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Characterization of human dendritic cell subsets by whole blood flow cytometry analysis

Thesis

for

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- Medical Section -

presented by

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from Guangxi, China

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Outline

Zusammenfassung

Ziel: Dendritische Zellen (DCs) spielen eine wichtige Rolle beim Fortschreiten von Herz-Kreislauf-Erkrankungen, ihre Funktionen hängen stark von den heterogenen Untergruppen mit ihren unterschiedlichen Abstammungslinien und Reifegraden ab. Es ist jedoch schwierig, seltene Zellpopulationen zu identifizieren. Hier haben wir ein 11-Farben-Durchflusszytometrie-Panel eingerichtet, um die Anzahl und den Phänotyp der zirkulierenden DC-Untergruppen bei Patienten mit schwerer Mitralinsuffizienz (MR) zu untersuchen, um das potenzielle Crosstalk von Herzinsuffizienz mit DCs aufzudecken. **Methoden:** Wir haben die Schritte zum Einrichten eines mehrfarbigen Durchflusszytometrie-Panels detailliert beschrieben. Damit untersuchen wir weiter zirkulierende DCs bei gesunden Kontrollen (n=9) und MR-Patienten (n=15). **Ergebnisse:** Wir haben 6 DC-Untergruppen und ihre Häufigkeit in Leukozyten klar unterschieden: pDCs (0,08%), mDCs (0,22%), mDC1 (0,01%), mDC2 (0,17%), $CD16^{+}$DC (0,12%) und $CD11b^{high}$DC (0,02%). Im Vergleich zu gesunden Kontrollpersonen hatten MR-Patienten signifikant verringerte Gesamt-DCs, pDCs, mDCs und mDC2, beide relativ (0,149 vs. 0,352%, P<0,001;0,017 vs. 0,075%, P<0,001; 0,084 vs. 0,22%, P<0,001; 0,049 vs. 0,173%, P=0,004) und absolute Zahlen (6,037 vs. 11,41/µl, P=0,012 für DCs, 0,747 vs. 2,454/µl, P=0,002 für pDCs, 3,359 vs. 7,097/ul, P=0,001 für mDCs, 1,993 vs. 4,632/ul, P=0,001 für mDC2). Wobei kein signifikanter Unterschied bezüglich mDC1 (0,003 vs. 0,006%, P=0,165 für die relative Zahl, 0,108 vs. 0,213/ul, P=0,209 für die

absolute Zahl) besteht. Eine erhöhte Expression von CD209 und CD11a auf mDC wurde im Vergleich zu Kontrollen festgestellt (mittlere Fluoreszenzintensität (MFI) $12{,}62x10^3$ vs. $1{,}80x10^3$, P=0,014 für CD209; MFI: $203{,}1x10^3$ vs. $91{,}0x10^3$, P<0,001 für CD11a). Die Expression von HLA-DR auf mDCs war reduziert (MFI: $46{,}50x10^3$ vs. $69{,}30x10^3$, P=0,002). Keine signifikanten Unterschiede für CD11a und HLA-DR auf pDCs. **Schlussfolgerungen:** Dieses robuste mehrfarbige Panel, das hier eingerichtet wurde, kann in unseren weiteren Forschungen verwendet werden, um die Rolle von DCs beim Fortschreiten von Herz-Kreislauf-Erkrankungen besser zu verstehen. Unser Befund legt nahe, dass die Veränderung der zirkulierenden DCs an der Pathophysiologie der MR-relevanten HF beteiligt sein könnte.

Abbreviation List

DC	Dendritic Cell
CVD	Cardiovascular Disease
pDC	Plasmacytoid Dendritic Cell
mDC	Myeloid Dendritic Cell
cDC	Conventional Dendritic Cell
NK cell	Natural Killer Cell
PBMC	Peripheral Blood Mononuclear Cells
TLRs	Toll-Like Receptors
FcR	Fc Receptors
XCR1	Chemokine (C Motif) Receptor 1
MI	Myocardial Infarction
ox-LDL	Oxidized Low-Density Lipoprotein
HF	Heart Failure
PAMPs	Pathogen Associated Molecular Patterns
DAMPs	Damage Associated Molecular Patterns
AGE	Glycation End Products

AGII	Angiotensin II
CAD	Coronary Artery Disease
ROS	Reactive Oxygen Species
DCM	Dilated Cardiomyopathy
LV	Left Ventricular
IFC	Imaging Flow Cytometers
CFSE	Carboxyfluorescein Succinimidyl Ester
FACS	Fluorescence-Activated Cell Sorting
MR	Mitral Regurgitation
PMVR	Percutaneous Edge-To-Edge Mitral Valve Repair
EDTA	Ethylene Diamine Tetra Acetic Acid
WBC	White Blood Cell
PBS	Phosphate Buffered Saline
FMO	Fluorescence Minus One
SI	Stain Index
RT	Room Temperature
SD	Standard Deviation

MFI	Median Fluorescence Intensity
SSC	Side Scatter
FSC	Forward Scatter
GFP	Green Fluorescent Protein
IL	Interleukin
NK	Natural Killer Cell
NKT	Natural Killer T Cell
NYHA	New York Heart Association
LVEF	Left Ventricular Ejection Fraction
6MWT	Six-Minute Walking Test
ICAM-3	Intracellular Adhesion Molecule 3
DC-SIGN	DC-Specific-ICAM-3-Grabbing Non-Integrin
APC	Antigen Presenting Cell

1. Introduction

Dendritic cells (DCs), named for their probing, “tree-like” or dendritic shapes, were first described in the skin by Langerhans in 1868 and were identified as antigen-presenting cells in 1973.[1] They originate from hematopoietic stem cells in the bone marrow and circulate as precursors in the blood stream, taking residence in target tissues at sites of potential antigen entry.[2] In peripheral blood, circulating DCs (known as “blood DCs”) represent the migratory form of DC precursors.[3] They and their antigen-presenting properties play a key role in the immune system.[4]

Atherosclerosis and atherosclerosis-related diseases such as cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide.[5,6,7] It has become evident that inflammation mediated both by innate and adaptive immunity plays an important role even in the earliest stages of the development of atherosclerotic lesions.[8,9] DCs were originally identified in arteries in 1995.[10] As key modulators of immune responses, they are likely to play a crucial role in directing innate or adaptive immunity against altered self-antigens present in atherosclerosis.[11] The diverse functions of DCs in immune regulation are reflected by the heterogeneous subsets with their different lineages and maturity, also functional plasticity.[12] Flow cytometry is an important tool for studying the immune system. However, a systematic flow cytometric approach to identify subsets of dendritic cells (DCs) accurately and consistently is not yet a routine experiment. Here we developed a panel of surface markers and an analysis strategy that accurately identifies all known populations of circulatory DCs.

1.1. Dendritic cells

1.1.1. Overview of human dendritic cell subsets

In humans, all DCs express high levels of MHC class II (HLA-DR) and lack typical lineage markers CD3 (T cell), CD19/20 (B cell) and CD56 (natural killer cell, NK cell). Three subsets of DCs can be identified in human peripheral blood: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) or conventional DCs (cDCs), which are further subdivided into two subsets based on the expression of the surface markers CD141 (BDCA-3) and CD1c (BDCA-1). (Table 1)

Table 1. Major DC subsets in peripheral blood

	pDCs	mDCs	
		MDC1(CD141$^+$ mDCs)	MDC2(CD1c$^+$ mDCs)
Frequency	0.01-0.4%	0.001-0.05%	0.01-0.3%
Markers	CD303(BDCA-2), CD304(BDCA-4/Neuropilin-1), CD123, CD4, CD45RA, CD141(BDCA-3)	Clec9a, XCR1, CD11c, CD13, CD33	CD1c, CD11c, CD123, CD13, CD33, CD32, CD64, FcεRI, CD2, CD45RO, CD141. A small portion also express CD14 and CD11b
Function	Upon pathogen encounter, produce large amounts of IFNs	Cross-presentation of dead cell antigens	Produce IL-12 upon TLR3 or TLR8 stimulation, Leading to TH1 CD4$^+$T cell polarization and priming of naive CD8$^+$T cells

The frequency of DC subsets is presented as the percentage of leukocytes (%).

1.1.2. Plasmacytoid dendritic cells (pDCs)

pDCs produce large amounts of type 1 IFNs, IFN-α, and IFN-β in response to binding nucleic acids typical of viruses and bacteria on toll-like receptors (TLRs). TLR7 recognizes single-stranded RNA, and TLR9 recognizes CpG DNA. IFNs have pleiotropic effects on several immune cells including T cells, NK cells, and mDCs, which makes pDCs critical respondents to viral infections. However, the antigen uptake capacity of pDCs is inferior to mDCs and at steady state, pDCs can induce tolerance rather than immune response.

Human pDCs are defined as CD303 (BDCA-2)$^{+}$, CD304 (BDCA-4/Neuropilin-1)$^{+}$, CD123^{+}, CD4^{+}, CD45RA^{+}, CD141 (BDCA-3)^{dim}, CD1c (BDCA-1)$^{-}$, and CD2^{-}. They lack expression of lineage markers (CD3, CD14, CD16, CD19, CD20, CD56) and express neither myeloid markers, such as CD13 and CD33, nor Fc receptors (FcR), such as CD16, CD64, or FcεRI.

