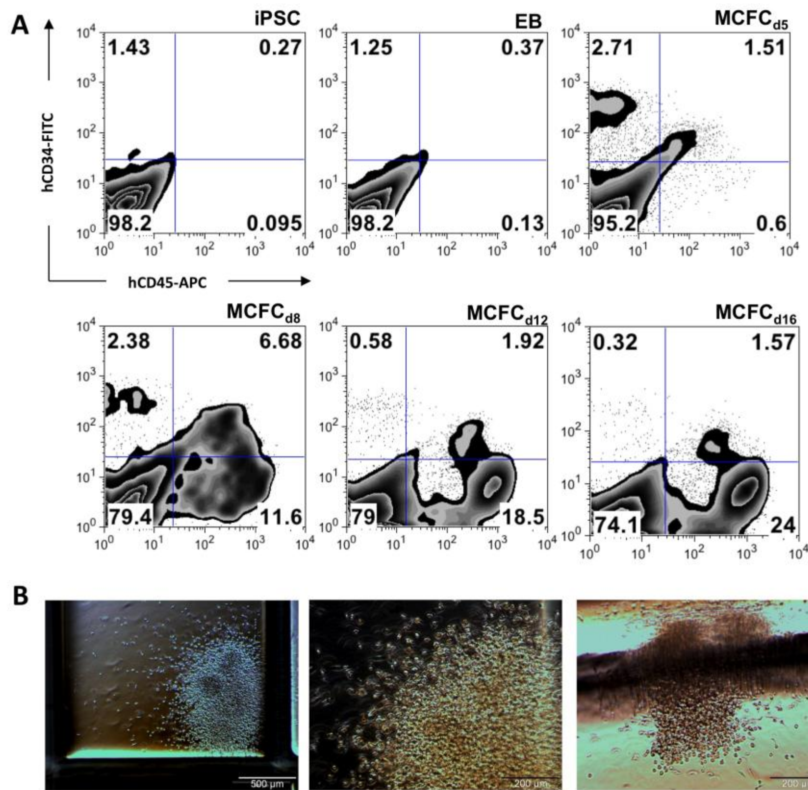


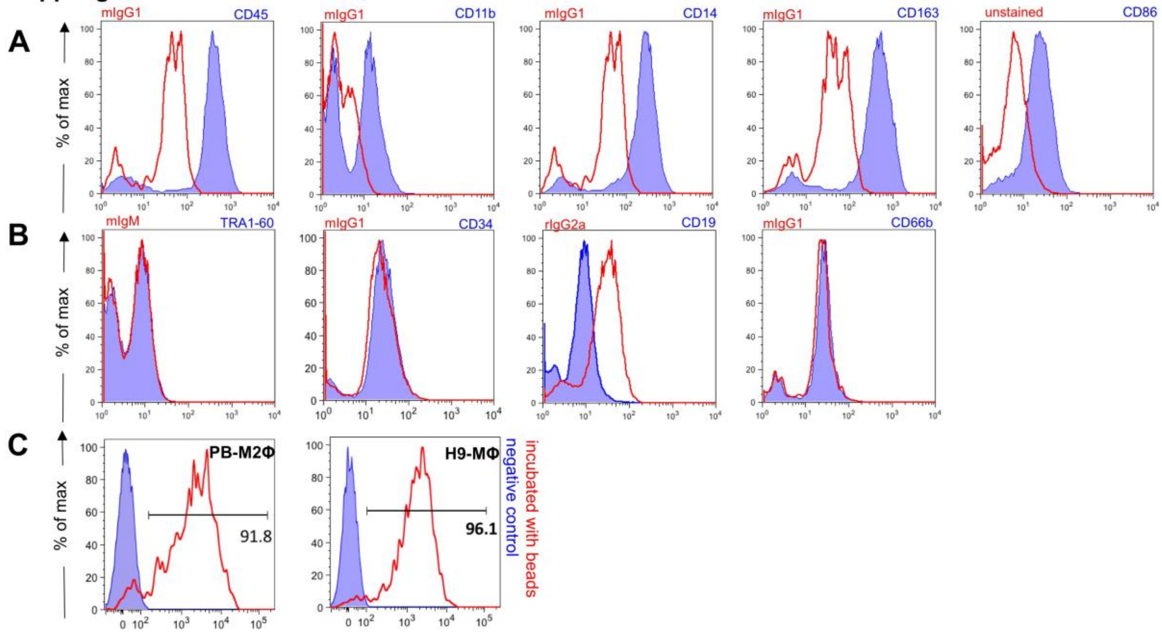
Supplementary Figure 1: Endothelial-like stroma cells, Related to Figure 1

