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Functional characterization and phenotypic monitoring of human hematopoietic stem cell expansion and differentiation of monocytes and macrophages by whole-cell mass spectrometry

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ABSTRACT

The different facets of macrophages allow them to play distinct roles in tissue homeostasis, tissue repair and in response to infections. Individuals displaying dysregulated macrophage functions are proposed to be prone to inflammatory disorders or infections. However, this being a cause or a consequence of the pathology remains often unclear. In this context, we isolated and expanded CD34+ HSCs from healthy blood donors and derived them into CD14+ myeloid progenitors which were further enriched and differentiated into macrophages. Aiming for a comprehensive phenotypic profiling, we generated whole-cell mass spectrometry (WCMS) fingerprints of cell samples collected along the different stages of the differentiation process to build a predictive model using a linear discriminant analysis based on principal components. Through the capacity of the model to accurately predict sample's identity of a validation set, we demonstrate that WCMS profiles obtained from *bona fide* blood monocytes and respectively derived macrophages mirror profiles obtained from equivalent HSC derivatives. Finally, HSC-derived macrophage functionalities were assessed by quantifying cytokine and chemokine responses to a TLR agonist in a 34-plex luminex assay and by measuring their capacity to phagocytise mycobacteria. These functional read-outs could not discriminate blood monocytes-derived from HSC-derived macrophages. To conclude, we propose that this method opens new avenues to distinguish the impact of human genetics on the dysregulated biological properties of macrophages in pathological conditions.

1. Introduction

$CD34^+$ hematopoietic stem cells (HSCs) circulating in peripheral blood of healthy donors are relatively rare, ~0.15% in average (Herbein et al., 1994). For this reason, HSC sources used for hematopoietic allogeneic transplantation following myeloablative regimens, are preferably performed either following G-CSF mobilization but also using cord blood for which HSCs frequency (~1%) with repopulation capacities are substantially higher (Kinniburgh and Russell, 1993; Wang et al., 1997). For basic research and clinical application purposes, much effort has been done to promote their expansion *in vitro* while maintaining their pluripotency and repopulating capacities using cocktails of cytokines and growth factors, notably Flt-3 ligand (FL), thrombopoietin (TPO), stem cell factor (SCF), interleukin-6 (IL-6), and/or interleukin-3 (IL-3) (Gammaitoni et al., 2003; Nakahata, 2001). More recently, approaches using additional small chemical inhibitors have been described reaching unprecedented expansion yields (Zhang and Gao, 2016). Consequently and despite their scarcity, circulating HSCs represent an accessible cell population that can be easily retrieved by immune-selection and expanded from otherwise discarded blood bank byproducts such as leuko-reduction filters (Peytour et al., 2013). Several reports described the expansion and differentiation of CD34+ HSCs progenitors into lymphoid and/or myeloid cell types including T cells, B cells, CD14+ monocytes as well as dendritic cell progenitors (Arrighi et al., 1999; Mytar et al., 2009; Payuhakrit et al., 2015; Stec et al., 2007). The generation of macrophages from CD34 precursors based on the adherence of macrophage progenitors within a mixture of expanding CD34 progenitors has been described (Clanchy and Hamilton, 2013). Instead, we are describing here a stepwise methodology of progenitor expansion, monocyte differentiation and purification before final differentiation into macrophages with M-CSF in the absence of additional growth factors. In addition, although different macrophage subsets can be readily derived from monocytes isolated from the periphery (Vogel et al., 2014), a report comparing phenotypically and

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functionally HSC-derived macrophages to *bona fide* autologous blood-monocyte-derived macrophages was still missing.

Macrophages are present throughout the body and therefore constitute a "widely dispersed organ system" (Gordon and Plüddemann, 2017). Tissue-resident macrophages and newly-recruited monocyte-derived macrophages infiltrating inflamed tissues regulate tissue homeostasis by ingesting dead, dying cells or toxic materials (Wynn and Vannella, 2016). This function is notably important for the prevention of neurodegenerative disorders such as Alzheimer's disease in which tissue-resident macrophages display depressed phagocytosis and clearance of amyloid peptides accumulating as brain deposits (Fiala et al., 2017). In addition, they play a complex role in response to tissue injury which requires temporal regulation of the M1-classical activation of macrophages that sustains inflammation and killing functions to prevent infection and M2 activation which pro-fibrotic and anti-inflammatory functions favor healing (Mills et al., 2015). These two types of functions inhibit each other and may lead to inflammatory disorders or promote cancers if unbalanced (Harris, 2014). Of particular importance, macrophages are "the chicken and the egg of immunity" (Mills and Ley, 2014). Through their innate capacity to sense conserved pathogen associated molecular patterns, engulf, process and present antigens derived from the ingested microbes, macrophages provide specific signals that direct T cell immunity towards the most adequate response.