1.1.3. CD141^{+} myeloid dendritic cells (MDC1)

CD141^{+}mDCs, sometimes also called cDC1 or MDC1, are a very rare subset of blood DCs representing less than 0.05%(0.001-0.05%) of leukocytes. They share several functional and phenotypical features with CD1c^{+}mDCs, such as IL-12 secretion and TLR8 expression, they are also characterized by IFN-λ secretion upon activation. CD141^{+}mDCs exhibit better cross-presentation of antigens derived from dead cells thanks to expression of the necrotic receptor Clec9a. This receptor and

the chemokine (C motif) receptor 1 (XCR1) are exclusive markers of this subset. XCR1 is also expressed by the corresponding mouse subset CD8α^{+}DCs.

CD141 (BDCA-3)$^{+}$ mDCs are Clec9a^{+}, XCR1^{+}, CD11c^{dim}, CD123^{-}, CD1c (BDCA-1)$^{-}$, and do not express lineage markers such as CD3, CD14, CD16, CD19, CD20, or CD56. They express myeloid markers, including CD13 and CD33, and are monocytic in appearance. In contrast to CD1c^{+} mDCs, CD141^{+} mDCs do not express CD2 and Fc receptors such as CD32, CD64, and FcεRI. They also differ in terms of toll-like receptor expression, cytokine production, and T helper cell polarization. The CD141 (BDCA-3) antigen is expressed at much lower levels on CD1c^{+} mDCs, pDCs, monocyte, and granulocytes in blood.

1.1.4. CD1c^{+} myeloid dendritic cells (MDC2)

CD1c^{+} mDCs, also called cDC2 or MDC2, account for 0.01-0.3% of Leukocytes. They produce IL-12 upon TLR3 or TLR8 stimulation with poly (I:C) or R848, which leads to polarization of TH1 CD4^{+} T cells and priming of naive CD8^{+} T cells. The CD1c (BDCA-1) antigen is a member of the CD1 protein family that are structurally related to MHC class I proteins and mediate the presentation of non-peptide antigens to T cells.

The CD1c antigen is specifically expressed on DCs that are CD11c^{high} CD123^{low}. CD1c^{+} mDCs have monocytic morphology and express myeloid markers such as CD13 and CD33, as well as Fc receptors, such as CD32, CD64, and FcεRI.

Furthermore, they are Lin (CD3, CD16, CD19, CD20, CD56)$^{-}$, CD2^{+}, CD45RO^{+}, CD141 (BDCA-3)^{low}, CD303 (BDCA-2)$^{-}$, and CD304 (BDCA-4/Neuropilin-1)$^{-}$. A minor proportion of CD1c^{+} mDCs expresses CD14 and CD11b. CD1c is also expressed by circulating B cells and by CD1a^{+} DCs generated ex vivo from monocyte or hematopoietic precursor cells. (Figure1)

Figure 1

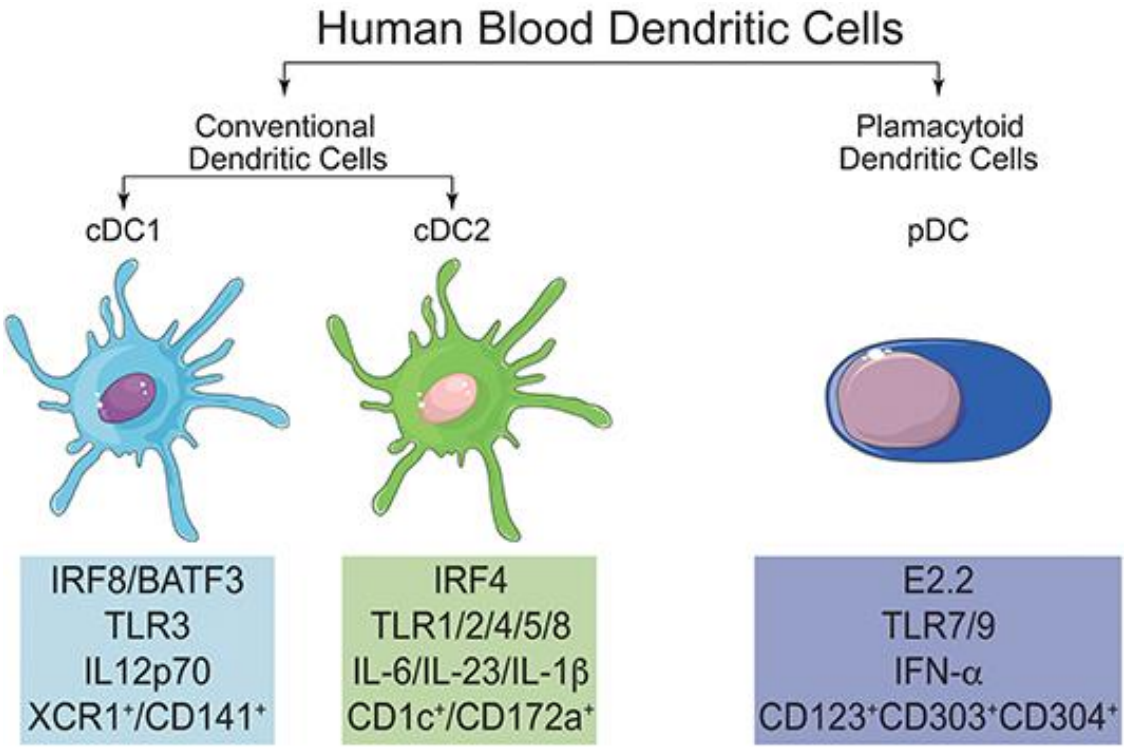

Figure 1. Main characteristics and differences of cDC1, cDC2, and pDC. In human blood, it is possible to find two main populations of DC, named conventional DC (cDC) and plasmacytoid DC (pDC). cDC can be further subdivided in cDC1 and cDC2. All three subtypes of DC can be differentiated by their signature transcription factors and by the expression of specific surface markers. (Patente etc. 2019, Figure 4).

1.2. Dendritic cells in cardiovascular diseases

1.2.1. Dendritic cells in atherosclerosis

Atherosclerosis is the dominant cause of CVDs leading to myocardial infarction (MI), heart failure (HF), or stroke.[13] The investigation of the underlying psychophysiology mechanisms shows that immune cells such as T cells, monocytes, and DCs invade the vascular wall stimulated by oxidized low-density lipoprotein (ox-LDL), TNF-α, and hypoxia,[14,15] which are often found in atherosclerotic lesions where they produce pro-inflammatory cytokines.[16] Both PAMPs and DAMPs can activate DCs[17] which subsequently mature, while further atherogenic factors in the vascular wall such as oxidized LDL cholesterol,[18] advanced glycation end products (AGE),[19] nicotine,[20] insulin,[21] and angiotensin II(AGII)[22] also have the capacity to induce the maturation of DCs. Mature, antigen presenting DCs migrate to secondary lymphoid tissue where they present antigens to T cells.[77] Mature DCs activate T cells and initiate the up regulation of DC licensing factors such as CD40L.[23] T cell activation leads to proliferation and differentiation of T cells into regulatory T cells (T_{regs}), T helper cells (Th), or killer T cells. In general, DCs can activate all types of effector T cells and regulate activation and regulation of immune responses, which are both involved in disease patterns of CVDs. These processes contribute to chronic vascular inflammation and form the basis for vascular obliteration.

Beyond DC tissue analysis, circulating DCs hold significant value in patients suffering from atherosclerosis, as supported by CAD patients having increased

number of DCs in the atherosclerotic vascular wall concomitant with decreased levels of circulating DCs in the blood.[23,24,25,26,27] While Yilmaz et al. reported a reduction of circulating mDCs, pDCs, and total DCs in patients with advanced CAD[28] and mDCs in patients with angina pectoris and MI,[24] Van Vre et al. found that absolute and relative numbers of circulating pDCs were lowered in 18 CAD patients compared to age- and sex matched controls.[25]

1.2.2. Hypertension and dendritic cells

Hypertension is one of the most common chronic diseases, which promotes atherosclerosis and represents a major risk factor for CVD-related death.[29] Several studies have suggested that immunological mechanisms, especially the inflammatory responses, are involved in hypertension.[30,31,32,33] Abbas et al. showed that hypertension activates DCs[28] and further confirmed that reactive oxygen species (ROS) produced by DCs through phagocyte oxidase caused lipid oxidation, which resulted in accumulation of proteins that were oxidatively modified by highly reactive-ketoaldehydes (isoketals). The isoketal-modified proteins behave like DAMPs and activate DCs, which start to express IL-6, IL-1, and IL-23 and the costimulatory CD80 and CD86. The isoketal-pulsed DCs induced T cell proliferation, particularly of $CD8^+$ and IFN and IL-17A, with the latter shown to elevate blood pressure.[34]

1.2.3. Dendritic cells in heart failure

Dendritic cells (DCs) are central to immune activation as their capacity to induce naive T cells activation so that to initiate adaptive immune response (Figure 2), as well as their critical role in innate immunity.[35]