(A) Brightfield images of endothelial like stroma cells surrounding the MCFC. **(B)** Surface marker staining of either stroma cells or MCFCs picked manually and dissociated using trypsin. Gates were pre-gated for living cells based on SSC/FSC (black line represents unstained controls; grey filled lines represent staining for respective antigene). Data shown for hCD34iPSC16.

Supp Figure 2

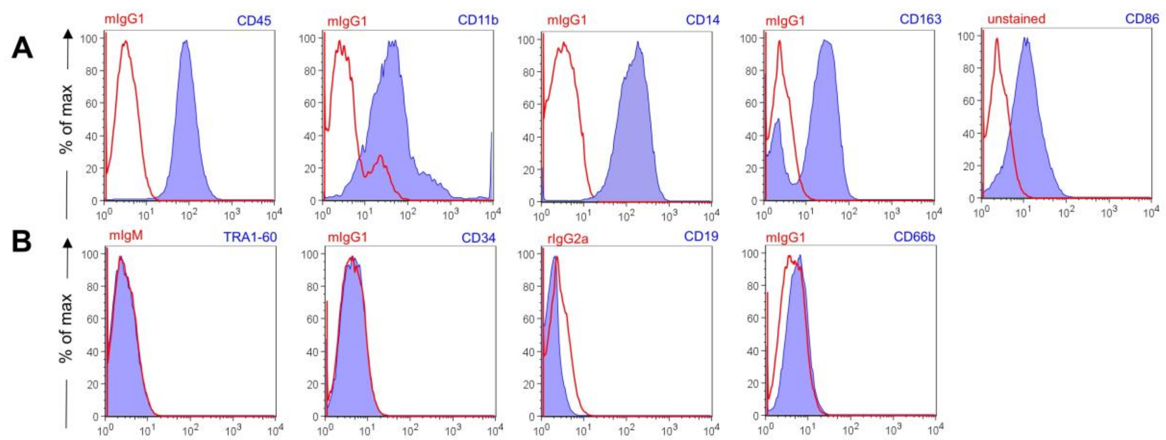


Supplementary Figure 2: Analysis of myeloid cell forming complex, Related to Figure 1 (A) Whole differentiation cultures (step 3) were dissociated and surface marker expression of CD45 and CD34 was analyzed at different time-points of hematopoietic differentiation by flow cytometry. **(B)** Light microscopy of hematopoietic colonies obtained after sorting day 8 MCFC for CD34/CD45$^+$ cells in methylcellulose (scale bar: 500 and 200 µM). Data shown for hCD34iPSC16.



