Distinguishing Human Peripheral Blood CD16⁺ Myeloid Cells Based on Phenotypic Characteristics.

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Summary

We distinguish human blood myeloid CD16⁺ dendritic cells from CD14^{dim}CD16⁺ monocytes by their surface phenotype, reinforcing that heterogeneity of cell preparations can confound functional analysis.

Running Title: Distinguishing Human CD16 Myeloid Cells

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Abbreviations

ADCC	Antibody dependent cellular cytotoxicity
aGVHD	Acute Graft versus Host Disease
Allo	Allogeneic
Auto	Autologous
DC	Dendritic Cell
f-MLP	N-formyl-methionine-leucine-phenylalanine
FMP	Influenza Matrix Protein
HCT	Hematopoietic Cell Transplant
HLA	Human Leucocyte Antigen
HLDA	Human Leucocyte Differentiation Antigen
Mo	Monocyte
MoDC	Monocyte derived DC
SLAN	6-Sulpho-LacNac
SPADE	Spanning-tree Progression Analysis of Density-Normalized Events
viSNE	Visualized Stochastic Neighbor embedding

Abstract

Myeloid lineage cells present in human peripheral blood include dendritic cells (DC) and monocytes (Mo). The DC are identified phenotypically as HLA-DR⁺ cells which lack major cell surface lineage markers for T cells (CD3), B cells (CD19, CD20), NK cells (CD56), red blood cells (CD235a), hematopoietic stem cells (CD34) and Mo which express CD14. Both DC and Mo can be phenotypically divided into subsets. DC are divided into plasmacytoid DC which are CD11c⁻, CD304⁺, CD85g⁺ and myeloid DC which are CD11c⁺. The CD11c⁺ DC are readily classified as CD1c⁺ DC and CD141⁺ DC. Mo are broadly divided into the CD14⁺CD16⁻ (Classical) and CD14^{dim}CD16⁺ subsets (Non-classical). A population of myeloid derived cells that have DC characteristics i.e. HLA-DR⁺ and lacking lineage markers including CD14, but express CD16 are generally clustered with CD14^{dim}CD16⁺ Mo. We used high dimensional clustering analyses of fluorescence and mass cytometry data, to delineate CD14⁺ Mo, CD14^{dim}CD16⁺ Mo (CD16⁺Mo) and CD14⁻CD16⁺ DC (CD16⁺DC). We sought to identify the functional and kinetic relationship of CD16⁺DC to CD16⁺Mo. We demonstrate that differentiation of CD16⁺DC and CD16⁺Mo during activation with IFN γ *in vitro* and as a result of an allo-hematopoietic cell transplant (HCT) *in vivo* resulted in distinct populations. Recovery of blood CD16⁺DC in both auto- and allo-(HCT) patients after myeloablative conditioning showed similar reconstitution and activation kinetics to CD16⁺Mo. Finally, we show that expression of the cell surface markers CD300c, CCR5 and CLEC5a can distinguish the cell populations phenotypically paving the way for functional differentiation as new reagents become available.

Introduction

Myeloid precursor-derived antigen presenting cells (APC) include dendritic cells (DC), monocytes (Mo) and macrophages. Each cell type has evolved to carry out unique functions that reflect their differentiation state, activation state and microenvironment, notably tissue location. APC react to their environment, maintaining peripheral immune tolerance, and responding to control inflammation. Consequently, they play a central role in disease i.e. the failed response in cancer, the inflammatory response in autoimmune disease and the rejection of transplants.

Characterization of the cell surface phenotype of human DC populations in a range of tissues is limited by availability of monoclonal antibodies (mAbs) to cell surface markers (1-7). The simplest phenotype used to identify DC typically involves expression of HLA-DR and exclusion of markers for the major cell lineages (Lin⁻) including T cells (CD3⁺), B cells (CD19⁺ and CD20⁺), NK cells (CD56⁺), erythrocytes (CD235a) and Mo (CD14⁺). This strategy results in mutually exclusive identification of CD304⁺ CD123⁺ plasmacytoid DC (pDC), and CD1c⁺, CD141⁺/XCR1⁺, and CD16⁺ myeloid DC populations (2, 5, 6). Such a gating strategy excludes Mo populations from analyses of DC.

The identification of human peripheral blood Mo using flow cytometry has broadly relied on their scatter profile and CD14 expression (8, 9). The expression of CD16 divided Mo into subsets with functional differences, particularly in their profile of cytokine secretion (9-11) and only very recently has the heterogeneity of monocytes been emphasized by high dimensional mass cytometry(12). Historically, Mo and DC were further distinguished by differences in their morphology and ability to stimulate primary T cell responses. However, their close relationship was demonstrated when plastic adherent Mo were cultured in GM-CSF/IL-4 to generate Mo derived DC (MoDC) (13), a population of cells that lost Mo markers and could stimulate an allogeneic MLR. More recent work, including transcriptome analysis has demonstrated the ontological relationship between different myeloid cell types including DC and Mo subpopulations from highly purified precursor stem cells from both human and mouse (14-18).

The relationship between the CD16⁺ population within the Lin⁻HLA-DR⁺CD14⁻ (CD16⁺DC) gate and the CD16⁺ CD14^{dim} HLA-DR⁺ cells within the Mo gate (CD16⁺Mo) is unclear. Studies of Mo subpopulations generally exclude CD16⁺DC in analyses due to CD14 expression being a pre-requisite for Mo

identification (9, 19, 20). Alternatively, CD16⁺DC are excluded in analyses by inadvertently grouping them with the total NK and granulocyte depleted CD16⁺ population i.e. when using pan CD16⁺ Mo isolation microbeads (21). Several studies have attempted to define CD16⁺ DC by comparing them to other mDC and not Mo (22-24). Meta-analysis of transcriptional profiling data of Mo and DC studies has been performed but the methodology used for cell preparations in different studies compromises these comparisons (22, 25, 26). Recent unbiased genomic classification performed using single cell RNA sequencing has now allowed further classification of DC and Mo from Lin⁻HLA-DR⁺ PBMC (27) into: DC1, CD141⁺; DC2, CD1c⁺_A; DC3, CD1c⁺_B; DC4, CD16⁺; DC5, Axl⁺Siglec6⁺ and DC6, pDC. Mo were divided into: Mono1, CD14⁺CD16⁻ Mo (CD14⁺Mo); Mono2, CD14^{dim}CD16⁺ Mo (CD16⁺Mo) and; Mono3, CD14⁺CD16⁺ Mo (CD14⁺CD16⁺ Intermediate Mo). These gene lists contain cell surface proteins that have been used to aid in their phenotypic identification (Reviewed in 7).

Transcriptional profiling has distinguished between cell populations but robust phenotypic distinction at the cell surface requires mAbs. The O-linked glycoprotein modification 6-Sulpho-LacNac (SLAN) on PSGL1 discriminates an inflammatory CD16⁺ myeloid cell type (24, 28). Gene expression profiling classifies SLAN⁺ cells with a subpopulation of CD14^{dim}CD16⁺Mo (9). As these studies were only powered to detect CD14⁺ cells, SLAN may still be expressed on the CD14⁻ population. Expression of CD64 (FcγRI) was found to differentiate a CD16⁺ Mo subset with DC characteristics (29).

The CD16⁺ myeloid cell population is clinically important in inflammation and transplantation. We endeavored to reconcile different approaches used to identify Mo and DC subsets through an extensive phenotypic analysis. We monitored the recovery of these populations within a clinical environment to establish their importance in predicting clinical outcomes in auto- and allo-hematopoietic cell transplantation (HCT). Our data indicates that early differentiation and activation of circulating CD16⁺DC in allogeneic HCT patients is predictive for the onset of acute graft versus host disease (GVHD).

Materials and Methods

Study Approval

Patient samples were collected with informed consent approved by the Sydney Local Health District Human Research Ethics Committee (HREC/11/CRGH61, HREC/12/CRGH/59, HREC/07/RPAH/28, HREC/15/RPAH/485), consistent with the declaration of Helsinki.

Isolation of DC and Mo from Human Samples

Venous blood or bone marrow samples from consenting volunteers were collected through the Department of Hematology, Concord Repatriation General Hospital (CRGH). Mononuclear cells were prepared by Ficoll-Paque density gradient centrifugation using the manufacturer's recommendations (GE Healthcare Life Sciences). CD14⁺Mo were isolated by a positive selection using CD14 microbeads (Miltenyi Biotec, Gladbach) and positively selected using an AutoMACS Pro (Miltenyi Biotec) using Program Possel (Fraction 1). DC and Non classical Mo were isolated using a two-step protocol by magnetic depletion of Lineage positive using mAbs to CD3, CD14, CD19, CD20, CD56, CD235, followed by labeling with anti-mouse IgG microbeads (Miltenyi Biotec) and depletion using an AutoMACs Pro Cell Separator using program Deplete025 (Fraction 2). Both fractions were then stained at 4°C for 20 minutes with fluorescently conjugated antibodies to CD3, CD19, CD20, CD56 – Brilliant Violet V450; CD14-PerCP Cy5.5, CD11c -Alexa Fluor700, HLA-DR-APC-H7, CD16-V500, CD304-APC all from BD Biosciences and CD1c-FITC (BDCA-1) from Miltenyi Biotec prior to sorting. Fraction 1 was sorted to obtain highly purified CD14⁺CD16⁻ Mo, while fraction 2 was sorted to obtain CD16⁺ Mo, CD16⁺DC, and CD1c⁺ DC. All sorts were performed using an Influx (BD Biosciences) fitted with a 100um nozzle at 20 PSI. Purities were routinely greater than 95%. Total PBMC, Lineage depleted pre-sort and post sort collections were further analyzed using the visualized stochastic neighbor embedding (viSNE) implementation in Cytobank (Cytobank Inc., Mountain View, CA)(30) to confirm purity and identity of sorted populations.

DC and Mo Morphology and Differentiation

Sorted DC and Mo subsets were immediately cytopun onto glass slides using a Thermo Shandon Centrifuge or adhered to Tissue-Tek chamber slides overnight. Cells were fixed in 100% methanol at

-20°C for 10 minutes prior to performing May-Grunwald Romanowsky (MGG) staining at room temperature. Images were acquired by an EVOS Imaging system (Life Technologies) using a 60X Plan Achromatic NA 1.42 objective with oil, and 6-10 fields of view were subsequently analyzed using Image J (NCBI) to determine cell size and circularity.