Figure 2

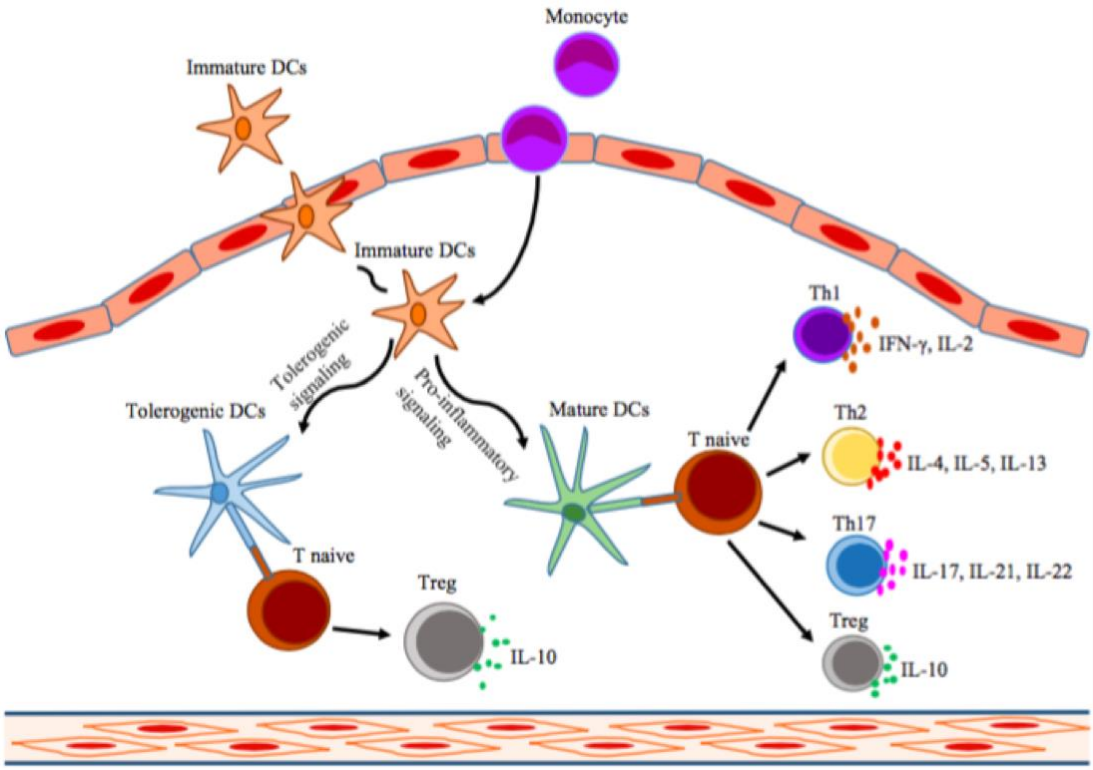

Figure 2. Role of dendritic cells(DCs) in inflammatory disease. DCs could recruit from circulation or proliferate from monocytes to the inflamed tissue. In the absence of inflammation, DCs may induce tolerance by presenting auto-antigens and environment antigens to the naive T cells via MHC class II molecules, leading release of IL-10. Meanwhile, DCs may progress to mature state with enhanced antigen presenting capacities, and up-regulate their production of cytokines and costimulatory molecules. With this, mature DCs can induce the naive T cells to differentiate into different types of $CD4^+$ T cells, which is responsible for adaptive immune response.

In the cardiovascular system, DCs do not only present in the myocardium. Studies by Steinman et al. firmly demonstrated that DCs localized in aortic wall and cardiac valves as well.[36] The turbulent flow by aortic wall and cardiac valves may lead DCs to accumulate and capture disease-related pathogens and present to T cells,[38] accordingly take part in the progress of relevant diseases. Animals studies demonstrated that DCs are responsible for cardiac fibrosis and involved in the progress of HF.[39,40,41]

In human, significant reduction of circulating DCs precursors has been reported in HF patients due to dilated and ischemic cardiomyopathy in all stages of disease severity.[42] Study with MI patients displayed the down-regulation of circulating DCs cause by their enhanced recruitment into the inflamed myocardial tissue,[43] however, this seems not to be the case in HF. Decreased numbers of DCs were found in a group of DCM patients who underwent heart biopsies, and this phenomenon indicates an unfavorable correlation with outcome in terms of HF and tissue fibrosis.[44] To the disappointing results, the authors assumed that possibly due to several reasons, for instance, the apoptosis of DCs, insufficient vascularization in the area of infected cardiomyocytes, and/or the immunomodulation led by regulatory T cells.[42]

In addition, immune cells take part in the progression of heart valve dysfunction.[45] In clinical studies, inflammatory response correlates negatively with LV parameters in valve dysfunction related HF.[46,47] The complex interactions of immune cells in heart valve may be involved in valvular disease related HF, and DCs might be a vital participator. However, with further studies are required to clarify the crosstalk between DCs and valvular disease related HF.

1.3. Flow Cytometry

Flow cytometry is a technology that provides rapid multi-parametric analysis of single cells in solution. Today, one of the most powerful tools for immunophenotypic study of the immune system is polychromatic flow cytometry.[48,49] Due to the development of flow cytometry, our knowledge of the immune system has greatly increased.[50] The polychromatic flow cytometry technique has become increasingly useful in identifying rare subsets of cells such as dendritic cells.[51,52]

Compared to 2 to 4-color assays, the amount of information provided by such a panel will aid in our understanding of the immune system, potentially defining cell subsets that might otherwise be missed.[53,54] In addition, using a multi-color flow cytometry panel can decrease the amount of blood needed for immunophenotyping, which is often limited especially during longitudinal studies.

Imaging flow cytometers (IFC) is one kind of flow cytometric instruments, it combines traditional flow cytometry with fluorescence microscopy. This allows for rapid analysis of a sample for morphology and multi-parameter fluorescence at both a single cell and population level.[78] They are particularly useful in multiple applications such as cell signaling, co-localization studies, cell to cell interactions, DNA damage and repair and any application that needs to be able to coordinate cellular location with fluorescence expression on large populations of cells.

1.3.1. Applications of flow cytometry

Flow cytometry has a wealth of techniques and applications that are suitable for multiple fields of study.[55,57,58,59,-60] Immunophenotyping is the most used application in flow cytometry. It utilizes the unique ability of flow cytometry to simultaneously analyze mixed populations of cells for multiple parameters. Antigen specific responses can be measured by stimulating cells with a specific antigen and then looking for cytokines production, proliferation, activation, memory, or antigen recognition through MHC multimers. Intracellular cytokines analysis is performed by treating cells with a protein transport inhibitor (Brefeldin A or Monensin) for 2-12 hours so that any cytokines produced by the cells can accumulate within the cell enabling better detection. Cell proliferation can be measured by flow cytometry using several different assays and markers. These assays use different methods to target proliferation related events such as incorporation of thymidine analogs (BrdU) into replicating DNA, generational tracking of inheritable permanent dyes (CFSE), and expression of proliferation related antigens (Ki67, PCNA). Apoptosis, or programmed cell death, is a phenomenon that is frequently examined in immunology and other fields of study. It is used to maintain the homeostasis of the immune system by removing cells without triggering an inflammatory response (necrosis). It is the mechanism of death for clonally expanded T cells following an immune response, for self-targeting T cells, for auto-reactive B cells, and multiple other cells in the immune system.

Cell sorting utilizes a flow cytometer with cell sorting capabilities to separate and purify cells or particles for further analysis. Essentially, any cell or particle that can be made fluorescent can be separated by a cell sorter. Cells can be sorted into 96 or 384 well plates, tubes, and slides. A few common types of samples are transfected cells expressing a fluorescent protein, stem cells, tumor infiltrating lymphocytes, tumor cells, and white blood cell populations. A major consideration with any cell sort is scaling up the amount of antibody needed for staining large amounts of cells.

1.3.2. Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS), sometimes called fluorescence-assisted cell sorting, is a specialized type of flow cytometry that uses fluorescent markers to target and isolate cell groups. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is commonly used in hematopoiesis, oncology, and stem cell biology research.

With the ability to separate cells based on surface markers as well as physical characteristics like size, granularity, and cytokine expression, FACS technology is highly versatile. It also has a high throughput, and FACS is now the standard in many clinical and research labs.

1.4. Aims of study

There is emerging evidence that immune response plays a vital role in the progress of heart diseases, and their functions highly depend on the heterogeneous subsets with their different lineages and maturity. However, difficulties to identify rare or heterogeneous cell populations can be limiting. Despite the popularity of flow cytometry to assess dendritic cell subsets proportion and function, there is a considerable variability in sample preparation and subset gating between studies which makes it difficult to compare findings between such studies. The aims of this study were,

1) To set up a 11-color flow cytometry panel that allows for simultaneous detection of the major circulating DC subsets. We analyzed the phenotypes of human DC subsets using this single 11-color flow cytometry panel, thus providing a method which allows researchers to have confidence in the use of this technique when assessing different samples.