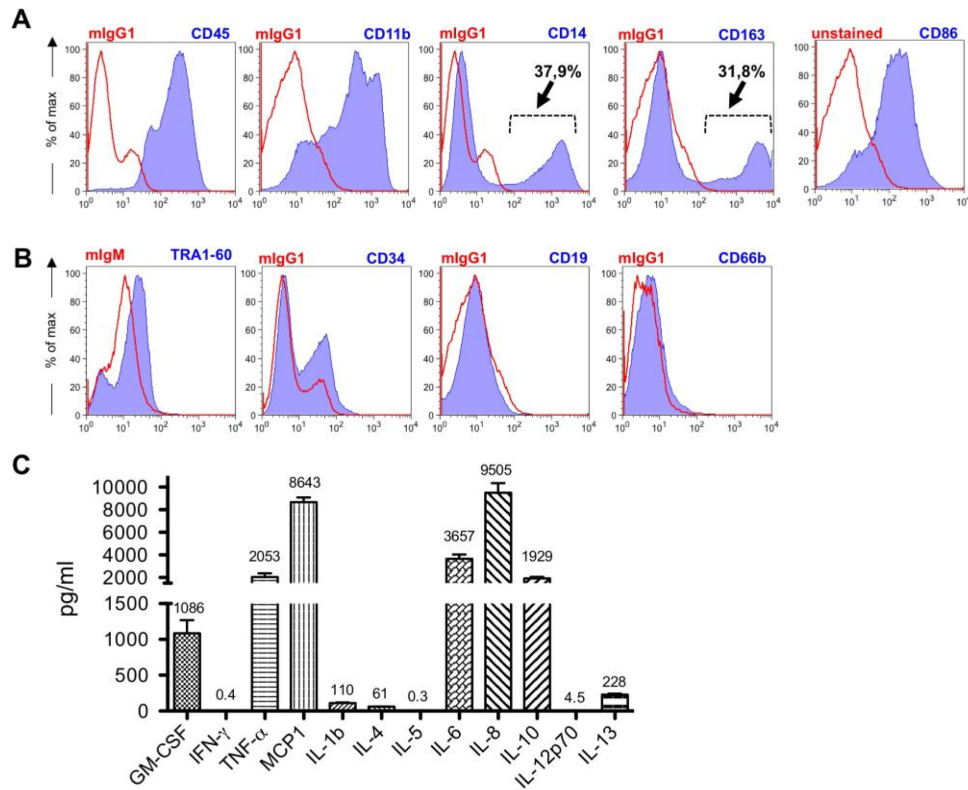
Supplementary Figure 3: Flow-cytometric analysis of H9 derived MΦ, Related to Figure 2 (A) Surface marker expression of typical MΦ maker (CD45, CD11b, CD14, CD163 and CD86; blue filled) on H9 derived MΦ (end of step 4) analysed by flow cytometry (red: respective isotype control). **(B)** Surface marker expression of TRA-1-60, CD34, CD19 and CD66b (blue filled) on harvested monocyte/macrophages (end of step 4) analysed by flow cytometry (red: respective isotype control). **(C)** Phagocytosis of fluorescent (FITC) labeled latex beads by PB-M2Φ or H9-M Φ (end of step 4) analysed by flow cytometry (representative experiment, n=3 of independent experiments; blue filled: untreated control, red: cells treated with 1µm beads,). Data shown for embryonic stem cell line H9.