For cell differentiation experiments sorted subsets were cultured in RPMI-1640 supplemented with 10% human Ab serum (Sigma-Aldrich), Penicillin, Streptomycin, Glutamax, Non-essential amino acids and Sodium Pyruvate and 2 Mercaptoethanol (Life Technologies). MoDC were generated by culturing 1×10^5 sorted cells in the presence of 800U/mL GM-CSF (Miltenyi Biotec, 130-095-372) and 1000U/mL IL-4 (Life Technologies, PHC0044) for five days as described previously (31). Macrophages were generated by culturing sorted cells in M-CSF (25ng/mL; Peprotech, Rehovot, Israel) for five days. After culture cells were gently removed using Versene (PBS/0.6mM EDTA) and re-plated onto TissueTek-II chamber slides (Pro-Sci Tek) for 24 hrs. Following stimulation, cell morphology was analyzed by MGG staining or phenotypically by flow cytometry with CD14-PerCp Cy5.5, CD16-V500, HLA-DR-APC-H7, CD11c-AF700, CD83-FITC and CD86-BV650 antibodies.

Flow Cytometry and Data Analysis

Cells were stained, according to standard protocols, at 4°C with directly conjugated specific mAbs or isotype controls as described previously (2, 32). Fluorescent antibodies submitted to the 10th Human Leucocyte Differentiation Antigen workshop (HLDA-10) (<http://HDCM.org>) were used as described (2, 32). Unlabeled mouse antibodies were detected with species specific Alexa Fluor (AF)-488 F(ab)₂ secondary reagents (all from ThermoFisher). Data were collected using a 5 laser Fortessa X20 or 3 laser Influx (BD Biosciences).

Spanning tree progression analysis of density normalized events (SPADE) was performed using Cytobank (30). FCS files were processed with FlowJo V9 to remove cell aggregates, DAPI⁺ dead cells and selected for lineage (CD3, 14, 19, 20, 56, 235)⁻ HLA-DR⁺ cells. Clustering was performed on ArcSinH transformed fluorescent parameters using 300 nodes and 10% down sampling from 5 donors to generate a unified SPADE tree based up expression of CD11c, CD14, CD16, CD1c, CD141, CD34 and HLA-DR as described (2). SPADE heat maps are displayed showing the median ArcSinH transformed fluorescence of indicated markers with the size of each node representative of the

number of cells (6). A threshold was set for nodes containing more than 0.05% total cells to maintain a coefficient of variation of less than 10% for each calculated mean (33). Hierarchical clustering using average linkage analysis of SPADE nodes and generation of heatmaps were performed using R3.31 (34) statistical programming environment with RStudio (35) as described (33, 36). Clusters were mapped back to original SPADE trees for population demarcation, gating confirmation and manual annotation.

PBMCs were stained for mass cytometry analysis as described (37, 38) with cisplatin (Fluidigm) for live/dead discrimination and the antibodies listed in Table S1. Antibodies were either purchased as metal-conjugated from Fluidigm, or without any carrier proteins from the companies specified and conjugated with MaxPar X8 labelling reagent kits (Fluidigm) according to the manufacturer's instructions. Cells were incubated with Cell-ID Intercalator-Ir (Fluidigm) prior to acquisition, allowing identification of DNA⁺ cells. Data were acquired on a CyTOF 2 Helios upgraded instrument (Fluidigm, Toronto, Canada).

Dimensionality reduction was performed using the viSNE implementation in Cytobank (39). SPADE clustering was performed as described using 300 nodes and 1% down-sampling and hierarchical clustering performed using R3.31 (34) as described (33).

Bioinformatics

Bioinformatic analysis was performed on CD1c⁺ DC, CD141⁺ DC, CD16⁺DC and CD304⁺ pDC (E-TABM-34(22) and GSE34515 (40)), CD14⁺CD16⁻ Mo (CM), CD14^{low} CD16⁺Mo (GSE16836 (41), GSE18565 (40)), retrospectively using publicly available Affymetrix gene expression arrays performed using HG_U133A_Plus arrays. Raw files from gene array data were analyzed using GenePattern bioinformatics suite hosted by the Broad Institute (42) . Briefly, Affymetrix files from public array data sets were compiled and normalized using RMA quantile normalization using the Expression File Creator. Data was pre-processed using the preprocess data module and Log 2 transformed. Principal component analysis was performed using the GenePattern suite. Comparative expression and hierarchical clustering was performed using Comparative Marker Selection (43) and GENE-E (44).

Gene Expression Assays

Total RNA extraction was performed on highly purified >95% DC and Mo as described previously (45, 46). Total RNA from tissues or purified cells was extracted using TRIzol (Thermo Fisher) as per the manufacturer's instructions (Thermo Fisher). Integrity and quantity of extracted mRNA was assessed using an RNA 6000 Nano bioanalyzer assay (Agilent Technologies). mRNA samples with RIN values greater than eight were treated with 10U DNase I (Thermo Fisher) before transcription into cDNA using the SuperScript III kit (Thermo Fisher). Validated TaqMan primers for CD14 (Hs02621496_s1), CD16a (Hs02388314_m1), CD16b (Hs04334165_m1), FPR1(Hs04235426_s1), FPR2 (Hs02759175_s1), FPR3 (Hs00266666_s1), SLC11A1 (Hs01105516_m1), EMR1(Hs00892591_m1), ABCC3 (Hs00978473_m1), HEG 1 (Hs00393516_m1) and GAPDH (Hs03929097_g1) were obtained from Applied Biosystems. Primers and FAM BHQuencher probes specific for human CD300e (5'GCGCGGGAAGACTGCTAAG^{3'}, 5'CGGGCATAGTTGGTGGTCTAAG^{3'}, probe: 5'^{FAM}AAGCGTTCAGAACCACGGACAGA-BHQ1-3') and Ubiquitin converting enzyme (UBC) (5'TGAAGAGAATCCACAAGGAATTGA^{3'}, 5'CAACAGGACCTGCTGAACACTG^{3'}, probe: 5'^{FAM}TGATCTGGCACGGGACCCTCCA^{BHQ1-3'}) were designed in house and purchased from LGC Biosearch Technologies (Petaluma, CA, USA). Amplification of duplicate samples of cDNA from three biological replicates was performed using Fast Taqman Master Mix (Thermo Fisher) and run on a 7500 Fast real-time PCR system (Applied Biosystems). Threshold cycle (Ct) values were normalized to the *GAPDH* housekeeping gene and are presented as fold changes to the mean of a reference CD16⁺Mo in the biological replicates using the 2^{-ΔΔCT} method. Error bars represent the range. Primer efficiencies were >98%.

Chemotaxis Assays

CD1c⁺ DC, CD16⁺DC and CD16⁺ Mo were sorted from peripheral blood venesections and were added to the upper chamber of 5μm Transwell plates (Corning), and the chemokines CXCL12 (100ng/mL; R&D Systems), formylated tripeptide –methionine-leucine-phenylalanine (f-MLP) (Sigma-Aldrich) (10 or 100 nM) or CCL5 (Miltenyi Biotec)(10 or 100 ng/mL) were added to the lower chamber. After 4 hrs incubation at 37°C and 5% CO₂, total migrated cells in the lower chamber were enumerated through simultaneous acquisition

of counting beads (Thermo Fisher) by flow cytometry using an Accuri C6 (BD Biosciences). Chemokine-specific migration was calculated as total cells migrating with chemokine minus migration with media only and is presented as a migration index compared to no chemokine controls.

Antigen Uptake and MHC Presentation

The kinetics of antigen uptake and degradation was measured using the self-quenched biotinylated BSA protein (DQ-BSA) (Thermo Fisher) by flow cytometry as previously described (47). Briefly, PBMC were loaded with 0.5 µg/mL DQ-BSA and incubated at 37°C. Kinetic analysis of DQ-BSA fluorescence as a measure of protein degradation was measured by flow cytometry in cell subsets by staining for DC and Mo subsets.

Capacity to present and cross present soluble antigen was tested by detecting HLA-A2 peptide complexes on the cell surface by flow cytometry. Sorted subsets (2×10^4 cells) were incubated with equimolar amounts of FMP₅₈₋₆₆, FMP₅₄₋₇₄, control WT-1₁₂₆₋₁₃₄ peptides (Mimitopes) for 16 hrs. Presentation of surface HLA-A2 peptide complexes were detected by flow cytometry using a HLA:A2:FMP₅₈₋₆₆-specific FITC antibody (Dendritics, Lyon, France). T cell assays were performed by culturing $1 - 5 \times 10^4$ sorted myeloid subsets with 2×10^4 CFSE labeled allogeneic naïve CD4⁺ T cells. Untouched naïve CD4⁺ T cells were purified from PBMC by negative selection using anti-CD11c, CD14, CD19 and CD20 -V450, CD56, CD8–Brilliant Violet (BV)421, CD33, and CD45RO-APC (BD Biosciences) followed by sorting on an Influx cell sorter. Purities were assessed by CD4-FITC and CD45RA-PECy7 (BD Biosciences) staining and analysis by flow cytometry, and were always >90%. Proliferation was monitored after five days by assessing CFSE dilution as described (3).

Cytology and Immunofluorescence and Microscope Image Acquisition

Sorted cells were cytopun onto poly-Lysine coated glass slides (Pro Sci Tech, Townsville Australia) at 1000 RPM for three minutes at low acceleration using a Thermo- Shandon cytopun centrifuge. Cytopun cell subsets were fixed with methanol for 10 minutes, MGG stained and visualized using an EVOS cell imaging system. Quantitative analysis of cell size and circularity were analyzed using Image J software (NIH) from 6-10 images taken at 40 x magnification per subset.

For immunofluorescence imaging, cytopun cells were fixed with -20°C acetone for 10 minutes and stored at -20°C . Slides were rehydrated in PBS 0.5% BSA for 30 minutes prior to staining with rabbit anti-human LAMP1 (ABNOVA) for 60 minutes in a humidified chamber. Rabbit antibodies were detected using a goat anti-rabbit Alexa594 (Life Technologies) for 60 minutes. Nuclear staining was performed using TO-PRO-3 (Life Technologies) prior to mounting with ProLong Diamond anti fade (Life technologies) and visualized using a Leica TCS SP8-X confocal microscope with a 63x oil immersion objective (NA1.4). Corrected total cell fluorescence was calculated using ImageJ on maximum intensity projection images from raw Z-stack images as described (48, 49). Images were processed using Huygens deconvolution software and maximum intensity projection images used for image display.