Consequently, several human pathogens evolved elaborated strategies to bypass macrophage microbicidal functions and proliferate. For instance, leishmania parasites and a variety of intra-cellular bacteria inhibit or escape phagosomal maturation in order to replicate inside macrophages (Liévin-Le Moal and Loiseau, 2016; Mitchell et al., 2016). From the host perspective, mendelian susceptibility to mycobacterial and Salmonella infection all refers to mutations affecting IL-12, IL-23 and IFN-γ cytokines or pathways which are key components of macrophage activation (Bustamante et al., 2014). In contrast, a substantial frequency of human exposed to *M. tuberculosis* clears the bacteria so efficiently that no traces of an immune memory can be detected (Cobat et al., 2009). This likely suggests that a particularly potent innate immune component prevails in these individuals. However, little is known about the genetic origin of natural resistance to infections. Aside, macrophages are also proposed to play an important role in the development of type 2 diabetes as an inflammatory disease (Donath and Shoelson, 2011) and diabetic patients are more susceptible to infections in general (Abu-Ashour et al., 2017). Evidence of impaired or exacerbated monocytes/macrophages functions in a variety of inflammatory disorders accumulates (Liu et al., 2014). Nonetheless, human clinical immunological studies almost always rely on cells from the blood. Consequently, it becomes difficult to disentangle from the observed altered functions the contribution of host genetics from the effect of obesity or hyperglycemia on the hematopoiesis itself, for instance, or simply from the fact that the most potent effector cells may have simply extravasated to an inflamed site at the time of phlebotomy. Finally, cellular immunotherapeutic intervention based on the pulmonary transplantation of genetically engineered macrophages revealed, in mice, promising potential for the treatment of hereditary pulmonary alveolar proteinosis (Suzuki et al., 2014). Taken together, the capacity to generate *ex vivo* HSC-derived macrophages from a particular individual would open several avenues: (i) gain mechanistic insights on the origin of the natural resistance to infections, (ii) dissect host genetics from environmental factors influencing macrophage functions, (iii) provide an immunotherapeutic tool.

In this report, we describe a method to generate macrophage from HSC-derived monocytic progenitors and compared them functionally and phenotypically to *bona fide* autologous blood-monocyte-derived macrophages. We assessed the reproducibility of our method by investigating comprehensive molecular signatures using whole-cell mass spectrometry biotyping, a method previously described to robustly discriminate immune cells as well as activation status of monocytes or macrophages (Munteanu et al., 2012; Ouedraogo et al., 2012; Portevin et al., 2015). We also demonstrate that HSC-derived macrophages show inflammatory and phagocytic functionalities comparable to their autologous *bona fide* monocyte-derived macrophages counterparts. We propose that such approach is particularly relevant in the context of immunotherapeutic intervention based on the transplantation of macrophages but also offer unprecedented avenues to bring mechanistic insights among individuals naturally resistant to a given pathogen and reciprocally, decipher whether dysfunctions of the monocyte/macrophage compartment observed under certain pathological conditions appeared as a consequence of the disease and/or have host genetic determinants.

2. Results

2.1. Generation of macrophages from HSC or blood monocytes progenitors

CD34 + hematopoietic stem cell progenitors were isolated on day 0 (HSCD0) from freshly processed peripheral blood mononuclear cells of healthy blood donors ($n = 12$) at an average yield of $1.75 * 10^5$ from $2 * 10^8$ PBMCs and an average purity of 91.65% +/− 4.31 (Fig. 1, panel A). HSCs were expanded for seven days (HSCD7) to an average amplification factor of 74 +/− 25.8 (Fig. 1, panel B). As depicted in panel C of Fig. 1, after one week of culture expanded HSCs maintained expression of CD34 and the stem cell growth factor receptor, CD117 and gained surface expression of CD45. As described previously (Stec et al., 2007), the replacement of thrombopoietin with macrophage colony stimulating factor for an additional 7 days of culture allowed partial differentiation and moderate amplification of HSCD7 into $CD14^+$/$HLA\text{-}DR^+$ monocytes (Fig. 1, panel D and E). At this stage, monocytes were either isolated from the expansion mix (HSCdMo) or from cryopreserved autologous peripheral blood mononuclear cells (BMo) using CD14 magnetic beads to an average purity of 94.13% +/− 4.32 and 96.02% +/− 0.93 respectively (Fig. 1, panel F). The monocyte fractions derived from HSCs progenitors or autologous blood PBMCs were then cultivated separately for 6 days in the presence of M-CSF as described here (Verreck et al., 2004), to generate HSC-derived macrophages (HSCDMac) and blood-derived macrophages (BMoDMac) respectively. Panel E of Fig. 1 depicts comparable expression levels of relevant antigen presenting cell's markers *i.e.* CD16, CD206, CD86 and HLA-DR assesseded on HSCDMac and BMoDMac.