Supp Figure 4



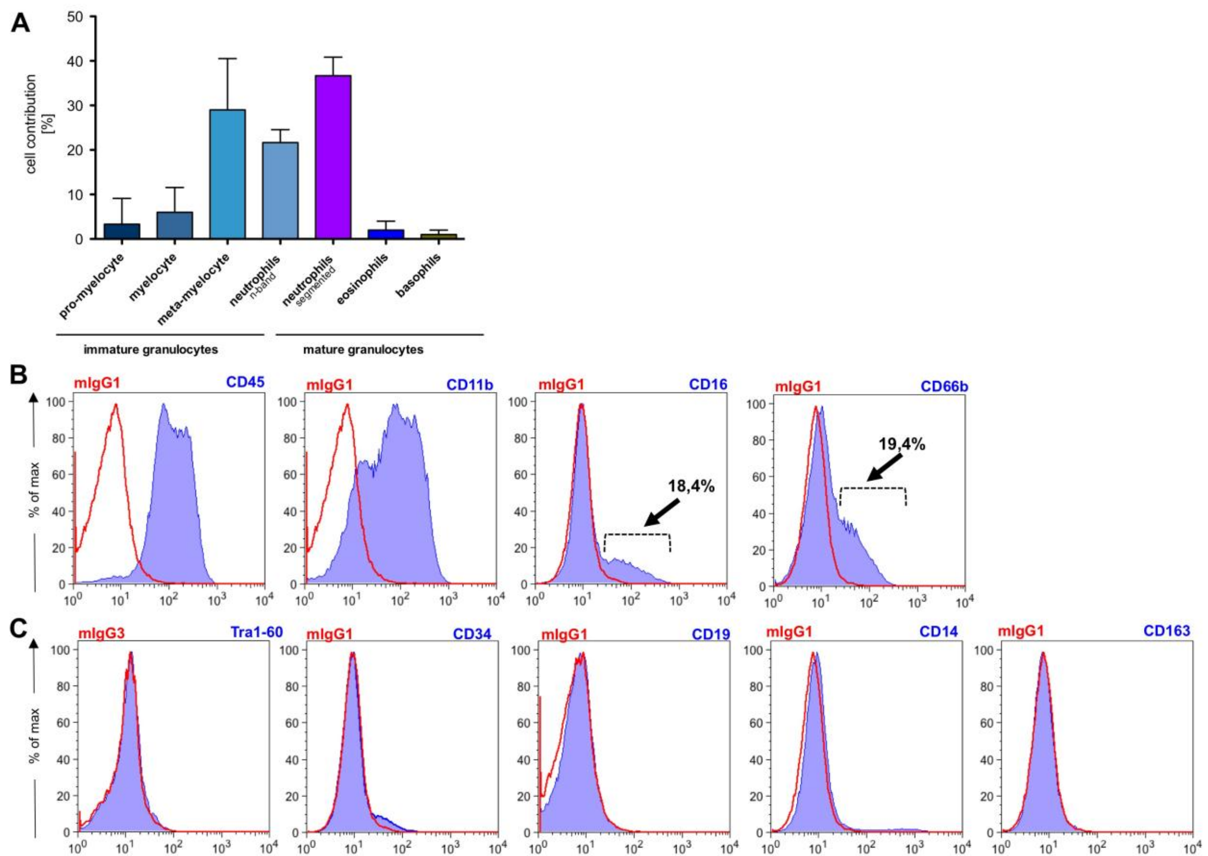
Supplementary Figure 4: Flow-cytometric analysis of hCD34iPSC11 derived MΦ Related to Figure 2 (A) Surface marker expression of typical MΦ maker (CD45, CD11b, CD14, CD163 and CD86; blue filled) on hCD34iPSC11 derived MΦ (end of step 4) analysed by flow cytometry (red: respective isotype control). **(B)** Surface marker expression of TRA-1-60, CD34, CD19 and CD66b (blue filled) on harvested monocyte/macrophages (end of step 4) analysed by flow cytometry (red: respective isotype control). Data shown for hCD34iPSC11.