Statistical Analysis

Statistical analysis was performed using Prism 7.0 (GraphPad Software). Standard error of the mean is shown unless otherwise stated. One-way or Two-way ANOVA with Tukey's correction for multiple comparisons was used as described in the figure legends. Differences with $p < 0.05$ were considered significant.

Supplemental Material

Table S1 lists antibodies and metal conjugates used in mass cytometry. Fig. S1 shows the gating strategy employed for identification of populations in viSNE analyzed CyTOF data and SPADE analysis of mass cytometry data used to generate heatmaps in Fig. 3. Fig. S2 shows the $\text{CD16}^+\text{Mo}$ and $\text{CD16}^+\text{DC}$ constituents of CD16^+ and $\text{CD14}^+\text{Mo}$ isolation techniques. Fig. S3 shows a compiled meta-analysis of publicly available gene arrays of human CD1c^+ , CD141^+ , pDC, $\text{CD16}^+\text{DC}$ as well as CD14^+ and $\text{CD16}^+\text{Mo}$. Principal component analysis of cell subsets and heatmap of statistically significant genes differentially expressed in $\text{CD16}^+\text{DC}$. qPCR analysis and membrane protein staining of candidate genes is also shown. Fig. S4 shows live cell morphology, MGG stains of cytopun cells and quantitation of cell morphology of cell subsets cultured for 48 hrs in either GM-CSF, GM-CSF/IL4, M-CSF or FLT3L. Fig. S5 shows an alternative gating strategy using CLEC5a to identify different CD16^+ myeloid subpopulations and confirmation using viSNE and the T cell stimulatory capacity of $\text{CD16}^+\text{Mo}$ and $\text{CD16}^+\text{DC}$ sorted using the CD300c marker.

Results

Phenotypic Definition of CD16⁺DC and the CD16⁺Mo Subpopulations in Human Peripheral Blood.

We first compared different approaches used to identify Mo and DC phenotypically. We performed comparative flow cytometry gating using conventional criteria to identify DC (Fig. 1A) and Mo (Fig. 1B). When we used CD14 as a separate gating parameter independent of the lineage (CD3, CD19, CD20, CD56, CD235a) exclusion gate used to define the Lin⁻ cells, we observed a clear separation of CD14⁺ and CD14⁻ events (Fig. 1C). As a result, we were able to discriminate conventional Mo subpopulations and DC. When the populations identified by DC gating or Mo gating were overlayed (Fig. 1D), we observed an inherent bias introduced by these analytical methods. This bias showed that both CD16⁺ DC (Fig. 1D, R3) and CD1c⁺ DC (Fig. 1D, R4) did not express CD14, exhibiting MFIs less than that of the CD16⁺ Mo subset.

To confirm our observations that CD14 and CD16 discriminated CD16⁺Mo and CD16⁺DC independently of biases introduced by differences in cellular auto-fluorescence and the potential for compensation artefacts due to the large multi-fluorescent panels, we employed a CyTOF approach using metal labelled antibodies detected by time of flight mass spectrometry. Our analysis of the data from the 36 parameter CyTOF panel that we used (Table S1), showed clear CD14⁺ and CD14⁻ populations within the Lin⁻ HLA-DR⁺ cells (Fig. 1E). CD14⁺ Mo, CD14⁺ CD16⁺Mo and CD16⁺Mo were readily identified within total CD14⁺ cells. The CD14⁻ population divided into CD11c⁻ CD304⁺, pDC and CD11c⁺ CD304⁻ myeloid DC. Notably the myeloid DC fraction contained a dominant CD1c⁺ and CD16⁺ population (Fig. 1E) consistent with conventional flow cytometry approaches (Fig. 1C).

CytoF data was analysed in an unsupervised manner with viSNE to reduce the dimensionality of the data (Fig.S1 and S2). Mapping conventional flow cytometry gates onto the viSNE representation distinguished all major Mo and DC subsets including CD16⁺Mo and CD16⁺DC. Moreover, this high dimensional cytometry approach separated the CD16⁺Mo and CD16⁺DC from rare contaminating HLA-DR⁺ NK cells. CD16⁺DC identified by conventional gating aided by viSNE showed no expression of other NK cell cytotoxicity receptors including NKp30 (CD337), NKp46 (CD335) (Fig. S1B-E) or the C-type lectin, NKG2D (data not shown).

We next investigated the cell populations isolated using criteria reported by others in the literature to isolate CD16⁺Mo (9, 21, 40, 41). The SLAN antigen is a carbohydrate modification of PSGL-1 which has been proposed to identify both a DC and Mo subset (28). Using the MDC-8 mAb, we showed that SLAN expression aligned to a population encompassing roughly equivalent fractions of CD16⁺Mo and CD16⁺DC subsets in a cohort of 5 healthy individuals (Fig. 2A-E). However, as a proportion of each subset, a greater proportion of CD16⁺ DC were SLAN⁺ compared to CD16⁺ Mo (32.9±5.5 vs. 12.1±3.0%, p<0.01; Fig 2F-G), consistent with previous studies describing lower CD14 expression by the SLAN⁺ population compared to non-classical Mo (50, 51). The use of magnetic bead depletion strategies followed by CD16 positive selection resulted in a large population of cells that phenotypically resemble CD16⁺DC and lacked expression of CD14 (Fig. S2A-B). These cells did not overlay onto the CD14^{dim}CD16⁺Mo population. Finally, we used a bioinformatics meta-analysis of different publicly available data sets to compare myeloid CD1c⁺, CD141⁺ and CD16⁺DC with both CD14⁺ CD16⁻ Mo and CD14^{dim} CD16⁺ Mo (22, 25, 26). This resulted in the separation of CD16⁺DC and CD16⁺Mo by their distinct transcriptional profiles (Fig. S3A-B).

Characterization of Myeloid Cell Surface Protein Signature.

As part of HLDA10 (52) we performed unbiased characterization of antibody binding to human APC (2, 6). In order to generate a “myeloid cell surface signature” we focused on C-type lectins and immune-modulatory molecules using SPADE to perform unbiased clustering of the dataset. To manage the data file size, we only clustered events within the Lin⁻ HLA-DR⁺ gate. The analysis clustered phenotypically similar groups of cells into 300 nodes based upon the core set of cell surface molecules employed in routine fluorescence analysis. The expression of 48 different cell surface proteins was then overlaid onto each node.

A continuum of CD14 expression from CD14⁺ Mo with the highest expression through CD14⁺ CD16⁺Mo to CD16⁺Mo was consistently observed. CD16⁺DC were consistently CD14⁻ and represented a discreet cluster of the myeloid arm (Fig. 3A). The generated SPADE trees were used to perform average linkage hierarchical clustering (Fig. 3B). First tier clusters segregated CD14⁺ and CD14⁺CD16⁺ Mo from all other cells. Second tier clusters were used for subset identification of CD14⁺CD16⁻ (Mo1), CD14⁺ CD16⁺ Mo (Mo2), CD14^{dim}CD16⁺ Mo, (Mo3) and; CD141⁺ (DC1), CD1c⁺A (DC2), CD1c⁺B, (DC3); CD16⁺ (DC4), and pDC (DC6) by mapping back to the original SPADE trees and two-dimensional flow cytometry plots for annotation. All canonical Mo and DC subsets were identified

using this approach. Notably, some observed clusters did not map to readily identifiable populations, which may represent the $Axl^+Siglec6^+$ (DC5) and other discrete populations.

Four main clusters of cell surface protein expression was observed across the major cell groups (Fig. 3B). These were: 1) a common myeloid cell signature, 2) a Mo specific signature that included all Mo subsets and excluded all DC subsets including the $CD16^+DC$, 3) a small cluster distinguishing myeloid and plasmacytoid DC, and 4) a cluster that lacked expression of these markers.

A core set of markers including CD14, the C-type lectins CLEC4D, CLEC5a and CLEC7a, and the formylated protein receptors FPR1 and FPRL1, and CD300 family served to identify all Mo subsets (Fig. 3B). Several of these candidates were detected in our bioinformatics analysis of the publicly available gene expression data sets (Fig. S3A-B) and independently verified by qPCR or flow cytometry analysis on multiple PBMC samples (Fig. S3C-H). Only a small number of cell surface proteins examined served to act as positive discriminators of DCs or specific DC subsets (e.g. cluster 3 in Fig. 3B). One of these, CCR5, is strongly expressed on $CD1c^+DC$, $CD141^+DC$ and pDC but is absent or barely detectable on $CD16^+DC$, while low to moderate expression was observed on Mo subsets.

The unique subset clusters that emerged in Fig. 3B include a major Mo like subset that was phenotypically similar to the $CD14^+CD16^+$ intermediate Mo. Cells within this population displayed increased expression of IL1RaCP, CLEC4D and reduced expression of CCR5. $HLA-DR^+CD11c^-$ cells were also detected that did not fall neatly into classically defined cell types. These included $CD34^+$ cells that we previously reported to have antigen presenting functions (5) and may indicate more primitive circulating progenitors.

To explore the relationship of $CD16^+DC$ within the myeloid lineage with $CD16^+$ monocytes, we performed similar SPADE clustering on mass cytometry data containing the same core clustering backbone along with a separate panel of 31 antibodies. A similar continuum of CD14 expression was observed within myeloid cells (Fig. 3C and Fig. S1D). Hierarchical clustering of CyTOF data again distinguished four discrete expression panels corroborating the clusters found by conventional flow cytometry: a pan myeloid (1), a Mo specific (2), a DC specific (3) and a negative expressed group (4)

(Fig. 3D). Notably CD16⁺DC formed a unique cluster separate from CD16⁺Mo (Fig. 3D) within the DC protein signature.

CD14⁺Mo constitute the largest fraction of PBMC within the Lin⁻HLA-DR⁺ population. Both CD16⁺Mo and CD16⁺DC are present at similar frequencies and are comparable in frequency to CD1c⁺DC (Fig. 3E). There was a significant difference in CD14 surface expression between both CD16⁺Mo and CD16⁺DC ($p < 0.05$) and no difference in CD16 levels between both subsets (Fig. 3F and G). A recent study showed that CD14^{very-dim} myeloid populations that appear negative for surface CD14 by flow cytometry can be separated from true CD14 negative populations using a CD14 magnetic sorting strategy (53). Implementing this strategy, we found that only a small proportion of cells defined as CD16⁺ DC were detected within the positive fraction of a MACS sort using CD14 microbeads, with the vast majority remaining in the negative fraction (Fig. S2C-E). The difference in CD14 expression between CD16⁺DC and CD16⁺ Mo were also observed at the mRNA level (Fig. 3I). The HLA-DR surface expression was determined to be significantly higher on the CD16⁺Mo than CD16⁺DC ($p < 0.05$) (Fig. 3H).