2) To investigate the relative and absolute numbers and phenotypes of circulating DC subsets in patients with severe mitral regurgitation (MR), and to show the changes compared with the healthy controls. Studying these major players of the immune system in one single panel may give us a broader view of the immune response during cardiovascular diseases.

2. Material and Methods

2.1. Study subjects

The study subjects consisted of 9 healthy adult donors, whose blood samples were served for panel setup and as controls. And 15 patients with severe MR (Mitral Regurgitation), who underwent PMVR (Percutaneous Edge-to-Edge Mitral Valve Repair) with MitraClip® system at the University Hospital, Department for Kardiologie/Angiologie/Intensivmedizin, University of Lübeck. All the subjects were informed and agreed before the blood taken, and the consent forms were obtained from all the patients. Heart failure patients had to be on optimal medical treatment for at least 3 months prior to MitraClip® treatment according to current guidelines.

2.2. Instrumentation

A CytoFlex S flow cytometry with four lasers (B75442, Beckman Coulter Inc. CA) was used for the study. Our instrument has been optimized to measure up to 13 fluorescent parameters. The violet laser can independently excite four fluorochromes (PB450, KO525, Violet610 and Violet660), the yellow laser can excite four fluorochromes (ECD/mCherry, PE/DsRed, PC5.5 and PC7), the red laser can excite three fluorochromes (APC, APC-A700, and APC-A750), and the blue laser can excite four fluorochromes (FITC, GFP, PerCP, and PC5). (Figure 3)

Figure 3

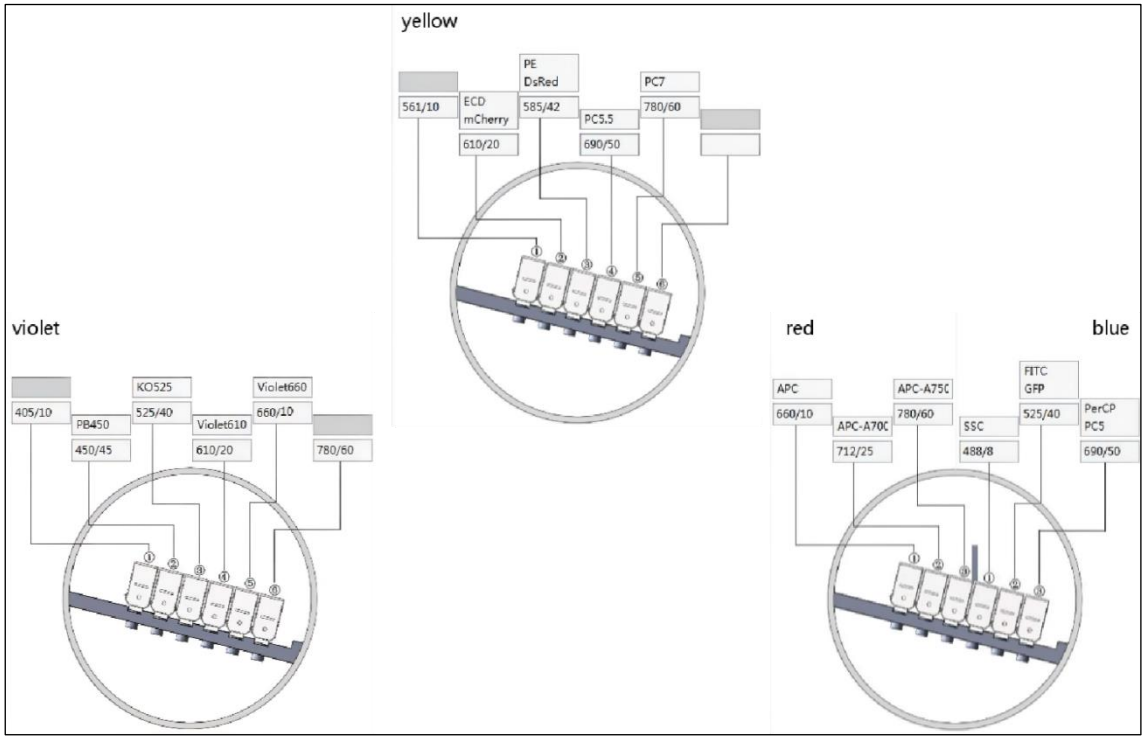

Figure 3. Detector Configuration of CytoFlex S flow cytometry. The instrument is equipped with 4 spatially separated lasers: violet(405mm), yellow(561mm), red(638mm), blue(488mm).

2.3. Sample preparation and staining for FACS analysis

Blood samples from the participants were collected into ethylene diamine tetra acetic acid (EDTA) tubes. The date of analysis for patients was the day of PMVR. The white blood cell (WBC) count was determined by a hemocytometer, Cell-Dyn Emerald (Abbott, Germany). And then blood samples were diluted with phosphate buffered saline (PBS) (pH ~7.4) to the concentration of ~$3x10^6$ WBC/ml. Antibody master mix was prepared. 300µl of diluted whole blood were used for per test, antibody cocktail was added to the blood (short spin with table centrifuge of master mix and vortex after added to blood), incubated at 4°C in the dark for 30 minutes. Re-suspended in 3.0 ml 1xRBC Lysis/Fixation solution (BioLegend), Gently mix each tube immediately by pipetting up and down. Incubated at room temperature, in the dark, for 15 minutes. Centrifuged at 350xg for 5 minutes at 7°C. Supernatant was removed carefully. Samples were washed with 3 ml ice-cold FACS buffer, by centrifuging at 350xg for 5 minutes (at 7°C). Supernatant was removed carefully. The cell pellet was re-suspended in 110µl ice cold FACS buffer. Samples were stored at 4°C, protected from light until analysis is performed (within 12 hours).

2.4. Antibody titration

All antibodies were titrated to determine the optimal concentrations. 7 serial dilutions from 1:5 down to 1:320 per antibody were performed: 20µl of stock antibody was diluted in 80µl of blood. This results in a 1:5 dilution.10µl of this was taken and diluted into 90µl of diluted blood (80ul blood + 10ul PBS), serial dilutions were performed until 1:320 dilution. Incubated for 20 minutes at room temperature, protected from light, washed two times with PBS, Samples were re-suspended in 500µl PBS. Acquired by CytoFlex S flow cytometer (Beckman Coulter Inc. CA).

2.5. Antibodies used for FACS analysis

The following monoclonal antibodies were used: FITC anti-human Lineage Cocktail (CD3/14/19/20/56) [clone UCHT1, HCD14, HIB19, 2H7, HCD56], PerCP/Cy5.5 anti-human HLA-DR (clone L243), PE/Cy7 anti-human CD11c (clone Bu15c), APC anti-human CD370 (CLEC9A/DNGR1) (clone 8F9), Alexa Fluor® 700 anti-human CD45 (clone 2D1), Brilliant Violet 510 anti-human CD16 (clone 3G8), Brilliant Violet 605 anti-human CD123 (clone 6H6), Brilliant Violet 650 anti-human CD1c Antibody (clone L161) (all from BioLegend, San Diego, CA); PE anti-human CD11a (clone HI111, BioLegend, San Diego, CA), PE/Dazzle 594 anti-mouse/human CD11b (clone M1/70, BioLegend, San Diego, CA), Brilliant Violet 421 anti-human CD209 (DC-SIGN) (clone 9E9A8, BioLegend, San Diego, CA).

2.6. Fluorescence minus one control

Fluorescence Minus One (FMO) control is the experimental cells stained with all the antibodies except one.[61,62] In our study, FMO control was performed for all the antibodies to improve the gate settings. The FMO control matrix is shown in Table 2.

Table 2 FMO control Matrix

Tube	FITC	PerCP/ Cy5.5	PE	PE-TR	PE-Cy7	APC	A700	BV-421	BV-510	BV-605	BV-650
Unstained	-	-	-	-	-	-	-	-	-	-	-
Full-stained	+	+	+	+	+	+	+	+	+	+	+
FMO-											
Lin	-	+	+	+	+	+	+	+	+	+	+
HLA-DR	+	-	+	+	+	+	+	+	+	+	+
CD11a	+	+	-	+	+	+	+	+	+	+	+
CD11b	+	+	+	-	+	+	+	+	+	+	+
CD11c	+	+	+	+	-	+	+	+	+	+	+
Clec9a	+	+	+	+	+	-	+	+	+	+	+
CD45	+	+	+	+	+	+	-	+	+	+	+
CD209	+	+	+	+	+	+	+	-	+	+	+
CD16	+	+	+	+	+	+	+	+	-	+	+
CD123	+	+	+	+	+	+	+	+	+	-	+
CD1c	+	+	+	+	+	+	+	+	+	+	-

Abbreviations: FITC: fluorescein isothiocyanate; PerCP/Cy5.5: Peridinchlorophyll/Cyanine5.5; TR: Texas Red; PE: phycoerythrin; Cy7: Cyanine-7; APC: allophycocyanin; A700: Alexa 700; BV: Brilliant Violet.

2.7. Compensation

Antibody-capture beads (VesaComp Antibody Capture Bead Kit, Beckman Coulter, Inc.) were used for single-color compensation controls, except of FITC-Lineage, PerCP/Cy5.5-HLA-DR, Alexa700-CD45, BV510-CD16 and BV605-CD123, we used cells for the compensation.