Supp Figure 5

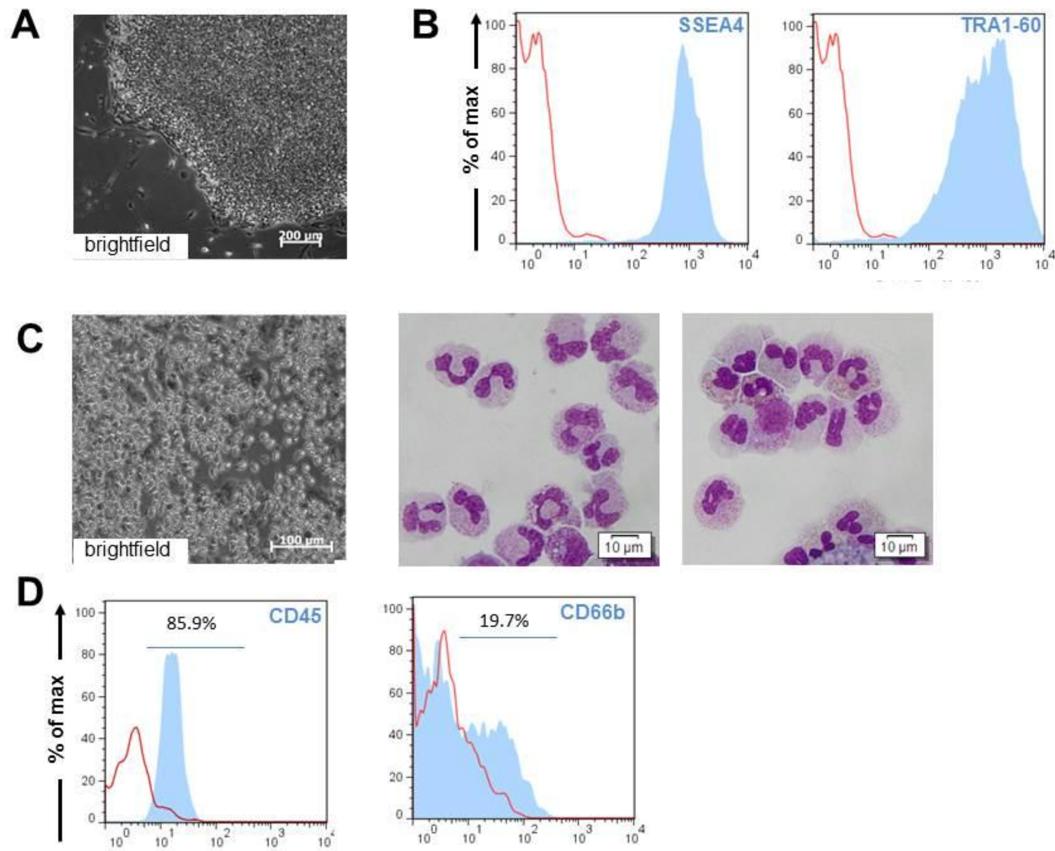


Supplementary Figure 5: Characterization of freshly harvested monocyte/macrophages Related to Figure 3 (A) Surface marker expression of typical MΦ maker (CD45, CD11b, CD14, CD163 and CD86; blue filled) on freshly harvested monocyte/macrophages (end of step 3) analysed by flow cytometry (red: respective isotype control)**. (B)** Surface expression of TRA-1-60, CD34, CD19 and CD66b (blue filled) on freshly harvested monocyte/macrophages (step 3) analysed by flow cytometry (red: respective isotype control). **(C)** Cytokine production of freshly harvested monocyte/macrophages (step 3) upon stimulation with lipopolysaccharid (LPS) (n=3 of technical replicates, mean ± SD). Data shown for hCD34iPSC16.

Supp Figure 6



Supplementary Figure 6: Characterization of freshly harvested granulocytes, Related to Figure 5 (A) Differential count of cytospins from iPSC-Gra (end of step 4) (n=3 of independent experiments; mean ± SD). **(B)** Surface expression of typical granulocytic maker (CD45, CD11b, CD16, CD66b; blue filled) freshly harvested granulocytes (end of step 3) by flow cytometry (red: respective isotype control). **(C)** Surface expression of TRA-1-60, CD34, CD19, CD14 and CD163 (blue filled) on freshly harvested granulocytes (end of step 3) by flow cytometry (red: respective isotype control). Data shown for hCD34iPSC16.



Supplementary Figure 7: Granulocytic differentiation of human fibroblast derived iPSC, Related to Figure 4 and 5. Human iPSC were derived from fibroblast biopsy and showed **(A)** typical morphology on brightfield images (scale bar: 200 µM) and **(B)** expression of pluripotency associated genes such as SSEA4 (blue) and TRA1-60 (blue filled, red shows respective isotype control). After final granulocytic differentiation (end of step 4) by 100ng/ml G-CSF, **(C)** cells showed round shaped morphology on brightfield images (left image), classical granulocytic morphology with segmented nuclei on cytospins (middle and right images, scale bar: 10 µM) and **(D)** surface marker expression of CD45 and CD66b (blue filled, red shows respective isotype control). Data shown for human fibroblast derived iPSC line.