2.2. Whole-cell MS biotyping reveals closely overlapping signatures of macrophages derived from HSC or blood monocytes progenitors

To appreciate further comprehensively how similar HSCDMac were to *bona fide* BMoDMac, we performed a whole-cell mass spectrometry approach to obtain molecular fingerprints composed of molecular entities with an apparent mass < 20 kDa present within each cell preparations. Across three independent donors, $2*10^5$ cellular aliquots were withdrawn along the different stages of the expansion and differentiation protocol of macrophages from HSCs or autologous blood monocytes. Cells were processed for whole-cell mass spectrometry (WCMS) profiling similar to a previous report (Portevin et al., 2015). Mass spectrometry data were processed, normalized and aligned using 'MALDIquant' (Gibb and Strimmer, 2012) to obtain a semi-quantitative molecular snapshot for all samples (median of detected peaks' number: 105, IQR: 98–110). An overlay of the averaged spectra in a restricted mass range for each cell derivatives and each donor is presented in panel A of Fig. 2 to illustrate the reproducible over-representation of particular masses for a given cell type and so, independently of the donor origin. For instance, signals with an apparent mass (m/z) of 11,076.6, 11,648 and 11,854.3 were reproducibly more abundant in macrophage samples of HSC or blood monocyte origin. Conversely, m/z signals at 11989.5 were consistently more abundant in freshly isolated or expanded HSCs. Consequently, we generated a heat

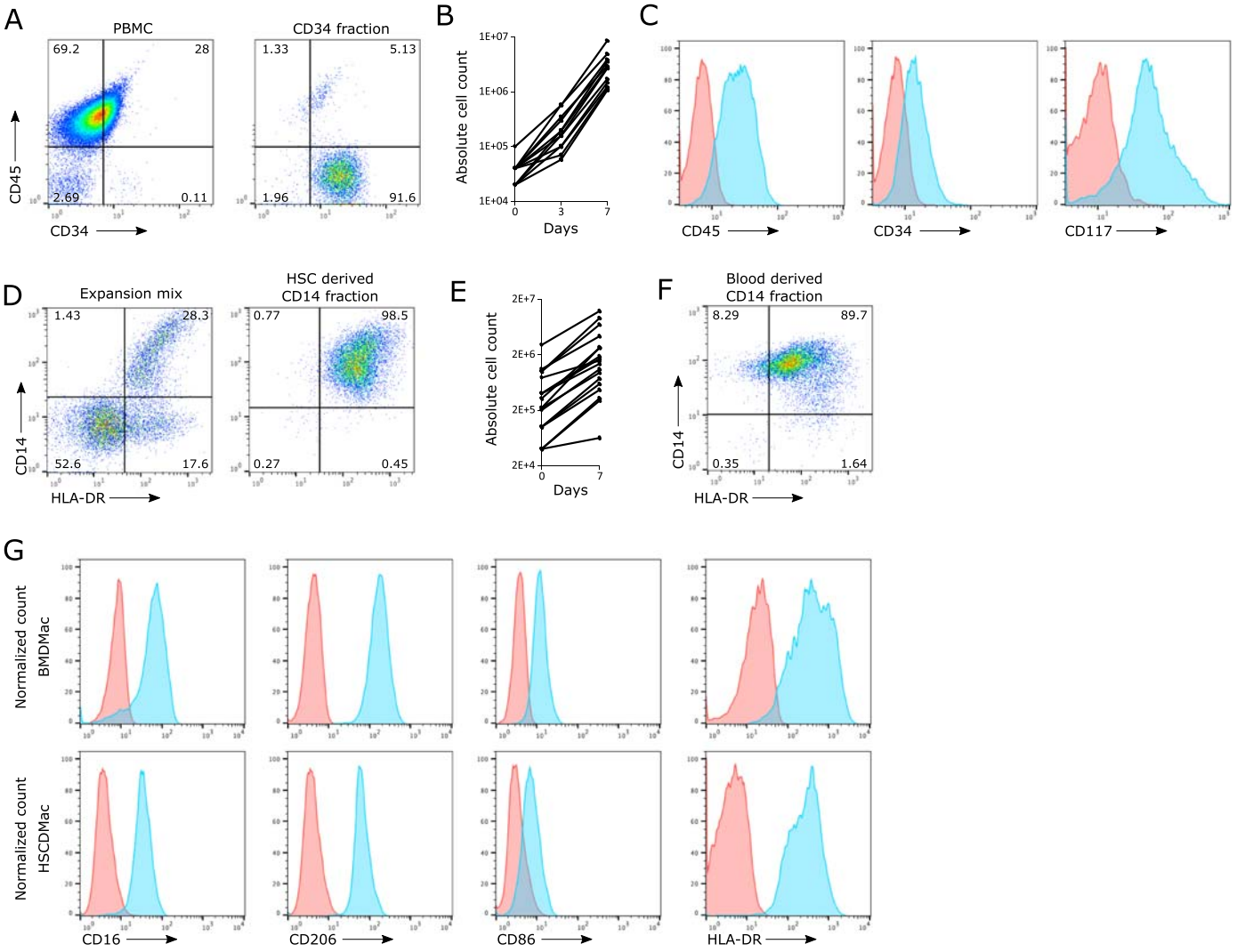

Fig. 1. Isolation, culture and differentiation of hematopoietic stem cells or blood monocytes into macrophages. A) Representative FACS plot showing substantial enrichment of $CD34^+$/$CD45^-$ HSCs (right panel) isolated from PBMCs (left panel). B) Graph depicting the absolute cell counts during HSC expansion across 12 independent donors. C) Representative FACS plot histogram of expanded HSCs after one week of culture (control in red overlaid with specified antibody in blue). D) Representative FACS dot plot of HCS expansion mix after one week of partial differentiation into $CD14^+$/$HLA\text{-}DR^+$ monocytes culture (left plot) and following magnetic bead selection of $CD14^+$ cells (right plot). E) Graph depicting the absolute cell counts during the differentiation of HSCs into monocytes ($n = 12$ independent donors). F) Representative FACS dot plot of $CD14^+$ selected cells from blood. G) Representative FACS plot histogram investigating the surface expression of CD16, CD206, CD86 and HLA-DR at the surface of macrophages derived from HSC (HSCDMac) and macrophages derived from blood monocytes (BMoDMac) (control in red overlaid with specified antibody in blue).