2.8. Data acquisition and sample analysis

Instrument calibration was checked daily by use of CytoFLEX Daily QC Fluorospheres kit (Beckman Coulter). After acquiring unstained and single-color control samples to calculate the compensation matrix, we acquired 100ul of 110ul samples to get as many events as we can. Data analysis was carried out with CytExpert 2.4 software (Beckman Coulter, Inc.). To avoid the methodical bias caused by the small cell population, we analyzed the blood sample from each volunteer for three times, and DC subsets numbers were described as the mean value of the resulting data. The percentage of DC subsets in leukocytes was determined as the relative number. The absolute numbers were described as cells per milliliter (cells/µl), which is based on the relative DCs numbers in relation to leukocytes count, and the leukocytes number was measured with an automated cell counter (Cell-Dyn Emerald, Abbott, Germany). Statistical analysis was performed with GraphPad Prism 9 software (GraphPad, LLC). Continuous variables are presented as mean ± standard error of the mean (SEM), For comparisons between groups, Mann-Whitney rank test was used for statistical comparison. The 2-tailed P value<0.05 was considered statistically significant.

3. Results

3.1. Baseline characteristics

The average age of the 15 MR patients and 9 healthy controls was 81.8 and 32.8 years, respectively. The time for PMVR was from June 2020 to October 2020. Table 3 shows the characteristics of study subjects. (Attachment 1 shows the raw data).

Table 3 Baseline characteristics of study subjects

	MR Patients(n=15)	Healthy Controls(n=9)
Mean Age (years)	75.2 (range, 72 to 92)	32.8 (range, 21 to 50)
Female gender (n, %)	10 (66.7%)	4 (44.4%)
NYHA-class (n, %)		
II	2(13.3%)	
III	6(40.0%)	
IV	7(46.7%)	
LVEF (n, %)		
≤35%	6 (40.0%)	
36-50%	3 (20.0%)	
>50%	6 (20.0%)	
MR severity (n, %)		
II°	1 (6.7%)	
III°	9 (60.0%)	
IV°	5 (33.3%)	
6MWT (m)	96.6±35.3	

Abbreviations: NYHA: New York Heart Association; LVEF: Left Ventricular Ejection Fraction; MR: Mitral Regurgitation; 6MWT: Six-Minute Walking Test. The severity of mitral regurgitation was determined according to the current European Association of Echocardiography guidelines.[37] The result of 6MWT is presented as mean ± SEM.

3.2. Panel design

The goal of this study was to design and develop a 11-color flow cytometry panel to enable the detection, enumeration, and analysis of the sub-populations of circulating dendritic cells. This multi-color panel design was based on fluorochrome brightness, antigen density and co-expression, fluorochrome spillover of immune-cell subsets of interest and reagent availability. A description of the panel is shown in Table 4.

Table 4. Panel description and Optimal antibody working volumes

Fluorophore	Antibody	Working volume(ul/test)
FITC	Lineage	10
PerCP/Cy5.5	HLA-DR	3.5
PE	CD11a	2.0
PE-TR/PE-Dazzle-594	CD11b	10
PE-Cy7	CD11c	4.0
APC	Clec9a	5.0
Alexa700	CD45	15
BV421	CD209	5.0
BV510	CD16	5.0
BV605	CD123	4.0
BV650	CD1c	7.0

Here are the antibodies we used in this study, with their matching fluorophores. The antibody working volumes were determined by the antibody titrations.

3.3. Selection of reagents and optimization of sample staining procedure

We chose the anti-Human Lineage Cocktail (BioLegend) which is composed of CD3, CD14, CD16, CD19, CD20, and CD56, instead of using 6 single antibodies. This allows us to exclude T cells, B cells, NK cells, monocyte, and neutrophils in one channel. Since blocking of FcR did not have a significant impact on the results (data not shown), it was not included in our staining protocol. For the red blood cell lysis, we tested two red blood cell lysis solutions and identified the BioLegend RBC Lysis/Fixation solution is the most efficient (data not shown).

We tested Live/Dead cells stained with Zombie aqua (BioLegend), but only a very small percentage of dead cells (<1%) was detected. Since by using the forward and side scatters areas, widths, and heights gating strategy, we can remove most of doublet cells, dead cells, and debris. More importantly, this always came out with a lot of spills over. So, we didn't include this in our study. (Figure 4)

Figure 4

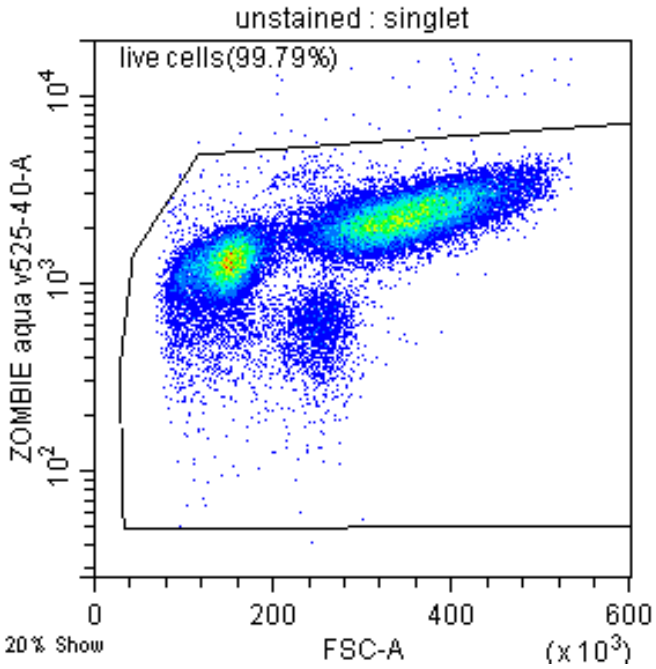

Figure 4. Live/Dead cells staining. First debris and doublets were removed by our gating strategy used in this study, then live cells were gated as Zombie Aqua negative cells. Here it shows that 99.79% cells are live cells.

3.4. Antibody titration

The optimal antibody concentrations were decided according to the Stain Index (SI). The population of interested was gated by standard gating strategy (Figure 5 A-C).

Figure 5

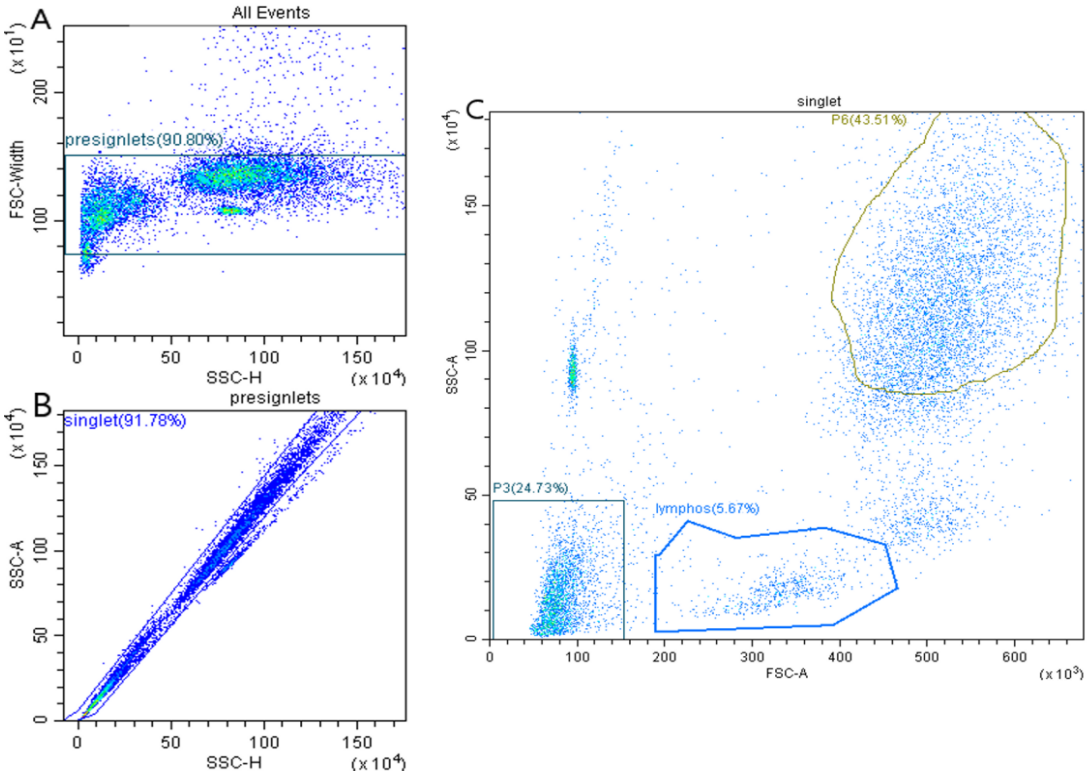

Figure 5. Gating strategy for antibody titration: Debris and doublets were excluded with FSC Width, SSC height and SSC Area (**A, B**), and then lymphocytes were gated with FSC-A and SSC-A (**C**).