Supplementary Methods

Human pluripotent stem cell culture

hiPSC lines were generated and cultured as described previously (hCD34iPSC11: Ackermann et al., 2014; hCD34iPSC16: Lachmann et al., 2014) (for human fibroblast derived iPSC: manuscript in preparation), using ESC medium (knock-out DMEM, 20% knock out serum replacement, 1 mM L-glutamine, 1% NEAA, 1% penicillin/streptomycin (all Invitrogen, Karlsruhe, Germany), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, United States), and 40 ng/ml bFGF (kindly provided by the Institute of Technical Chemistry, Hannover University, Germany). Work with human ESC lines were approved by the german authorities and cultured as described above.

Isolation monocytes from peripheral blood and differentiation in macrophages

All healthy donors gave written informed consent according to the local ethical committee at Hannover Medical School. PBMCs were isolated from the peripheral blood of healthy volunteers by gradient centrifugation using Biocoll Separating Solution (40 min, 400 g; Biochrome, Billerica, MA). Monocytes were isolated from PBMCs by counters selection using MACS Monocyte Isolation Kit II isolation kit (Miltenyi). $CD14^+$ cells were subsequently cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% penicillin-streptomycin (all Invitrogen), and 100 ng/ml hGM-CSF (M1 macrophages) or hM-CSF (M2-macrophages) (Peprotech), respectively.

Isolation of granulocytes from peripheral blood

All healthy donors gave written informed consent according to the local ethical committee at Hannover Medical School. Human neutrophils were isolated from the peripheral blood of healthy volunteers using Polymorphprep (Axis-Shield PoC AS, Oslo, Norway) according to the manufacturer's recommendations as previously described (Janciauskiene et al., 2004) .Purity of neutrophils was ≥90%, judged by examination of cytospins, and cell viability exceeded 97% according to staining with 0.4% trypan blue solution (Sigma-Aldrich).

Chemotaxis assay (modified Boyden chamber method)

For analysis of chemotactic potential of granulocytes 500 µl of the reaction medium (RPMI/1% BSA) with or without 10nM formyl-Met-Leu-Phe (fMLP; Sigma-Aldrich) or 25 ng/ml rhIL-8 (CellSystems) was placed into each well of 24-well plate, and the cell culture insert (3.0-µm pores; Greiner bio-one) was gently placed into each well to divide the well into upper and lower sections. Cells were suspended in the reaction medium at $1x10^5$/ml, and a 500-µl cell suspension was added to the upper well, allowing the cells to migrate from the upper to the lower side of the membrane for 2 h at 37°C. After incubation, cell in the lower chamber were collected and counted.

Reactive Oxygen Species (ROS) assay

Cells were transferred to FACS tubes and filled HBSS (Invitrogen), supplemented with 0.5 %

BSA and 7.5 mM glucose (HBSSBG), and centrifuged at 300 g for 5 minutes at RT. 1000 U of catalase (Sigma Aldrich) and 0.25 µg DHR 123 (Invitrogen) were added to each sample. Samples were vortexed and incubated for 5 minutes at 37° C. Granulocytes stimulation was performed with 0.2 µM PMA (Sigma-Aldrich). Samples were vortexed and incubated for 25 minutes at 37° C shaking in the dark at 175 rpm. Alternatively, cells were labeled with 2',7'-dichlorofluorescein di-acetate (DCFDA, Sigma Aldrich),. Labeled cells were re-suspended in HBSS and incubated with increasing concentrations of PMA (8, 16 and 32 nM) (Sigma Aldrich), a well-described inducer of ROS, or left untreated (Control). DCF fluorescence (excitation at 498 nm, emission at 522 nm) was monitored for 1 h at 37°C using Infinite® M200 microplate reader (Tecan, Männedorf, Schweiz).