map from the distance matrix computed with the peak intensities for each signals (SNR > 4) within each individual spectra across the full mass range (m/z 4000–20,000). As depicted in Fig. 2B, the heat map revealed very low distances between samples of the same cell type and in particular for HSC-derived macrophages of blood monocyte derived macrophages indicating further that both macrophage products are phenotypically extremely similar. In order to test whether the proteomic signatures could be reproducibly detected across additional samples, we subjected the peak intensity matrix to a supervised discriminant analysis based on principal components. The scatter plot visualization of the first two discriminant functions further demonstrated that WCMS profiles could readily discriminate HSC expanded or not or HSC derived monocytes or blood monocytes. However, signatures from macrophage samples derived from HSC or blood monocyte overlapped indicating that information in the spectra is missing to segregate macrophage origin (Fig. 2C). The predictive properties of the discriminant functions were finally validated with a validation sample set. Excepting one monocyte sample derived from HSC, the 29 remaining samples were properly matched to their expected identity (Fig. 2D). Interestingly, two macrophage sample from HSC and blood monocyte origin were properly identified as macrophage but from the other origin sustaining further that proteomic profiles from both cell derivatives are finely interlaced and the two populations potentially identical.

2.3. Functionalities of macrophages derived from HSC and bona fide blood monocytes derived macrophages are comparable

Having demonstrated that HSCDMac and BMoDMac are phenotypically similar, we aim to study whether both cell derivatives would also present similar functional capacities. We first investigated the capacity of both macrophage preparations to phagocytose *M. bovis* BCG, the live vaccine strain against tuberculosis. For this purpose, we used a GFP transformed *M. bovis* BCG to monitor intra-cellular uptake qualitatively by microscopy and quantitavely using flow cytometry. Macrophages from three independent donors were seeded at equal density and incubated with mycobacterial suspension at a multiplicity of infection of 1:1. After 24 h, cells were washed and mounted in DAPI mounting medium to distinguish infected from non-infected cells (Fig. 3A). The frequency of macrophage containing green bacteria was very similar between HSCDMac and BMoDMac (Fig. 3B). Nonetheless,