The expression of antibodies was shown in histogram. A gate was set to separate the population into positive and negative groups with the unstained sample (Figure 6A). Then the Standard Deviation of the negative population (SD_{neg}) and the Median Fluorescence Intensity of both negative (MFI_{neg}) and positive population (MFI_{pos}) were calculated by CytExpert 2.4. The stain index is calculated as $SI=(MFI_{pos}-MFI_{neg})/SD_{neg}$. SI is plotted against dilution to draw the titration curve (Figure 6C). All the optimal antibody concentrations used in this study were shown in Table 4. (Attachment 2 shows the raw data for antibody titration).

Figure 6

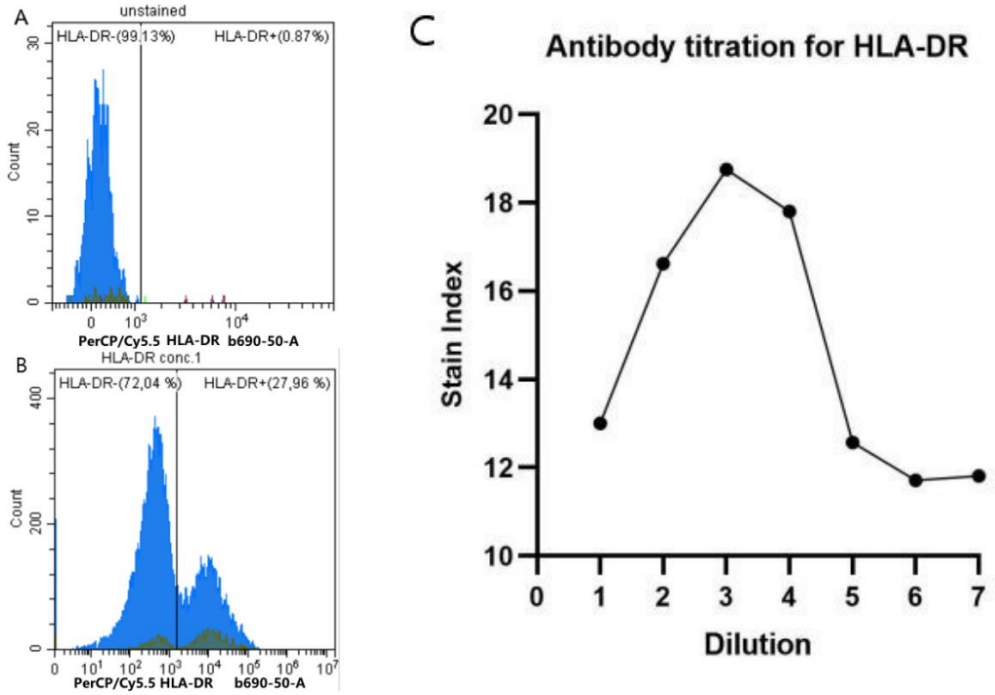

Figure 6. Antibody titration for HLA-DR. Histogram (PerCP/Cy5.5 HLA-DR vs. Count) of unstained sample (**A)** and dilution 1 **(B); C:** Titration curve for HLA-DR. The stain index is plotted with dilution. The series dilution 1, 2, ..., 7 represents dilution in 1:5, 1:20, ..., 1:320. In this case, the optimal stain index was observed at dilution 3.

3.5. Fluorescence minus one control

FMO control is important when building multi-color flow cytometry panels as they will help you determine where the gates should be set. In this study we performed FMO control to assess the spread of all the fluorophores and set every gates accordingly. Figure 7 shows how to set gate for CD11c positive and CD11c negative population by taking into account the fluorescence spread. And how the results were improved by FMO control.

Figure 7

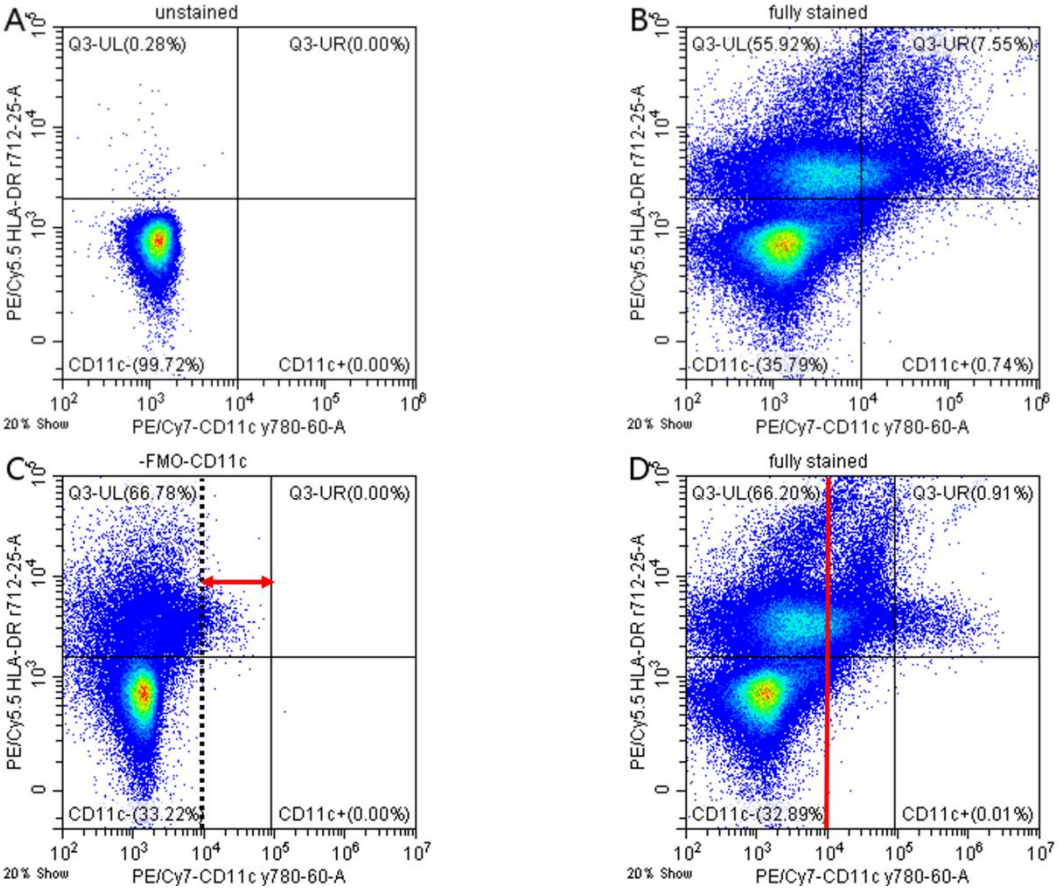

Figure 7. FMO control for CD11c. Dot plots showing the fluorescence spillover into the PE-Cy5.5 channel. **A.** unstained control; **B.** Gating for CD11c according to (A); **C.** CD11c FMO control: red double arrow represents the spread between the two channels; **D.** Gating for CD11c according to (C): black line represents FMO gating boundary compared to unstained boundary in red.

3.6. Compensation

Fluorescence compensation is critical for poly-chromatic flow cytometry analysis. It can ensure that there was no overlap between band pass filters and that the diachronic filters separate the two contiguous band pass filters. Figure 8 shows the "bad" and "good" compensation by using beads and cells for BV605-CD123, respectively, and how the results were impacted by compensation.