Quantitative Reverse-Transcriptase PCR

Analysis of mRNA was performed as previously described (Pfaff et al., 2013). The following pre-designed *SYBRgreen assays were obtained from Quantitect Primer Assays* (Qiagen, Hilden, Germany): HS_GAPDH (QT QT00079247), Hs_Brachyury (T, QT00094430), Hs_POU5F1 (QT00210840), Hs_RUNX1 (QT00026712) and Hs_PTPCR (QT00028791).

Cytospins

Cytospins were generated utilizing a shandon cytocentrifuge (Thermo Scientific, Waltham, MA, USA). Slides were stained for 5 min in May-Grünwald stain and 10 min in 5% of Giemsa Azur-Eosin-Methylenblue solution and washed extensively in aqua dest.

Flow Cytometry

Flow cytometric analysis was carried out as described (Lachmann et al., 2013). For macrophages PBS supplemented with 10% FCS was used to block unspecific binding. Cells were rinsed with FACS buffer, analyzed with a FACScalibur machine (Beckton & Dickinson, Heidelberg, Germany) and raw data were analyzed using the software FlowJo (TreeStar, Ashland, OR). Used antibodies from eBioscience San Diego, CA, United States: hTra-1-60-PE (Cat-No: 12-8863-80), hCD11b-APC (Cat-No: 17-0118-41), hCD14-PE (Cat-No: 12-0149-42), hCD163-APC (Cat-No: 17-1639-41), hCD86-PE (Cat-No: 12-0869-41), hCD19-APC (Cat-No: 17-0199-41), hCD16-FITC (Cat-No: 11-0168-41), hCD34-FITC (Cat-No: 11-0349-41) and isotype-controls: mouse-IgG1a-PE (Cat-No: 12-4714-41), FITC (Cat-No: 11-4714-41) or APC (Cat-No: 17-4714-41), mouse-IgG3-FITC (Cat-No: 11-4742-41) and rat-IgG2a-PE (Cat-No: 12-4321-81). Used antibodies from Biolegend San Diego, CA, United States: hCD86-APC (Cat-No: 305411), hCD66b-FITC (Cat-No: 305104) or hCD45-PE (Cat-No: 304007).

Cytokine secretion assays (Luminex)

Luminex® analyses were performed with a Cytokine Human 14-Plex Panel (Millipore, Schwalbach, Germany) for analyses of (GM-CSF, IFN-γ, TNF-α, MCP-1, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12). Cytokine standards supplied by the manufacturer were run on each

plate together with the test samples. Samples were treated accordingly to manufacturer's instructions. For cytokine detection, data were acquired on a Luminex-200 System and analyzed with the Xponent software v.3.0 (Life Technologies).

Electron microscopy

The late endosome/Lysosome fusion assay was performed as previously described (Kuehnel et al., 2001) with minor modifications. Briefly, in order to label the late endocytic organelles/lysosomes of macrophages with 5 BSA-gold particles, cells were grown under adherent conditions and incubated with gold particles in Culture medium (OD520: 5) for 15 minutes, followed by a chase of 45 minutes in culture medium. Afterwards the different macrophages were allowed to phagocytose 1µm Latex beads (OD660:0.1) for 1 hour. Not internalized Latex beads were removed by intense washing with PBS, followed by an incubation of 1 hour in medium. Afterwards cells were fixed with1.5% Paraformaldehyde and 1.5% Glutaraldehyde in 200mM Hepes pH 7.35, harvested by scraping and embedded in EPON (Kuehnel et al., 2001). BSA-gold particles used were prepared fresh by using tannic acid in sodium citrate to reduce gold chloride as described previously (Slot and Geuze, 1985).

Statistics

GraphPad Prism 6 was applied to perform unpaired Student's *T* test or analysis of variance (ANOVA). Unless otherwise stated, s.e.m. is indicated. Asterisks mean: * $P < .05$; ** $P < .01$; *** $P < .001$.

Supplementary References

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