Given that the Fc Receptor, CD16 (FcγRIII) is a major functional hallmark of both CD16⁺ Mo and CD16⁺ DC we assessed broad FcγR phenotype of the cells with available mAbs. As expected, the CD16⁺Mo and CD16⁺DC showed similar levels of expression of CD16 (FcγRIIIa). CD16⁺Mo exhibited higher expression of CD64 (FcγR1) and CD89 (FcαRI) than the CD16⁺DC. The undetectable CD14 levels on CD16⁺DC differentiate them from the previously described CD64⁺CD16⁺ DC-like Mo subset that express CD14 (29). Interestingly, analysis of CD32 (FcγRIIa/b) indicated that while both CD16⁺ populations expressed CD32, there was very little inhibitory CD32b expressed by the CD16⁺DC population (Fig. 3J). The activating CD32a form was the most abundant on both CD16⁺ Mo and CD16⁺DC, in contrast to CD1c⁺ DC and B cells, that express predominantly CD32b (Fig. 3K).

Finally, since CD16 is essential for mediating antibody dependent cellular cytotoxicity (ADCC) we compared the populations' ability to mediate ADCC. No significant difference was observed in the capacity of sorted CD16⁺Mo or CD16⁺DC to mediate ADCC against the KM-H2 target via a human anti-human CD83 antibody (3C12C) (Fig. 3M) (54, 55).

Morphology and Differentiation Capacity

Highly purified populations of CD14⁺Mo, CD16⁺Mo and CD16⁺DC were sorted based on strict expression of CD14. Purity of these populations was validated by flow cytometry and viSNE to map their different position in high dimensional space (Fig. 4A and data not shown). Each subset showed differences in gross morphology of MGG stained cytopins (Fig. 4B). CD14⁺Mo displayed folded eccentric nuclei, blue grey cytoplasm and frequent vacuoles. CD16⁺Mo displayed irregular folded nuclei, clear basophilic cytoplasm with small and large vacuoles and occasional cytoplasmic projection. CD16⁺DC displayed irregular complex folded nuclei frequently containing nucleoli and open chromatic complexes, a predominantly basophilic cytoplasm with numerous small vacuoles and cytoplasmic projections (Fig. 4B). Quantitative analysis identified significant differences in cell area between all subsets. CD16⁺DC ($210.5\mu\text{m}^2 \pm \text{SEM } 3.42$) were the largest, followed by CD14⁺Mo (mean $185\mu\text{m}^2 \pm \text{SEM } 4.81$) whilst CD16⁺Mo ($176.3\mu\text{m}^2 \pm \text{SEM } 3.64$) were the smallest. There were no differences in circularity (data not shown).

In order to determine whether CD16⁺DC maintained the same differentiation potential as other Mo and DC we cultured highly purified CD14⁺Mo, CD16⁺Mo and CD16⁺DC populations for five days with GM-CSF/IL4 to induce MoDC, or M-CSF to induce macrophages. All three subsets differentiated into MoDC like cells upon culture with GM-CSF and IL4, with characteristic dendritic morphology (Fig. 4C), losing expression of CD14 from CD14⁺Mo and CD16⁺Mo derived cells (data not shown) and maintaining or inducing expression of CD16 (data not shown). Quantitative analysis of morphology demonstrated that CD16⁺DC differentiated into cells larger than CD16⁺Mo derived cells with increased area (mean CD16⁺DC $274.5\mu\text{m}^2 \pm \text{SEM } 4.21$; mean CD16⁺Mo $242.5\mu\text{m}^2 \pm \text{SEM } 5.83$; $p < 0.001$) but remained more circular ($P < 0.0001$). Cells resulting from cultures of CD16⁺Mo and CD14⁺Mo were more similar in both size and shape.

Culture in the presence of M-CSF to induce macrophage differentiation (Fig. 4D) resulted in CD14⁺Mo adopting typical macrophage morphology. CD16⁺Mo failed to induce CD14 (data not shown) and were significantly smaller in area than CD14⁺Mo derived macrophages (Fig. 4C; mean CD16⁺Mo $213.2\mu\text{m}^2 \pm 5.83$; mean CD14⁺Mo $402.6\mu\text{m}^2 \pm 12.95$; $p < 0.001$). Whilst similar to CD16⁺DC differentiated cells in area (CD16⁺DC mean $274.5\mu\text{m}^2 \pm \text{SEM } 4.2$; CD16⁺Mo $242.5\mu\text{m}^2 \pm \text{SEM } 5.83$, $p = 0.8512$), differentiated CD16⁺Mo were more elongated than CD16⁺DC (Data not shown, $p < 0.001$).

To determine whether cell types differentiated into MoDC or macrophages proceeded along similar kinetics we cultured each subset in the presence of either GM-CSF, GM-CSF/IL4, M-CSF or FLT-3L for up to 48 hours. No significant morphological differences were observed between CD14⁺Mo, CD16⁺Mo or CD16⁺DC in their response to any cytokine combination at either 24 (data not shown) or 48 hrs (Fig. S4A-B). We observed that CD16⁺Mo and to some extent CD16⁺DC formed prominent cell clusters in culture as early as 24 hrs in culture independent of cytokine addition. The clusters formed by CD16⁺Mo cultured cells were more numerous and consistently contained more cells than CD16⁺DC ($p < 0.001$) (Fig. S4C and D).

Chemotactic Capacity

Our phenotyping identified differences in three chemokine receptors CCR5, FPR1 and FPRL1. To test if these differences contributed towards migratory specialization, we performed chemotaxis assays on highly purified sorted cell subsets toward CCL5 that acts through CCR5 and the formylated tripeptide f-MLP, a potent granulocyte chemoattractant that acts through the formylated peptide receptors FPR1 and FPRL1. All subsets migrated to the CXCR4 binding chemokine CXCL12, with CD1c⁺DC showing significantly greater migration to all subsets (Fig. 5A; $p < 0.001$). Despite differences in surface expression of CCR5 between CD16⁺Mo, CD16⁺DC and CD1c⁺DC all subsets migrated equally to varying concentrations of CCL5 (Fig. 5A). Similarly, despite there being abundant differences in surface protein expression of FPR1 and FPRL1 between subsets, no significant differences were observed in the capacity of any subset to migrate to f-MLP at either 100 μ M or 10 μ M (Fig. 5B) in transwell assays.

Antigen Processing and Presentation

Given the capacity for both Mo and DC to phagocytose, degrade and present extracellular antigens we asked whether the subsets were equally capable of uptake and proteolysis of extracellular soluble protein. Firstly, we analyzed uptake of the carbohydrate dextran over a period of two hours. CD14⁺Mo rapidly accumulated dextran-FITC followed by CD16⁺Mo. Both CD16⁺DC and CD1c displayed a reduced rate of uptake and a trend to reduced total uptake over the time tested (Fig. 5C). To assess whether differential capacity to uptake antigen translated into different levels of antigen degradation, we employed a self-quenched bodipy protein, DQ-BSA that requires proteolytic

cleavage to become fluorescent. Bodipy fluorescence was observed as early at 15 minutes in CD14⁺Mo and CD16⁺Mo populations and gradually accumulated over time in different cellular compartments (Fig. 5D). CD14⁺ Mo were the first cells to begin degrading antigen, followed by CD16⁺Mo while both CD16⁺DC and CD1c⁺ were the slowest in their capacity to process DQ-BSA. The delayed kinetics observed in DC may relate to characteristic differences in endocytic vesicles. To determine this, we stained purified subsets for intracellular lysosomes using LAMP1. Both CD14⁺Mo and CD16⁺Mo showed abundant cytoplasmic LAMP staining around the perinuclear space whilst CD16⁺DC and CD1c⁺DC displayed significantly less LAMP staining (Fig. 5E and F) consistent with the delayed kinetics of DQ-BSA degradation.

In order to determine if delayed kinetics of antigen degradation observed in CD16⁺DC reflected a change in capacity to present processed antigen, we tested whether CD16⁺DC or CD16⁺Mo showed the same capacity to cross-present antigen to MHC Class I. The FMP₅₈₋₆₆ peptide which binds in equilibrium displacement to existing HLA-A*0201 complexes showed positive staining in all subsets (Fig. 5G and H). Cross presentation of the FMP₅₈₋₆₆ epitope contained within the FMP₅₄₋₇₄ long peptide was most apparent in CD1c⁺DC which presented antigen more efficiently than all other subsets ($p < 0.05$). Detectable levels of cross-presented FMP₅₈₋₆₆ was observed in all subsets compared to cells pulsed with the irrelevant Wilms tumor antigen peptide (WT₁₂₆₋₁₃₄). No statistical difference between the ability of CD16⁺Mo and CD16⁺DC to cross present soluble antigen was observed (Fig. 5H). We next addressed the capacity of CD16⁺Mo and CD16⁺DC to stimulate allogeneic naïve CD4⁺ T cells. Both CD16⁺Mo and CD16⁺DC displayed similar stimulatory capacity across a range of stimulator to responder ratios. This was surprising given the lower HLA-DR surface expression, lower antigen uptake and degradation capacity of CD16⁺DC, compared to CD16⁺Mo and suggests that the CD16⁺DC are in fact, very efficient at uptake and presentation of allo-antigen onto their cell surface. CD1c⁺DC were able to induce much greater allogeneic T cell proliferation than any other population at both 1:2 and 1:4 stimulator to responder ratios ($p < 0.05$)(Fig. 5I).