Figure 8

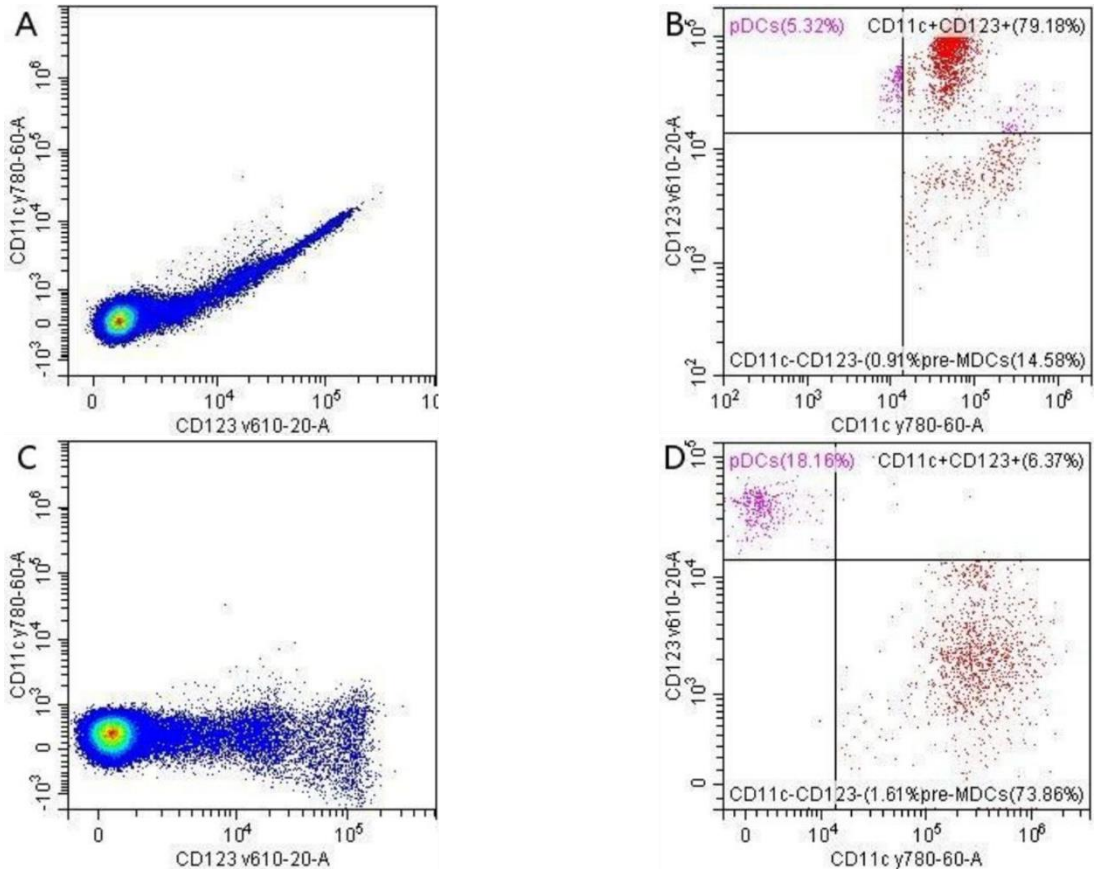

Figure 8. Compensation for BV605-CD123. A: "bad" compensation by using beads: the "tail" was up(under compensated); **C:** "good" compensation by using cells; We can see the population was separated more clearly with the compensation by using cells**(D)** than by beads**(B)**.

In this study, compensation matrix obtained by staining Antibody-capture beads Compbeads (VesaComp Antibody Capture Bead Kit, Beckman Coulter, Inc.) with each of the antibodies used in the panel, with the exception of FITC, PerCP/Cy5.5, Alexa700, BV510 and BV605, we used cells for the compensation. Table 5 shows the compensation matrix used in this study.

Table 5. Compensation matrix

	FITC	PerCP/ Cy5.5	APC	A700	BV-421	BV-510	BV-605	BV-650	PE	PE-TR	PE-Cy7
FITC		-0.03	0.10	1.47	0.93	1.18	0.06	0.11	0.80	0.24	0.34
PerCP/ Cy5.5	0.95		2.07	5.15	2.00	0.17	7.66	3.41	3.88	17.61	0.60
APC	0.01	1.78		0.50	0.40	0.08	0.21	26.02	0.00	0.05	0.01
A700	0.01	18.83	30.53		0.11	0.16	0.15	13.34	0.00	0.12	0.03
BV-421	0.00	0.16	0.32	0.16		10.30	4.28	6.74	0.05	0.11	0.09
BV-510	1.00	0.12	0.08	0.38	13.90		0.55	0.93	0.03	0.05	0.15
BV-605	0.17	0.16	0.15	0.20	1.59	39.10		33.49	1.14	3.68	0.08
BV-650	0.06	2.69	6.17	0.14	0.40	11.25	41.08		0.20	0.80	0.00
PE	0.03	0.03	0.10	0.08	0.12	0.10	60.71	0.85		27.72	1.15
PE-TR	0.04	0.12	0.77	0.16	0.12	0.58	89.05	8.30	30.21		0.44
PE-Cy7	0.02	17.24	8.61		0.32	0.03	8.45	6.34	0.95	5.08	

Spectral overlap or spillover between fluorochromes was calculated by CytExpert 2.4.

3.7. Gating strategy for DCs and DC subsets

First, clumps (greater FSC(A) relative to FSC(H)) and debris (very low FSC) were removed from cells that have an equal area and height (Figure 9A, B). The major leukocyte population was gated based on SSC/FSC properties (Figure 9C) and as $CD45^+$ cells (Figure 9D). DCs were broadly gated from leukocytes based on their SSC/FSC properties (they have an intermediate size that falls between monocyte and lymphocytes, Figure 9E). Then DCs were identified as $HLA\text{-}DR^+$ $Lineage^-$ cells to exclude $CD3^+$ T lymphocytes, $CD14^+$ monocyte, $CD20^+$ B lymphocytes, and $CD56^+$ NK cells (Figure 9F).

Figure 9

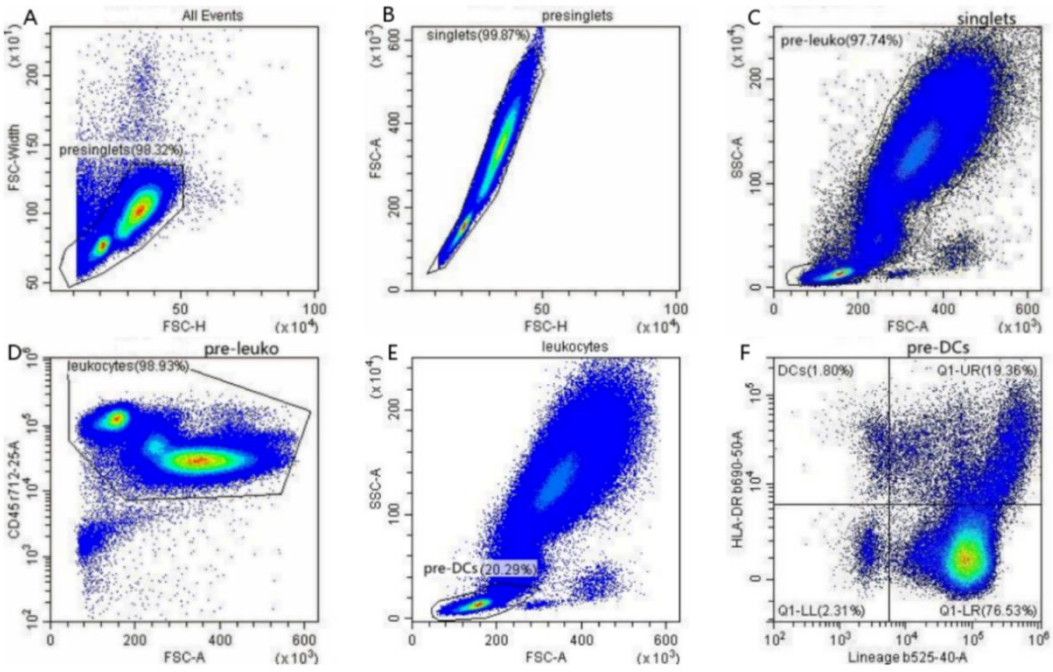

Figure 9. DCs gating strategy. (A) FSC(H) vs. FSC(W) and **(B)** FSC(H) vs. FSC(A) plot: Gating the cells that have an equal area and height, thus removing clumps and debris. **(C)** FSC(A) vs. SSC(A) plot: Broad selection of leukocytes based on their SSC/FSC properties. (**D**) FSC(A) vs. CD45 plot: Gating to select leukocytes. **(E)** FSC(A) vs. SSC(A) plot: Broad selection of DCs based on their SSC/FSC properties. **(F)** Lineage vs. HLA-DR plot: Gating to select DCs.

The major DC subsets were gated as pDC ($CD123^{+}CD11c^{-}$) (Figure 10A), mDC ($CD123^{+}CD11c^{+}$) (Figure 10B). We distinguished two DC subsets highly expressed CD16 ($CD11c^{+}CD16^{+}$, Figure 10C) and CD11b ($CD11c^{+}CD11b^{high}$, Figure 10D). $CD16^{+}$ DCs were classified as "DC4" and have recently been described as transcriptomically distinct from non-classical monocyte.[63,64] mDCs were further subdivided into two populations based on Clec9a, CD1c expression: mDC1 ($CD11c^{+}Clec9a^{+}$, Figure 10E) and mDC2 ($CD11c^{+}CD1c^{+}$, Figure 10F).