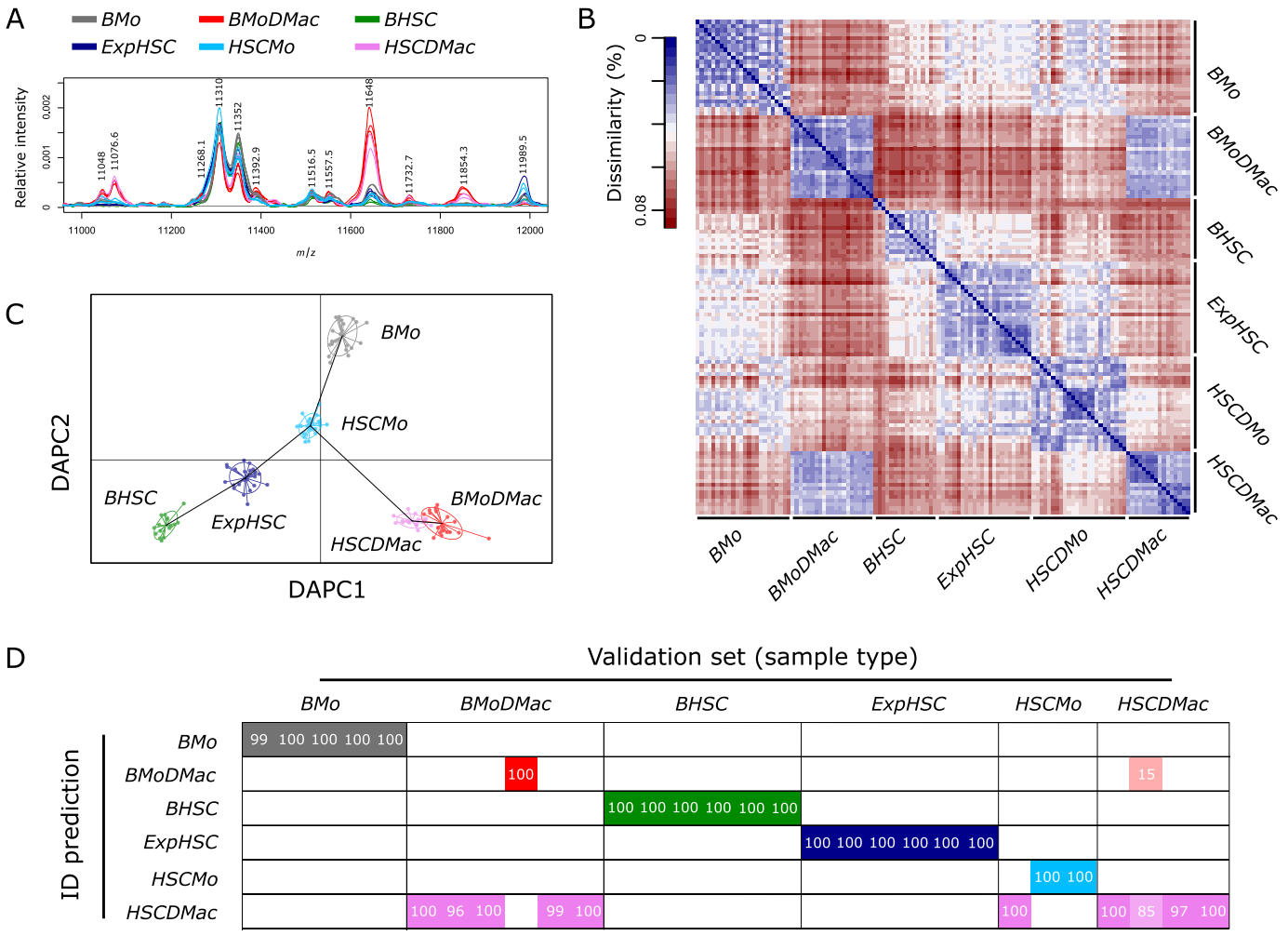

Fig. 2. Whole-cell mass spectrometry proteomic profiles of macrophages derived from HSC are highly similar to macrophages derived from *bona fide* blood monocytes. A) Whole cell mass spectrometry profiles of the indicated cell types and derivatives (test set) showing that in the selected mass range particular masses are specifically enriched in macrophages derived from HSC (HSCDMac, pink) and macrophages derived from blood monocytes (BMoDMac, red) or HSC isolated from blood (BHSC, green) and expanded HSC fractions (ExpHSC, blue), and so, independently of the donor origin ($n = 3$). B) Heat-map of Manhattan dissimilarities between the indicated cell types and derivatives calculated from the peak list intensities obtained by whole-cell MS. C) PCA plots of a supervised machine learning approach using discriminant analysis based on principal components (DAPC) illustrating the potential of whole-cell mass spectrometry fingerprints obtained from the test set to distinguish HSC (expanded or not) from monocytes (derived from HSC or isolated from blood) and to a lesser extent macrophages (derived either from HSC or from blood monocytes). D) Heat-map of the cell type identity prediction of a validation set (independent donors, $n = 6$) using the DAPC tool built from the test set (prediction score ranging from 0 to 100 depicted for each individual sample).

macrophage may uptake multiple bacteria as depicted in the insets of Fig. 3A and therefore the total amount of ingested micro-organisms was estimated by looking at the mean fluorescence intensity increase of infected macrophage compared to uninfected ones. Again, no significant difference could be observed between HSCDMac and BMoDMac demonstrating comparable phagocytic properties of both cell derivatives (Fig. 3C). Another important function of macrophages relies on their capacity to release inflammatory mediators in response to microbial stimulation. We therefore investigated the capacity of both macrophage preparations to produce inflammatory cytokines and chemokines in response to LPS stimulation, a TLR4 agonist produced by gram negative bacteria. Again, no significant difference could be observed between HSCDMac and BMoDMac demonstrating comparable inflammatory properties of both cell derivatives (Fig. 4).

3. Discussion

We showed here that human macrophages can be successfully derived from hematopoietic stem cells (HSCs) through an intermediate stage of differentiation of HSC-derived monocytes. During the first week of expansion of HSCs, we observed that CD34 marker expression was maintained. So was logically CD117, the receptor for the stem cell growth factor (SCF) present in the amplification medium. Interestingly, amplified HSCs quickly gained expression of tyrosine phosphatase, CD45. Although, we did not investigate the precise nature of the CD45 isoform, the expression of this key regulator of immune cell signaling is known to be progressively gained during the maturation of the lymphoid and myeloid lineage from pluripotent HSCs (Hermiston et al., 2003).

Partial differentiation of CD34+ progenitors into CD14+ monocytes is consistent with the heterogeneity of *in vitro*-cultured HSCs isolated from peripheral blood with potentially distinct erythroid/lymphoid/myeloid lineage commitment (Jobin et al., 2015; Kondo et al., 2003). Consequently, we implemented a CD14 enrichment step to ascertain the purity of the monocyte fraction subjected to macrophage differentiation and have comparable culture conditions between HSC-derived monocytes and blood-derived monocytes. For the sake of simplifying the method and reduce costs, it should be possible to simply substitute the monocyte with the macrophages differentiation medium as only macrophages adhere to the plates and contaminants may be washed off easily. We successfully used M-CSF as a maturation factor for the differentiation of HSCs into monocytes as well as monocyte