CD16⁺DC and CD16⁺Mo show similar reconstitution kinetics and CCR5 activation post-HCT

To compare the differentiation pathway of CD16⁺DC to that of the CD16⁺Mo or CD1c⁺DC lineages, we monitored the absolute blood counts in six patients undergoing autologous (auto-) hematopoietic cell transplants (HCT)(Table 1). We endeavored to minimize biases due to conditioning and alloreactivity by tracking Mo and DC reconstitution in multiple myeloma patients receiving

autografts with the same melphalan conditioning regimen. Kinetic studies showed rapid reconstitution of circulating myeloid cells from day seven post-transplant with all subsets becoming detectable over this time (data not shown). We monitored differences in the rate at which progenitor stem cell reconstituted myeloid lineages by performing sampling in three patients commencing from when a diagnostic white cell count of $0.1\text{cell}/\mu\text{l}$ was detected. $\text{CD14}^+\text{Mo}$ and $\text{CD16}^+\text{Mo}$ showed rapid early development and rose to normal levels between days 7-10 (Fig. 6A). Comparison of $\text{CD16}^+\text{Mo}$ and $\text{CD16}^+\text{DC}$ revealed that development of each subset were detectable early following transplant and their recovery were closely paralleled. The correlation of reconstitution kinetics between these subsets was evident when comparing all time-points in six patients through regression analysis ($R^2 = 0.8594$, Fig. 6B). This contrasted with the regression analysis of $\text{CD16}^+\text{DC}$ versus $\text{CD1c}^+\text{DC}$ reconstitution, which showed no correlation ($R^2 = 0.1229$; not shown).

Our previous studies correlated the activation of mDC, particularly $\text{CD16}^+\text{DC}$, with the onset of GVHD following allo-HCT (56, 57). To determine whether this was limited to $\text{CD16}^+\text{DC}$ or was reflected in altered reconstitution of other myeloid subsets we analyzed patients receiving allo-HCT (Table 1) for reconstitution of $\text{CD1c}^+\text{DC}$, $\text{CD16}^+\text{Mo}$ and $\text{CD16}^+\text{DC}$. In six patients examined, we observed spikes in the absolute counts of $\text{CD16}^+\text{Mo}$ starting from 15 days post-transplant that were paralleled by $\text{CD16}^+\text{DC}$ (Fig. 6C). The correlation between levels of $\text{CD16}^+\text{DC}$ and $\text{CD16}^+\text{Mo}$ ($R^2 = 0.7799$, Fig. 6D) but not $\text{CD16}^+\text{DC}$ and $\text{CD1c}^+\text{DC}$ ($R^2 = 0.2049$; not shown) at all time-points in six patients was also made apparent by regression analysis. Together, the auto- and allo-HCT patient data suggests that $\text{CD16}^+\text{DC}$ follows a developmental path linked with $\text{CD16}^+\text{Mo}$.

The expression of CCR5 on $\text{CD16}^+\text{DC}$ is a useful biomarker for the development of GVHD (56). While CCR5 proved to be a useful discriminator between $\text{CD16}^+\text{Mo}$ and $\text{CD16}^+\text{DC}$ in the steady state (Fig. 6E), we saw enhanced upregulation of CCR5 in $\text{CD16}^+\text{DC}$ in allo-HCT patients with GVHD (Fig. 6F-G). In healthy donors, CCR5 on both $\text{CD16}^+\text{Mo}$ and $\text{CD16}^+\text{DC}$ was upregulated by $\text{IFN}\gamma$ (Fig. 6H) consistent with an inflammatory state. Interestingly, while CCR5 expression was directly modulated by $\text{IFN}\gamma$ on both $\text{CD16}^+\text{Mo}$ and $\text{CD16}^+\text{DC}$, its expression was less tightly regulated on $\text{CD16}^+\text{Mo}$. CCR5 could be induced on $\text{CD16}^+\text{Mo}$ by short term cell culture alone whereas $\text{CD16}^+\text{DC}$ could not (Fig. 6I). Monitoring a cohort of ten patients post allo-HCT (Table 1), we observed that CCR5 was often induced 2 standard deviations above the fluorescence minus one baseline in $\text{CD16}^+\text{DC}$ prior to

clinical diagnosis of GVHD or infections (Fig. 6J). CD16⁺ Mo in these patients expressed higher overall levels of CCR5, but its' up- and downregulation reflected that on CD16⁺DC ($R^2 = 0.712$, Fig. 6K). This correlation was not apparent when comparing CCR5 induction on CD16⁺DC to CD1c⁺DC ($R^2 = 0.0956$, Fig. 6L). This data demonstrates a potential linkage between the activation of CD16⁺Mo and CD16⁺DC subsets during inflammatory events.

Alternative Phenotypic Markers that Differentiate CD16⁺Mo from CD16⁺DC in the Steady State.

In order to further distinguish CD16⁺Mo from CD16⁺DC we sought to identify markers that would allow discrimination of the subsets independently of CD14 and CD16. Of the surface proteins examined in this study, we identified CD300c, CLEC5a and CCR5 (Fig. 3A and B, Fig. 6 and Fig. S3E and H), markers that had the potential to separate all three CD16⁺ subpopulations, i.e. CD14⁺ CD16⁺ Mo, CD16⁺Mo and CD16⁺DC. In each case, three different CD16⁺ populations could be identified on the basis of expression of CD14 combined with CD300c, CCR5 (Fig. 7A and E) or CLEC5a (Fig. S5A), respectively. We overlaid each of these populations onto viSNE maps of total Lin⁻ HLA-DR⁺CD11c⁺CD16⁺ (Fig. 7B, F and Fig. S5B) cells to determine how each population compared with the original phenotypic definition of CD14⁺CD16⁺ (Intermediate) Mo, CD16⁺Mo, and CD16⁺DC (Fig. 1). The overlap of each CD16⁺ population based on either CD14, CD300c, CCR5 or CLEC5a expression in scatter plots (Fig. 7C and G and Fig. S5C) or in viSNE plots (Fig. 7D, H and Fig. S5D) demonstrated that these markers have potential as alternative cell surface markers distinguishing the CD16⁺Mo from CD16⁺DC. Finally we sorted the CD16⁺Mo and DC subsets based on discrimination by CD300c (Fig. S5E-F) and tested their capacity as stimulators in an MLR (Fig. S5G). Similar to the MLR experiment using the original gating strategy (Fig. 5I), the CD16⁺CD300c^{hi} and CD16⁺CD300c^{lo} populations (relating to CD16⁺Mo and CD16⁺DC, respectively) were equivalent in their capacity to stimulate a T cell proliferative response.

Discussion

Cellular based immune therapy for cancer has long been regarded to have great potential. To date, most clinical trials have used MoDC, generated from peripheral blood Mo, with limited success(58). The heterogeneity of the Mo population from which MoDC are generated will influence the outcome. An alternative to MoDC is a DC population purified *ex vivo* from peripheral blood (59, 60). To develop the platforms to purify DC for immune therapy requires the ability to select distinct populations based on cell surface phenotype. It is well accepted that there are three populations of Mo in human peripheral blood; the CD14⁺CD16⁻ classical, CD14⁺CD16⁺ intermediate and CD14^{dim}CD16⁺ non-classical. Similarly, peripheral blood DC consist of pDC and the myeloid DC populations CD141⁺ (DC1), CD1c⁺ (DC2 and DC3), and a population of CD16⁺ cells (DC4) (2, 5, 27). However, the relationship between the non-classical CD14⁺CD16⁺ Mo and the CD16⁺DC4 has been regularly debated. In this study we have used both fluorescence and CyTOF approaches to probe the total human mononuclear phagocytic system from peripheral blood. We compared the CD16⁺Mo and CD16⁺DC populations using high dimensionality reduction approaches to generate phenotypically similar clusters across combined datasets. This allowed us to visualize heterogeneity in the cell surface phenotype of CD16⁺ cells without preconceived notions of cell identity. We demonstrated that these approaches provide distinct cell surface protein signatures able to segregate the closely related CD16⁺DC from the CD14⁺CD16⁺Mo. A number of other studies have been used to segregate the cell populations including transcriptomic (22, 27, 41) and proteomic(61) studies, but these have been biased by the strategy used to purify cell subsets for analysis. A recent paper described CyTOF analysis of human DC subsets from blood, tonsil, spleen, and skin using mAbs to 47 different molecules (62) whilst two other papers used systems biology approaches to look at inflammatory myeloid populations in SLE (63) and coronary heart disease(12). In this body of work, we report a large phenotypic study using mAbs to over 100 independent molecules to allow distinction of the different subsets and address the similarities and differences in subset phenotypes.

The lack of consensus distinguishing CD16⁺Mo and CD16⁺DC in peripheral blood has resulted in part from the historical context in which observations were made and in part as a consequence of technology. Mo have been defined phenotypically as those cells expressing CD14, albeit in some cases low levels, whilst on the other hand, DC are defined as those cells that are HLA-DR⁺ but lack the common lineage markers including CD14. The use of unbiased approaches has shown distinction between CD16⁺ cells that are CD14⁺ to dim and those cells that lack surface CD14. These subsets have been corroborated with transcriptomic data that recently distinguished a CD16⁺DC (DC4) from CD16⁺Mo (27). In particular the DC4 cells were enriched in type I interferon signaling pathway and

response to virus whilst CD16⁺Mo were enriched in leukocyte migration and immune system process genes. Our aim was to compare and contrast the phenotype and properties of CD16⁺Mo and CD16⁺DC populations with respect to those of Mo and DC.

Single cell transcriptomic analysis has been powerful in clustering cell populations and indeed have validated the presence of three CD16⁺ myeloid populations(27). The ranking of discriminators from transcriptomics allows for inclusion of transcripts encoding proteins from all cellular compartments. Our interests have been in understanding the cell surface proteins that have potential to allow selection of cell for functional analysis. Our studies identified CD300c, CLEC5a and CCR5 as discriminating surface membrane molecules to which validated mAbs are available. However, the transcriptomic analysis did not identify any of these transcripts as being selective for one population over another, emphasizing the discrepancy between transcript and membrane surface protein expression.

CD16⁺Mo are described as the DC precursors in peripheral blood and indeed, reverse transmigration experiments demonstrated their differentiation into a DC like cell (64). MoDC-like cells are evident in numerous inflammatory environments (20). Our studies found no membrane molecules that were expressed more on CD16⁺DC than on the CD16⁺Mo. Instead, there were numerous markers that were present at higher density on the CD16⁺Mo than the CD16⁺DC adding to the assumptions that CD16⁺Mo may well differentiate into CD16⁺DC. Amongst the mAbs that differentially bound the two populations were HLA-DR, CD300c and chemokine receptors including FPR1, FPRL1 and CCR5. Our studies showed minimal differences in cell migration to ligands of the chemokine receptor candidates at the chemokine concentrations used.

Phagocytic and antigen processing capacity has been closely linked to the capacity to present antigen in the context of HLA. The phagocytic ability of CD16⁺Mo and CD16⁺DC distinguished the two populations with the former showing an increased capacity over time. We were intrigued to see that CD16⁺DC had antigen uptake and processing more akin to CD1c⁺DC than to other Mo subsets. Similarly, the capacity of CD16⁺DC to cross present soluble antigen was intermediate between CD1c⁺DC at the upper end and CD14⁺Mo and CD16⁺Mo at the lower end.

We have previously demonstrated that monitoring activation of DC subsets (57) including CD16⁺ DC predicts for the development of GVHD in patients who have received an allo-HCT (56). Our previous studies with a larger cohort demonstrated a correlation of CCR5 expression on CD16⁺DC with GVHD development and its potential as a diagnostic indicator (56). CCR5 is constitutively expressed in CD14⁺Mo, CD16⁺Mo and CD1c⁺DC and can be upregulated both in CD16⁺DC and CD16⁺Mo (65, 66). The low background expression of CCR5 by CD16⁺DC in the steady state and its IFN driven upregulation (56) serve to make CD16⁺DC a relevant target for monitoring inflammation and GVHD development. How CCR5 serves to functionally distinguish CD16⁺Mo and CD16⁺DC is yet to be determined. While differences in cell surface chemokine receptor expression did not result in altered chemotactic responses in the steady state in our experiments, other cues may be required to initiate chemotaxis. Interestingly, our data indicates that CCR5 regulation on CD16⁺Mo and CD16⁺DC may be linked in an inflammatory setting. Studies targeting CCR5 using maraviroc, a receptor antagonist in allo-HCT purportedly to target T cells in phase I/II clinical trials has shown some efficacy in reducing the incidence of GVHD (65-67) leading to its use as a prophylactic clinically. As targeting activated DC has shown promise in both mouse and human xenograft studies of GVHD, the mechanism of action of maraviroc may include targeting activated CD16⁺DC (54, 68).

While we strive to understand the roles of DC and Mo in human disease, the robustness of clinically applicable methods to identify different subsets without ambiguity has thus far been problematic. We have provided a comprehensive unified look at CD16⁺Mo and CD16⁺DC in healthy donors and shown how the continuum of expression of the so called “lineage markers” currently used to identify are biased towards the preconceived idea of the cell classification system used. Further, we have demonstrated that during inflammation associated with allo-HCT the identity of these subsets can still be unambiguously identified and that they can be used as biomarkers in disease monitoring. We predict that CCR5, CD300c and Clec5a are three cell membrane markers that will provide functional discrimination between CD16⁺Mo and CD16⁺DC and these targets are now the focus of our studies to monitor these cells during inflammation. The recent publications highlighting the use of CD163, CD88 and CD89(63) or CD9 and CD93(12) emphasize the heterogeneity of human myeloid populations and the need for researchers to be cognizant of the different technologies used to isolate human myeloid populations for studies before comparing their data to that of others.

Author Contributions

P.D.F., P.A.S. and G.J.C. designed, performed experiments, analyzed data and wrote the manuscript. J.L.H. performed flow cytometry of clinical samples, M.S.P, T.H.L, W-H.H. and P.A.S. performed chemotaxis and ADCC experiments, C.E.B. and J.G., I.C., E.N. recruited patients, A.R, B.K, E.A, M.S.P. performed phenotyping experiments, A.M. performed microscopy, H.M.M. and B.F.S.G. organized and performed CyTOF. D.N.J.H. designed, analyzed data and wrote the manuscript.

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Conflict of Interest Disclosure

G.J.C. is a Director of DendroCyte BioTech Pty Ltd. There are no conflict of interest disclosures.

References

1. Hart DN. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood*. 1997 Nov 1;90(9):3245-87.
2. Fromm PD, Kupresanin F, Brooks AE, Dunbar PR, Haniffa M, Hart DN, et al. A multi-laboratory comparison of blood dendritic cell populations. *Clin Transl Immunology*. 2016 Apr;5(4):e68.
3. Bryant C, Fromm PD, Kupresanin F, Clark G, Lee K, Clarke C, et al. A CD2 high-expressing stress-resistant human plasmacytoid dendritic-cell subset. *Immunol Cell Biol*. 2016 May;94(5):447-57.
4. Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, et al. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *The Journal of experimental medicine*. 2010 Jun 7;207(6):1247-60.
5. MacDonald KPA, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DNJ. Characterization of human blood dendritic cell subsets. *Blood*. 2002 December 15, 2002;100(13):4512-20.
6. Clark GJ, Kupresanin F, Fromm PD, Ju X, Muusers L, Silveira PA, et al. New insights into the phenotype of human dendritic cell populations. *Clin Transl Immunology*. 2016 Jan;5(1):e61.
7. Clark GJ, Silveira PA, Hogarth PM, Hart DNJ. The cell surface phenotype of human dendritic cells. *Semin Cell Dev Biol*. 2018 Mar 27;In Press, Accepted March 3, 2018.
8. Passlick B, Flieger D, Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood*. 1989 Nov 15;74(7):2527-34.
9. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity*. 2010 Sep 24;33(3):375-86.
10. Boyette LB, Macedo C, Hadi K, Elinoff BD, Walters JT, Ramaswami B, et al. Phenotype, function, and differentiation potential of human monocyte subsets. *PloS one*. 2017;12(4):e0176460.
11. de Pablo-Bernal RS, Canizares J, Rosado I, Galva MI, Alvarez-Rios AI, Carrillo-Vico A, et al. Monocyte Phenotype and Polyfunctionality Are Associated With Elevated Soluble Inflammatory Markers, Cytomegalovirus Infection, and Functional and Cognitive Decline in Elderly Adults. *The journals of gerontology Series A, Biological sciences and medical sciences*. 2016 May;71(5):610-8.

12. Hamers AAJ, Dinh HQ, Thomas GD, Marcovecchio P, Blatchley A, Nakao CS, et al. Human Monocyte Heterogeneity as Revealed by High-Dimensional Mass Cytometry. *Arterioscler Thromb Vasc Biol.* 2019 Jan;39(1):25-36.
13. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *The Journal of experimental medicine.* 1994 Apr 01;179(4):1109-18.
14. Lee J, Breton G, Oliveira TY, Zhou YJ, Aljoufi A, Pühr S, et al. Restricted dendritic cell and monocyte progenitors in human cord blood and bone marrow. *The Journal of experimental medicine.* 2015 Mar 9;212(3):385-99.
15. Breton G, Zheng S, Valieris R, Tojal da Silva I, Satija R, Nussenzweig MC. Human dendritic cells (DCs) are derived from distinct circulating precursors that are precommitted to become CD1c+ or CD141+ DCs. *The Journal of experimental medicine.* 2016;213(13):2861-70.
16. Satpathy AT, Kc W, Albring JC, Edelson BT, Kretzer NM, Bhattacharya D, et al. Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *The Journal of experimental medicine.* 2012 Jun 04;209(6):1135-52.
17. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annual review of immunology.* 2013;31:563-604.
18. Shortman K, Naik SH. Steady-state and inflammatory dendritic-cell development. *Nature reviews Immunology.* 2007 Jan;7(1):19-30.
19. Wong KL, Tai JJ, Wong WC, Han H, Sem X, Yeap WH, et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood.* 2011 Aug 4;118(5):e16-31.
20. Wong KL, Yeap WH, Tai JJ, Ong SM, Dang TM, Wong SC. The three human monocyte subsets: implications for health and disease. *Immunologic research.* 2012 Sep;53(1-3):41-57.
21. Zhao C, Zhang H, Wong WC, Sem X, Han H, Ong SM, et al. Identification of novel functional differences in monocyte subsets using proteomic and transcriptomic methods. *Journal of proteome research.* 2009 Aug;8(8):4028-38.

22. Lindstedt M, Lundberg K, Borrebaeck CA. Gene family clustering identifies functionally associated subsets of human in vivo blood and tonsillar dendritic cells. *Journal of immunology*. 2005 Oct 15;175(8):4839-46.
23. Vuckovic S, Gardiner D, Field K, Chapman GV, Khalil D, Gill D, et al. Monitoring dendritic cells in clinical practice using a new whole blood single-platform TruCOUNT assay. *Journal of immunological methods*. 2004 Jan;284(1-2):73-87.
24. Schakel K, Mayer E, Federle C, Schmitz M, Riethmuller G, Rieber EP. A novel dendritic cell population in human blood: one-step immunomagnetic isolation by a specific mAb (M-DC8) and in vitro priming of cytotoxic T lymphocytes. *European journal of immunology*. 1998 Dec;28(12):4084-93.
25. Piccioli D, Tavarini S, Borgogni E, Steri V, Nuti S, Sammiceli C, et al. Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets. *Blood*. 2007 Jun 15;109(12):5371-9.
26. Robbins SH, Walzer T, Dembele D, Thibault C, Defays A, Bessou G, et al. Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome biology*. 2008;9(1):R17.
27. Villani AC, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science*. 2017 Apr 21;356(6335).
28. Schakel K, von Kietzell M, Hansel A, Ebling A, Schulze L, Haase M, et al. Human 6-Sulfo LacNAc-Expressing Dendritic Cells Are Principal Producers of Early Interleukin-12 and Are Controlled by Erythrocytes. *Immunity*. 2006;24(6):767-77.
29. Grage-Griebenow E, Zawatzky R, Kahlert H, Brade L, Flad H, Ernst M. Identification of a novel dendritic cell-like subset of CD64(+) / CD16(+) blood monocytes. *European journal of immunology*. 2001 Jan;31(1):48-56.
30. Kotecha N, Krutzik PO, Irish JM. Web-based analysis and publication of flow cytometry experiments. *Curr Protoc Cytom*. 2010 Jul;Chapter 10:Unit10 7.
31. Vuckovic S, Fearnley DB, Mannering SI, Dekker J, Whyte LF, Hart DN. Generation of CMRF-44+ monocyte-derived dendritic cells: insights into phenotype and function. *Experimental hematology*. 1998 Dec;26(13):1255-64.