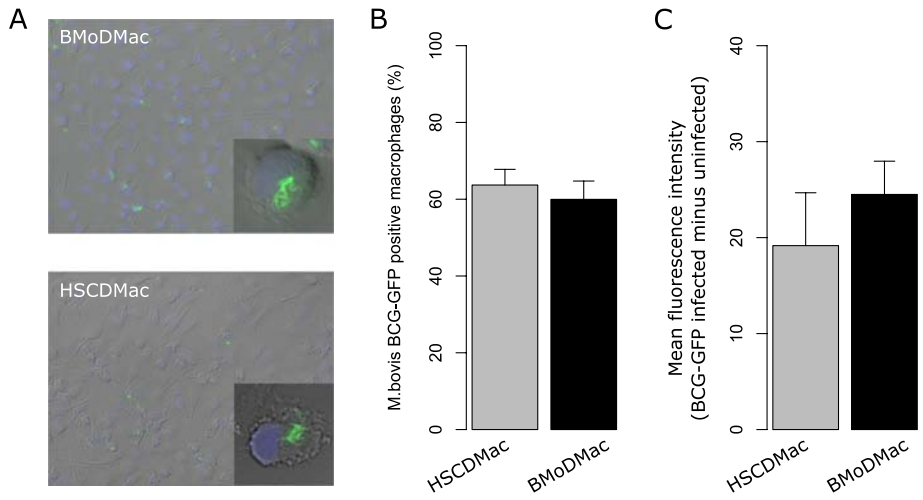

Fig. 3. Comparable phagocytic capacities of macrophages derived from either HSC (HSCDMac) or *bona fide* blood monocytes (BMoDMac). HSCDMac and BMoDMac were infected with *M.bovis* BCG-GFP for 24 h and analyzed by microscopy and flow cytometry A) Representative fluorescence microscopy field and inset of an infected cell depicting the presence of multiple bacteria in the intra-cellular compartment of macrophages. B) Histogram of the mean frequency of *M.bovis* BCG-GFP positive cells +/− SEM across macrophages preparations from 3 independent donors. C) Histogram of the mean fluorescence intensity delta between *M.bovis* BCG-GFP infected and non- infected macrophages +/− SEM across experimental triplicates.

into macrophages. Nonetheless, GM-CSF may also substitute to M-CSF for the maturation of this myeloid lineage and side by side comparison should deserve further investigation.

We phenotypically compared HSDMac and BModMac by flow cytometry and could not detect differences between the two cell populations for any of the investigated cell surface markers. Whole-cell mass spectrometry fingerprinting (WCMS) constitutes a cheap, fast and therefore widely used method in clinical microbiology. This method has recently been applied to the field of mammalian cells, mostly cell lines and primary cells from blood origin (Munteanu and Hopf, 2016; Ouedraogo et al., 2010). Interestingly, semi-quantitative analysis of WCMS fingerprints was shown to correlate with apoptosis induction and discriminate immune cell activation (Dong et al., 2011; Ouedraogo et al., 2012; Portevin et al., 2015). We therefore used a WCMS approach to assess whether different signatures may indicate the HSC or blood origin of the macrophage population. We observed that among the various differentiation steps, BMoDMac and HSCDMac samples were the most similar (Fig. 2B). Using these WCMS signatures, we built a predictive model to demonstrate the validity of the signatures to predict the nature of an independent set of samples (validation set,