32. Clark GJ, Kupresanin F, Fromm PD, Ju X, Muusers L, Silveira PA, et al. New Insights into the Phenotype of Human Dendritic Cell Populations Clinical and Translational Immunology. 2015;5:e61.
33. Diggins KE, Ferrell PB, Jr., Irish JM. Methods for discovery and characterization of cell subsets in high dimensional mass cytometry data. Methods. 2015 Jul 1;82:55-63.
34. R Core Team. R: A Language and Environment for Statistical Computing. 2016.
35. RStudio Team. RStudio: Integrated Development for R. Boston, MA: RStudio, Inc, 2016.
36. Ferrell PB, Jr., Diggins KE, Polikowsky HG, Mohan SR, Seegmiller AC, Irish JM. High-Dimensional Analysis of Acute Myeloid Leukemia Reveals Phenotypic Changes in Persistent Cells during Induction Therapy. PloS one. 2016;11(4):e0153207.
37. Bendall SC, Simonds EF, Qiu P, Amir el AD, Krutzik PO, Finck R, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science (New York, NY). 2011 May 06;332(6030):687-96.
38. Newell EW, Sigal N, Bendall SC, Nolan GP, Davis MM. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8+ T cell phenotypes. Immunity. 2012 Jan 27;36(1):142-52.
39. Amir E-aD, Davis KL, Tadmor MD, Simonds EF, Levine JH, Bendall SC, et al. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nat Biotech. 2013 06//print;31(6):545-52.
40. Frankenberger M, Hofer TP, Marei A, Dayyani F, Schewe S, Strasser C, et al. Transcript profiling of CD16-positive monocytes reveals a unique molecular fingerprint. European journal of immunology. 2012 Apr;42(4):957-74.
41. Ancuta P, Liu KY, Misra V, Wacleche VS, Gosselin A, Zhou X, et al. Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16+ and CD16- monocyte subsets. BMC genomics. 2009;10:403.
42. Reich M, Liefeld T, Gould J, Lerner J, Tamayo P, AMesirov JP. GenePattern 2.0. Nat Genetics. 2006;38(5):500-1.
43. Gould J, Getz G, Monti S, Reich M, Mesirov JP. Comparative gene marker selection suite. Bioinformatics (Oxford, England). 2006 Aug 01;22(15):1924-5.

44. Gould J. GENE-E. [Website] 2016 [cited 2015 Aug;15 2015]; Visualisation and analysis platform for genomics data]. Available from: <https://software.broadinstitute.org/GENE-E/index.html>
45. Lo TH, Silveira PA, Fromm PD, Verma ND, Vu PA, Kupresanin F, et al. Characterization of the Expression and Function of the C-Type Lectin Receptor CD302 in Mice and Humans Reveals a Role in Dendritic Cell Migration. *Journal of immunology*. 2016 Aug 01;197(3):885-98.
46. Ju X, Silveira PA, Hsu WH, Elgundi Z, Alingcastre R, Verma ND, et al. The Analysis of CD83 Expression on Human Immune Cells Identifies a Unique CD83⁺-Activated T Cell Population. *Journal of immunology*. 2016 Dec 15;197(12):4613-25.
47. Tel J, Schreiber G, Sittig SP, Mathan TS, Buschow SI, Cruz LJ, et al. Human plasmacytoid dendritic cells efficiently cross-present exogenous Ags to CD8⁺ T cells despite lower Ag uptake than myeloid dendritic cell subsets. *Blood*. 2013 Jan 17;121(3):459-67.
48. Burgess A, Vigneron S, Brioudes E, Labbe JC, Lorca T, Castro A. Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance. *Proc Natl Acad Sci USA*. 2010 Jul 13;107(28):12564-9.
49. McCloy RA, Rogers S, Caldon CE, Lorca T, Castro A, Burgess A. Partial inhibition of Cdk1 in G 2 phase overrides the SAC and decouples mitotic events. *Cell Cycle*. 2014;13(9):1400-12.
50. Hofer TP, Zawada AM, Frankenberger M, Skokann K, Satz AA, Gesierich W, et al. slan-defined subsets of CD16-positive monocytes: impact of granulomatous inflammation and M-CSF receptor mutation. *Blood*. 2015 Dec 10;126(24):2601-10.
51. Siedlar M, Frankenberger M, Ziegler-Heitbrock LH, Belge KU. The M-DC8-positive leukocytes are a subpopulation of the CD14⁺ CD16⁺ monocytes. *Immunobiology*. 2000 May;202(1):11-7.
52. Engel P, Boumsell L, Balderas R, Bensussan A, Gattei V, Horejsi V, et al. CD Nomenclature 2015: Human Leukocyte Differentiation Antigen Workshops as a Driving Force in Immunology. *Journal of immunology*. 2015 Nov 15;195(10):4555-63.
53. Richter L, Landsverk OJB, Atlasy N, Bujko A, Yaqub S, Horneland R, et al. Transcriptional profiling reveals monocyte-related macrophages phenotypically resembling DC in human intestine. *Mucosal Immunol*. 2018 Sep;11(5):1512-23.
54. Seldon TA, Pryor R, Palkova A, Jones ML, Verma ND, Findova M, et al. Immunosuppressive human anti-CD83 monoclonal antibody depletion of activated dendritic cells in transplantation. *Leukemia*. 2016 Mar;30(3):692-700.

55. Li Z, Ju X, Lee K, Clarke C, Hsu JL, Abadir E, et al. CD83 is a new potential biomarker and therapeutic target for Hodgkin lymphoma. *Haematologica*. 2018 Apr;103(4):655-65.
56. Shahin K, Sartor M, Hart DN, Bradstock KF. Alterations in chemokine receptor CCR5 expression on blood dendritic cells correlate with acute graft-versus-host disease. *Transplantation*. 2013 Oct 27;96(8):753-62.
57. Lau J, Sartor M, Bradstock KF, Vuckovic S, Munster DJ, Hart DN. Activated circulating dendritic cells after hematopoietic stem cell transplantation predict acute graft-versus-host disease. *Transplantation*. 2007 Apr 15;83(7):839-46.
58. Bryant CE, Sutherland S, Kong B, Papadimitriou MS, Fromm PD, Hart DNJ. Dendritic cells as cancer therapeutics. *Semin Cell Dev Biol*. 2018 Mar 21;In Press, Accepted March 21, 2018.
59. Fromm PD, Papadimitriou MS, Hsu JL, Van Kooten Losio N, Verma ND, Lo TH, et al. CMRF-56(+) blood dendritic cells loaded with mRNA induce effective antigen-specific cytotoxic T-lymphocyte responses. *Oncoimmunology*. 2016 Jun;5(6):e1168555.
60. Hsu JL, Bryant CE, Papadimitriou MS, Kong B, Gasiorowski RE, Orellana D, et al. A blood dendritic cell vaccine for acute myeloid leukemia expands anti-tumor T cell responses at remission. *Oncoimmunology*. 2018;7(4):e1419114.
61. Worah K, Mathan TSM, Vu Manh TP, Keerthikumar S, Schreibelt G, Tel J, et al. Proteomics of Human Dendritic Cell Subsets Reveals Subset-Specific Surface Markers and Differential Inflammasome Function. *Cell Rep*. 2016 Sep 13;16(11):2953-66.
62. Alcantara-Hernandez M, Leylek R, Wagar LE, Engleman EG, Keler T, Marinkovich MP, et al. High-Dimensional Phenotypic Mapping of Human Dendritic Cells Reveals Interindividual Variation and Tissue Specialization. *Immunity*. 2017 Dec 19;47(6):1037-50 e6.
63. Dutertre CA, Becht E, Irac SE, Khalilnezhad A, Narang V, Khalilnezhad S, et al. Single-Cell Analysis of Human Mononuclear Phagocytes Reveals Subset-Defining Markers and Identifies Circulating Inflammatory Dendritic Cells. *Immunity*. 2019 Aug 27.
64. Randolph GJ, Sanchez-Schmitz G, Liebman RM, Schakel K. The CD16(+) (FcγRIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. *The Journal of experimental medicine*. 2002 Aug 19;196(4):517-27.

65. Moy RH, Huffman AP, Richman LP, Crisalli L, Wang XK, Hoxie JA, et al. Clinical and immunologic impact of CCR5 blockade in graft-versus-host disease prophylaxis. *Blood*. 2017 Feb 16;129(7):906-16.
66. Huffman AP, Richman LP, Crisalli L, Ganetsky A, Porter DL, Vonderheide RH, et al. Pharmacodynamic Monitoring Predicts Outcomes of CCR5 Blockade as Graft-versus-Host Disease Prophylaxis. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation*. 2018 Mar;24(3):594-9.
67. Reshef R, Luger SM, Hexner EO, Loren AW, Frey NV, Nasta SD, et al. Blockade of lymphocyte chemotaxis in visceral graft-versus-host disease. *N Engl J Med*. 2012 Jul 12;367(2):135-45.
68. Wilson J, Cullup H, Lourie R, Sheng Y, Palkova A, Radford KJ, et al. Antibody to the dendritic cell surface activation antigen CD83 prevents acute graft-versus-host disease. *The Journal of experimental medicine*. 2009 Feb 16;206(2):387-98.

Figure Legends

Fig. 1. A Differential analysis of Mo and DC subsets using cell surface phenotype. A) DC identified as (i) HLA-DR⁺ Lin (CD3, CD14, CD19, CD20, CD56)⁻ include (ii) CD304⁺ pDC and CD11c⁺ mDC which separate into (iii, iv) CD1c⁺, (iii) CD141⁺ and (iv) CD16⁺ populations. **B)** Within an HLA-DR⁺ Lin negative (i) (CD3, CD19, CD20, CD56) population are classical and non-classical Mo subsets are determined by (ii) CD14 and CD16 expression. **C)** Within an HLA-DR⁺ Lin negative (i) (CD3, CD19, CD20, CD56) population, identification of (ii) CD14⁻ cells discriminates (iii) CD1c⁺ DC and CD16⁺ DC. **D)** Overlaying Lin⁻HLA-DR⁺ cell populations from (Bii) onto CD16⁺ cells from (Ciii) distinguishes CD16⁺CD14^{dim} Mo (gate 2) and CD16⁺CD14⁻ mDC (gate 3). The cells in gate 3 have similar CD14 expression to CD1c⁺ DC in gate 4. Gate 1 marks the classical CD14⁺ monocytes. **E)** CyTOF was used to characterize expression of myeloid markers in healthy donors using a 31 parameter stain. Lineage (CD3, CD19, CD20, CD56)⁻, HLA-DR⁺ cells were analyzed using conventional biaxial gating to confirm cell subsets identified by fluorescence based approaches (n=5).

Fig. 2. SLAN glycoprotein modification does not distinguish CD16⁺ Monocyte and CD16⁺ DC subsets. **A)** SLAN expression was measured by flow cytometry on total Lin⁻ HLA-DR⁺ cells and **B)** overlaid onto CD14⁺ and CD16⁺ bivariate gating showing all Lin⁻ HLA-DR⁺ cells. **C)** Canonical Mo and DC subsets were identified and **D)** overlaid along with SLAN⁺ cells. **E)** Proportion of Mo and DC subsets making up the SLAN⁺ cells; **F)** SLAN expression on CD16⁺ Mo and CD16⁺ DC were compared and **G)** quantified using a non-parametric Mann-Whitney Test.

Fig. 3. Characterization of myeloid cells with peripheral blood using unsupervised clustering. A) SPADE clustering of Lineage (CD3, CD19, CD20, CD56, CD235a)⁻, HLA-DR⁺ cells from freshly isolated PBMC by fluorescence cytometry. 127 samples from 5 HD were used to construct the SPADE tree. Expression of test markers were overlaid onto nodes, CD14 and CD16 expression is shown. **B)** A heatmap and average linkage hierarchical clustering of SPADE nodes based on expression of 48 myeloid cell surface proteins. Marker intensity displayed represents the median arcSinh transformed fluorescence. Population annotation was determined by referencing original SPADE nodes with conventional gating. The four main expression clusters are highlighted on the right. **C)** CD14 and CD16 expression is shown on SPADE cluster analysis of Lineage (CD3, CD19, CD20, CD56, CD235a)⁻, HLA-DR⁺ PBMC from mass cytometry. Myeloid populations were defined using gating strategy

defined in Fig. S1A. **D)** Average linkage analysis of 31 cell surface protein expression on PBMC from mass cytometry analysis from 3 healthy donors. **E)** The total proportion of each subset in PBMC (n=22), median fluorescence intensity of **F)** CD14 (n=9) and **G)** CD16 (n=9), **H)** HLA-DR (n=9). **I)** mRNA expression of CD14 and CD16 isoforms (n=3) for each defined myeloid subset. **J)** Analysis of Fc receptor expression pattern in Mo and DC subsets was performed by flow cytometry. **K)** The ratio of activating FcγRIIa was compared to total amount of FcRIIa/b was calculated for DC and Mo subsets and compared to control B cells. N=6. *p<0.05, ****p<0.0001, One way ANOVA with Tukey's Multiple comparisons. **L)** ADCC capacity was measured by ability to lyse anti-CD83 (3C12C) coated CFSE labelled KM-H2 cells by flow cytometry (n=3) and **J)** fold increase in cytotoxicity quantified was quantified. 1 way ANOVA with Tukey's multiple comparison test,*p<0.05, ***p<0.001, ****p<0.0001.

Fig. 4. CD14⁻CD16⁺ myeloid populations maintain differential potential to differentiate into DC and macrophages. **A)** CD14⁺ Mo, CD16⁺ Mo, and CD16⁺ DC were sorted to >90% purity, and **B)** rested for 2 hrs or cultured for 5 days in **C)** GM-CSF/IL4 or **D)** M-CSF (n=3) cytopspun and assessed morphologically following MGG staining. Cell size and circularity were analyzed from 6-10 bright field images per donor. One way ANOVA with Dunnet correction, **, p<0.001, *** p<0.0001. One representative experiment is shown.

Fig. 5. Characterization of CD16⁺Mo and CD16⁺DC reveal differential antigen uptake and processing between CD16⁺Mo and CD16⁺DC but no difference in migration, cytotoxic or stimulatory effector capacity. **A)** Sorted myeloid subsets were seeded in transwell plates and allowed to migrate to control CXCL12 (n=6), CCL5 (n=3) or **B)** f-MLP (n=3). The proportion of migrated cells was calculated after 6hrs and enumerated by flow cytometry. **C)** Macropinocytosis of dextran-FITC and **D)** antigen uptake and degradation of DQ-BSA was monitored kinetically in whole PBMC (n=3) up to six hrs and quantified in myeloid cell subsets by flow cytometry. **E).** Maximum intensity projections of confocal Z-stacked images were used to determine LAMP expression and localization in Mo and DC subsets. A representative image from 3 independent experiments is shown. **F)** Quantitation of intracellular LAMP using Image J from combined data from three donors. *p<0.05, *** p<0.0001. One way ANOVA with Tukey's multiple comparisons. **G)** Cross-presentation of soluble Influenza matrix protein (FMP) by sorted Mo and DC subsets was performed by pulsing cells with short control WT-1₁₂₆₋₁₃₄,

FMP₅₈₋₆₆ or a long FMP₅₄₋₇₄ peptide containing the FMP₅₈₋₆₆ immunodominant epitope. Cross-presentation was measured after 16 hrs by detection of HLA-A2 presented FMP₅₈₋₆₆ peptides on the cell surface by flow cytometry using a specific antibody. One representative experiment of four is shown. **H)** Quantification of cross presentation was determined using by Super enhanced DMAX subtraction compared to control WT-1₁₂₆₋₁₃₄ pulsed cells. n=4, One way ANOVA with Tukey's multiple comparisons; * p<0.05. **I)** The capacity for class II antigen presentation was compared by culturing sorted cell subsets with allogeneic untouched CD4 T cells in different stimulator: responder ratios for 5 days. Proliferation was monitored by CFSE dilution by using flow cytometry. One representative experiment of three is shown. 2 way ANOVA with Tukey's multiple comparison test, *p<0.05.

Fig. 6. CD16⁺ DC and CD16⁺ Mo display similar kinetics of hematopoietic reconstitution and activation following HCT. A) Absolute blood counts of CD16⁺ Mo and CD16⁺ plus CD1c⁺ DC subsets in peripheral blood of patients undergoing auto-HCT following a melphalan bone marrow conditioning regime or **B)** patients undergoing allo-HCT. Patients were monitored following transplant (day 0) using true count beads by flow cytometry. ND indicates not detected. Dashed lines mark lower 95% confidence interval of CD16⁺ Mo and DC levels determined in a healthy hospital cohort of 10

individuals used to establish reconstitution. Three of six representative auto- and allo-HCT patients shown. Non-linear regression analysis of positive CD16⁺ Mo and CD16⁺ DC blood counts in all **C)** auto-HCT and **D)** allo-HCT patients. CCR5 expression was monitored prospectively in CD16⁺ Mo and CD16⁺ DC in patients undergoing allo-HCT. A representative plot showing CCR5 expression in CD16⁺ Mo and CD16⁺ DC subsets in peripheral blood of **E)** healthy donors (n=6), **F)** Allo-HCT at GVHD onset (n=6), **G)** Allo-HCT patients without GVHD (n=4) and **H)** in healthy donor blood following overnight stimulation with 10ng/mL IFN γ by flow cytometry (n=3). **I)** Kinetics of IFN γ induced CCR5 expression on myeloid subsets detected by flow cytometry (n=3). **J)** Kinetic analysis of CCR5 expression during hematopoietic reconstitution following allo-HCT. Solid arrows indicate clinical onset of aGVHD or chronic GVHD; dashed arrows indicate clinical diagnosis of infection indicated. Dotted line and shaded area represent the median plus two standard deviations above fluorescence minus one controls respectively. Non-linear regression analysis of CCR5 expression in CD16⁺ DC versus **K)** CD16⁺ Mo or **L)** CD1c⁺ DC at all collected timepoints in 10 patients.

Fig. 7. CD300c, and CCR5 help to refine overlapping heterogeneity within CD16⁺ myeloid cells in the steady state. **A)** Separation of total Lin⁻ HLA-DR⁺CD11c⁺CD16⁺ myeloid cells by CD300c expression. **B)** High dimensional mapping using viSNE was performed by clustering on CD11c, CD14, CD16, CD1c, CD141, CD34 and HLA-DR. Analysis of Lin⁻ HLA-DR⁺ CD16⁺ myeloid cells demonstrate spatial separation of unique cell clusters. **C)** Back gating of conventional annotated CD16⁺ Mo and CD16⁺ DC onto populations identified using CD300c. **D)** Identification of subset heterogeneity by viSNE representations of CD16⁺ Mo, CD16⁺ DC and CD300c⁺ populations. Heatmap represents CD300c expression. **E)** Separation of total Lin⁻ HLA-DR⁺ CD16⁺ myeloid cells by CCR5 expression. **F)** High dimensional mapping of Lin⁻ HLA-DR⁺ CD16⁺ myeloid cells using viSNE to identify discrete cell populations. **G)** Back gating of conventional annotated CD16⁺Mo and CD16⁺DC onto populations identified using CCR5. **H)** Identification of subset heterogeneity of viSNE representations of CD16⁺ Mo, CD16⁺ DC and CCR5⁺ populations. Heatmap represents CCR5 expression. The unified viSNE landscape was produced from 10 independent samples. One representative experiment of three for each test antibody is shown.

Table 1. Auto and Allo-HCT Patient Characteristics.

Patient Number	Disease	Transplant Type	Conditioning Regimen	GVHD within 100 days	Day of GVHD onset
1-6	MM	Auto-HCT	Melphalan	N	n/a
7	AML	Matched unrelated donor	Busulfan/ Cyclophosphamide	Y	41
8	AML	Matched unrelated donor	Busulfan/ Cyclophosphamide	N	n/a
9	AML	Matched unrelated donor	Fludarabine/ Busulfan 4	Y	27
10	MM	Sibling haploidentical	Fludarabine/ Busulfan 4	Y	71
11	AML	Sibling haploidentical	Fludarabine/ Busulfan 4	N	n/a
12	AML	Matched unrelated donor	Fludarabine/ Busulfan 4	Y	49
13	ALL	Matched unrelated donor	Fludarabine/ Busulfan 3	Y	25
14	MDS	Matched unrelated donor	Busulfan/ Cyclophosphamide	N	n/a
15	AML	Matched unrelated donor	Fludarabine/ Busulfan 4	Y	27
16	ALL	Matched unrelated donor	Fludarabine/ Busulfan 4	N	n/a
17	MM	Matched unrelated donor	Melphalan/ Busulfan	N	n/a
18	AML	Matched sibling	Fludarabine/ Melphalan	Y	41
19	ALL	Matched sibling	Fludarabine/ Melphalan	Y	27

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