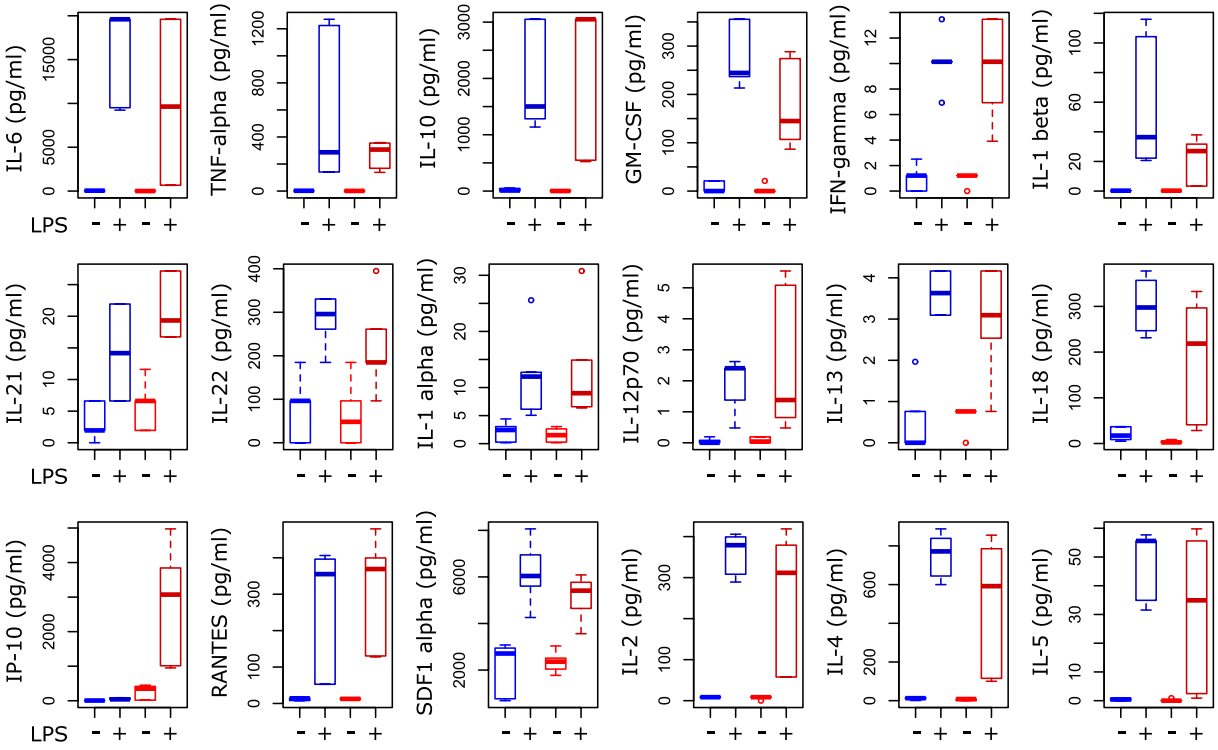

Fig. 4. Comparable inflammatory responses of macrophages derived from either HSC (HSCDMac) or *bona fide* blood monocytes (BMoDMac). HSCDMac and BMoDMac were stimulated or not with LPS, a TLR 4 agonist, for 24 h and supernatants assessed for 34 different cytokines or chemokines. Results from 3 independent donors are summarized and presented as box-and-whisker plots for the 18 detected analytes. No significant differences in the ability to release inflammatory mediators were detected between HSCDMac and BMoDMac in response to LPS (Student's *t*-Test, $p > 0.05$).

Fig. 2D). Given the successful identity prediction based on WCMS fingerprints, we suggest that this analytical pipeline could be of great use for quality insurance purposes in the context of iPS or bone marrow-derived samples which can be of particular clinical relevance for immunotherapeutic intervention of Alzheimer's disease for instance (Magga et al., 2012).

Finally, no significant differences between HSCDMac and BMoDMac could be observed in the phagocytosis of *M. bovis* BCG or the inflammatory response to LPS. These samples being all derived from healthy blood donors; we expected and demonstrated that beyond phenotypic similarities, HSCDMac harbor functional capacities that are tightly comparable to macrophages derived from blood monocytes. Under steady state conditions, all circulating monocytes are derived from CD34+ progenitors; nonetheless, the hematopoietic niche in which CD34 differentiate into monocytes *in vivo* cannot rigorously be compared with *in vitro* culture conditions and as such, the fact that CD34 cell-derived macrophages and macrophages derived from peripheral blood monocytes are indistinguishable both phenotypically and functionally was not trivial. However, the monocyte compartment that gives rise to infiltrating macrophages is subjected to profound qualitative changes in response to pathological stresses such as rheumatoid arthritis (Kawanaka et al., 2002), atherosclerosis (Schlitt et al., 2004), diabetes (Min et al., 2012), HIV infection (Thieblemont et al., 1995), bacterial sepsis (Fingerle et al., 1993) and pulmonary tuberculosis (Vanham et al., 1996). In addition, monocytes from tuberculosis patients showed defective abilities to differentiate into dendritic cells (Balboa et al., 2013). We propose that side by side functional comparison of HSDMac and BMoDMac from similar cohorts of patients could distinguish the genetic determinants which may drive the disease from the physiological consequences of the respective pathology but also enlighten the cellular origin of the natural resistance to intra-cellular parasites.

4. Methods

4.1. Ethics statement

Fresh blood packs (buffy coat) were purchased anonymously from the Blutspendezentrum SRK beider in Basel, Switzerland. In compliance with the Helsinki Declaration, signed informed consents stating specifically that "the donation or certain components thereof be used for medical research after definitive anonymization" was obtained prior blood donation. Consent form can be found here: http://blutspendebasel.ch/blutspende-downloads.html (accessed on August 29th 2017).

4.2. Blood processing for PBMC isolation

Peripheral Blood Mononuclear Cells (PBMCs) were freshly isolated from blood packs (see ethics statement) by density centrifugation using Greiner Bio-One Leucosep® tubes according to the manufacturer's recommendations. PBMCs rings were collected, washed twice in RPMI-1640 and counted. Part of the collected PBMCs were used directly for HSC isolation, and the remaining cells suspended at $20 * 10^6$ cells/ml in ice-cold freezing medium (50% RPMI-1640, 40% FCS, 10% DMSO) and transferred at −80 °C in cryotubes and Nalgene® Mr. Frosty for 24 to 72 h before long-term storage in liquid nitrogen.

4.3. Hematopoietic stem cell derived macrophages

Hematopoietic stem cell progenitors were isolated from 100 to 200 $* 10^6$ freshly isolated PBMCs using CD34 microbeads (Miltenyi Biotec GmbH). CD34+ cells were seeded and passed after 72 h at $6 * 10^4$ cells/-cm^2 in XVivo10 medium (Lonza) containing FCS (4%), SCF (50 ng/ml), TPO (15 ng/ml), IL-3 (30 ng/ml) and Flt-3L (30 ng/ml). On day 7, expanded HSCs were transferred in IMDM medium (Lonza) containing SCF (25 ng/ml), M-CSF (30 ng/ml), IL-3 (30 ng/ml), Flt-3 Ligand (30 ng/ml) and FCS (20%) at $4 * 10^4$ cells/cm^2, cytokines were added at the same concentration 3 days later. On day 7, cells were subjected to CD14 selection using CD14 microbeads (Miltenyi Biotec GmbH) and differentiated in tissue culture treated plates or dishes at a density of $14 * 10^4$ cells/cm^2 in RPMI-1640 (Sigma-Aldrich), 10% heat-inactivated FCS (Gibco Life Technologies™), and M-CSF (50 ng/ml) for 7 days at 37 °C in a humidified atmosphere with 5% CO_2. Blood monocytes were isolated from PBMCs using CD14 microbeads and differentiated into macrophages as indicated for HSC-derived monocytes. Macrophages were harvested after incubation with Trypsin/EDTA solution (Sigma-Aldrich) for 20 min at 37 °C.

4.4. Flow cytometry analysis

Staining of cell aliquots were performed in PBS 0.5% FCS containing combination of the following antibodies: anti-CD14 FITC (MΦP9, Becton Dickinson), anti-CD16-PE (Leu11c, Becton Dickinson) and anti-HLA-DR (MDC8, Miltenyi Biotec) or anti-CD45-FITC (HI30, BD Pharmingen), anti-CD34-PE (581, BD Pharmingen) and anti-CD117-APC (104D2, Biolegend) for 15 min on ice. After washing, antibody labeled cell preparations were fixed in CellFix buffer (BD Biosciences), fluorescence acquired on a BD FACSCalibur apparatus and data analyzed using Flowing Software 2.5.1 (University of Turku).

4.5. Cytokines and chemokines profiling

Analyte concentrations from macrophage culture supernatants stimulated or not with LPS (O111:B4, 10 ng/ml final, Sigma-Aldrich) for 24 h were assessed using human cytokine and chemokines 34-plex (ProcartaPlex™, affymetrix eBioscience), fluorescence acquired on a Luminex® 200™ System and data analyzed with nCal R package.

4.6. Macrophage phagocytosis of M. bovis BCG-GFP

Exponentially growing *M. bovis* BCG-GFP culture washed with PBS-Tween80 (0.1%), resuspended in PBS and centrifugated at 260 g for 10 min to separate clumps from single cell bacteria was frozen in 50% glycerol and bacterial counts estimated by serial dilution on 7H11 agar plates complemented with OADC supplement (BD biosciences). Macrophage seeded within 16-well glass chamber slides (Lab-Tek®) at a density of $3 * 10^5$ cells/cm^2 were infected with *M. bovis* BCG-GFP at an MOI 1:1. After 24 h, supernatants were discarded and slides mounted in DAPI-containing Vectashield medium (Vector Laboratories). Two independent fields were acquired on a Leica DM5000B for each sample and 199 cells counted in average (IQR, 104–290). Alternatively, macrophages were recovered after 20 min digestion with Trypsin solution (Sigma) and fixed with BD CellFix solution (BD biosciences), fluorescence acquired on a BD FACSCalibur apparatus and data analyzed using Flowing Software 2.5.1 (University of Turku).

4.7. Whole-cell mass spectrometry

Cryopreserved cell pellets were washed with 70% ethanol and briefly vortexed before centrifugation (10 min, 16,000 RCF). Supernatants were discarded, cell pellets dried for 1 min at room temperature and finally solubilized with 10 μl formic acid 10%, mixed with 2 volumes of a saturated sinapinic acid solution (40 mg for 1 ml of acetonitrile 60%/H20 37%/TFA 3%) and spotted in quadruplicates on a MALDI-TOF chip. External calibration was performed using ribosomal corresponding *m/z* signals from whole-cell *E. coli* (DH5α). Mass spectra (m/z mass range: 3000 to 30,000) were acquired on a Shimadzu Biotech Axima Confidence.

4.8. Mass spectrometry data analysis

Mass spectra exported as mzXML files with MALDI-MS Shimadzu Biotech Launchpad 2.8.1 (Kratos Analytical Ltd) were imported in R

using MALDIquant Foreign package. Mass spectra were processed applying successively square-root transformation, Savitzky-Golay smoothing, SNIP baseline correction, standardization of intensities based on TIC and aligned using conserved m/z peaks at 5665.5 and 11,310 and a tolerance of 0.032 using MALDIquant R package. Peak detection was performed with a signal-to-noise ratio of 4 and HalfWindowSize of 60 and binned with a tolerance of 0.016. Peak list and their respective intensities were retrieved to build a matrix used for subsequent linear discriminant analysis and prediction using adegenet R package.

Acknowledgments

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Conflict of interest

VP and GV are employed at Mabritec AG, a service laboratory in the field of MALDI-TOF mass spectrometry. The authors declare that they have no conflict of interest.

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