Figure 10

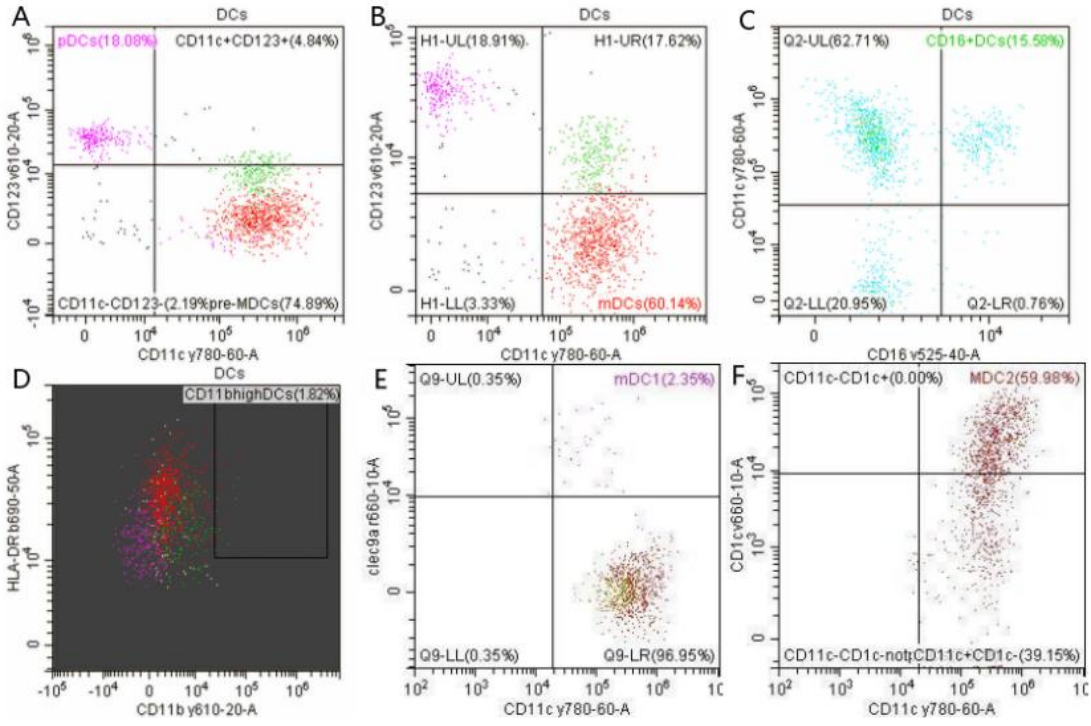

Figure 10. Gating strategy for DC subsets. Selected DCs redisplayed to gate the DC subsets: **(A)**pDC: $CD11c^{-}CD123^{+}$, **(B)** mDC: $CD11c^{+}CD123^{-}$, **(C)** $CD16^{+}$DC: $CD11c^{+}CD16^{+}$ and **(D)** $CD11b^{high}$DC: $CD11b^{high}HLA\text{-}DR^{+}$. The selected mDCs were subdivided into **(E)** mDC1: $CD11c^{+}Clec9a^{+}$ and **(F)** mDC2: $CD11c^{+}CD1c^{+}$. Results presented here were from one donor and representative of nine donors.

3.8. Relative and absolute numbers of DC subsets

Using the gating strategy previously described, we find a normal distribution of DC subsets in humans that are in agreement with previous studies.[28,48] The average number of total DCs accounts for 0.35% leukocytes, the average pDCs and mDCs number is 0.08% and 0.22% of leukocytes, respectively. 0.01% is mDC1, 0.17% is mDC2, 0.12% is $CD16^{+}$DCs, and 0.02% is $CD11b^{high}$DCs. The relative and absolute numbers of DC subsets are shown in Table 6. (Attachment 3 shows the raw data of FACS analysis)

Table 6 Normal distribution of human circulating DC subsets

	Total DCs	DC subsets					
		pDCs	mDCs	mDC1	mDC2	$CD16^{+}$ DCs	$CD11a^{high}$ DCs
Relative number (%)	0.35 ±0.010	0.08 ±0.005	0.22 ±0.025	0.01 ±0.001	0.17 ±0.046	0.12 ±0.025	0.02 ±0.012
Absolute number (/ul)	11.41 ±1.192	2.45 ±0.269	7.10 ±0.653	0.21 ±0.065	4.63 ±0.951	3.08 ±0.375	0.70 ±0.404

Data was obtained from nine healthy adult donors by an automated hematology blood analyzer and CytoFlex S flow cytometry. The relative number of DCs is presented as percentage of leukocytes(%), the absolute number is presented as cells per milliliter(/μl); Data is presented as mean ± SEM.

3.9. FACS analysis of circulating DC subsets on patients with MR

Table 7 shows the comparative analysis of the main circulating DC subsets between patients with MR and healthy controls. P<0.05 is considered statistically significant.

Table 7 Comparison of DC subsets between MR patients and healthy controls

	relative number (%)			absolute number (/ul)		
	patients	controls	P	patients	controls	P
DCs	0.149±0.025	0.352±0.010	<0.001	6.037±1.164	11.41±1.192	0.012
pDCs	0.017±0.006	0.075±0.005	<0.001	0.747±0.310	2.454±0.269	0.002
mDCs	0.084±0.012	0.222±0.025	<0.001	3.359±0.636	7.097±0.653	0.001
mDC1	0.003±0.001	0.006±0.001	0.165	0.108±0.031	0.213±0.065	0.209
mDC2	0.049±0.010	0.173±0.046	0.004	1.993±0.524	4.632±0.951	0.001

Data were obtained from patients with MR(n=15) and healthy adult donors(n=9). The relative number of DCs is shown as the percentage of leukocytes(%), the absolute number is shown as cells per milliliter(/μl); Data is presented as mean ± SEM. P<0.05 is considered statistically significant.

The frequency of total DCs was significantly decreased in patients compared to healthy controls (0.149 vs. 0.352%, P<0.001, Figure 11A). Such a reduction was not only seen in relative number, but also in absolute number (6.037 vs. 11.41/µl, P=0.012, Figure 11B), hence excluding a possible dilution effect. For the major DC subsets, a reduced pDCs relative number (0.017 vs. 0.075%, P<0.001) and absolute number (0.747 vs. 2.454/µl, P=0.002) were observed in patients as compared to controls (Figure 11C, D). Similar to pDCs, the relative number of mDCs was significantly decreased in patients compared to healthy controls (0.084 vs. 0.222%, P<0.001, Figure 11E), with a similar tendency in absolute number (3.359 vs. 7.097/ul, P=0.001, Figure 11F).

Figure 11

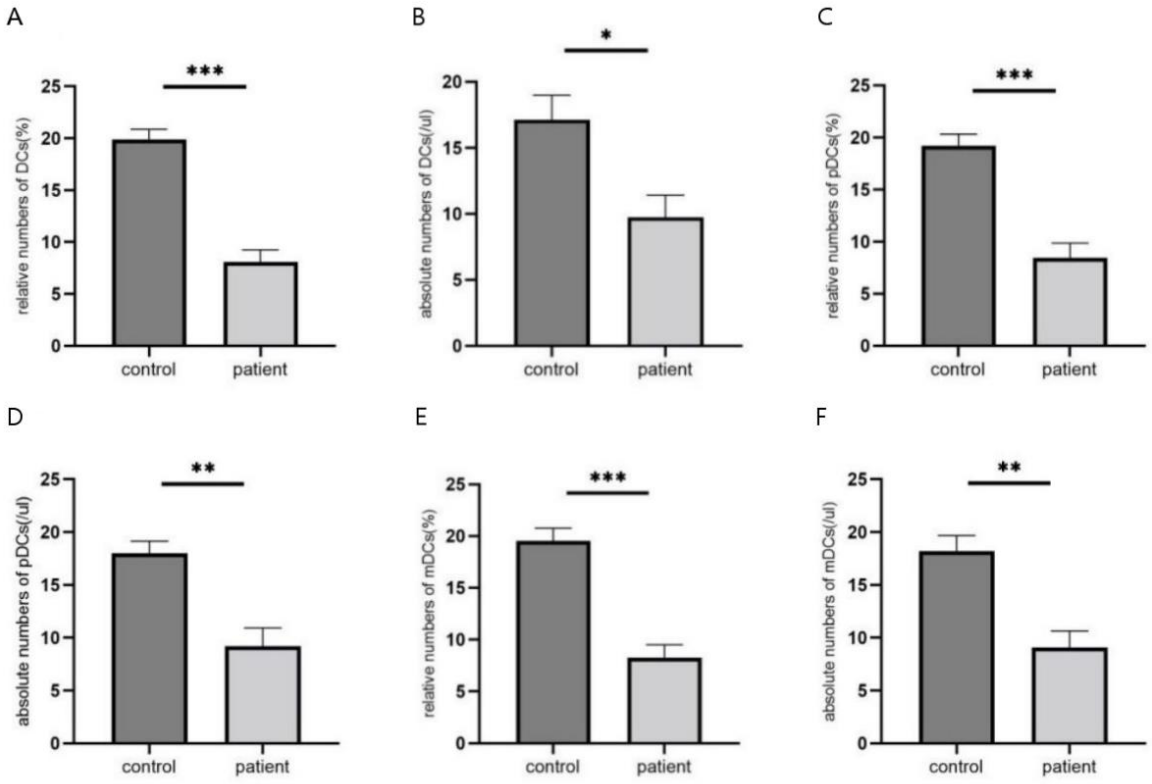

Figure 11. Relative and absolute numbers of major DC subsets in control group (n=9) and patients with MR (n=15). **(A)** Relative number of total DCs; **(B)** Absolute number of total DCs; Circulating pDCs are shown in **(C)** relative number and **(D)** absolute number;The relative number of mDCs is shown in **(E)**, the absolute number in **(F).** Data is presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Similar to the findings for pDCs, the relative number of mDC2 was significantly decreased in MR patients compared to the controls (0.049 vs. 0.173%, P=0.004, Figure 12C), with a similar tendency in absolute number (1.993 vs. 4.632/ul, P=0.001, Figure 12D). No significant changes of mDC1 were detected in MR patients compared to controls. (0.003 vs. 0.006%, P=0.165 for relative number, 0.108 vs. 0.213/ul, P=0.209 for absolute number; Figure 12A, B).

Figure 12

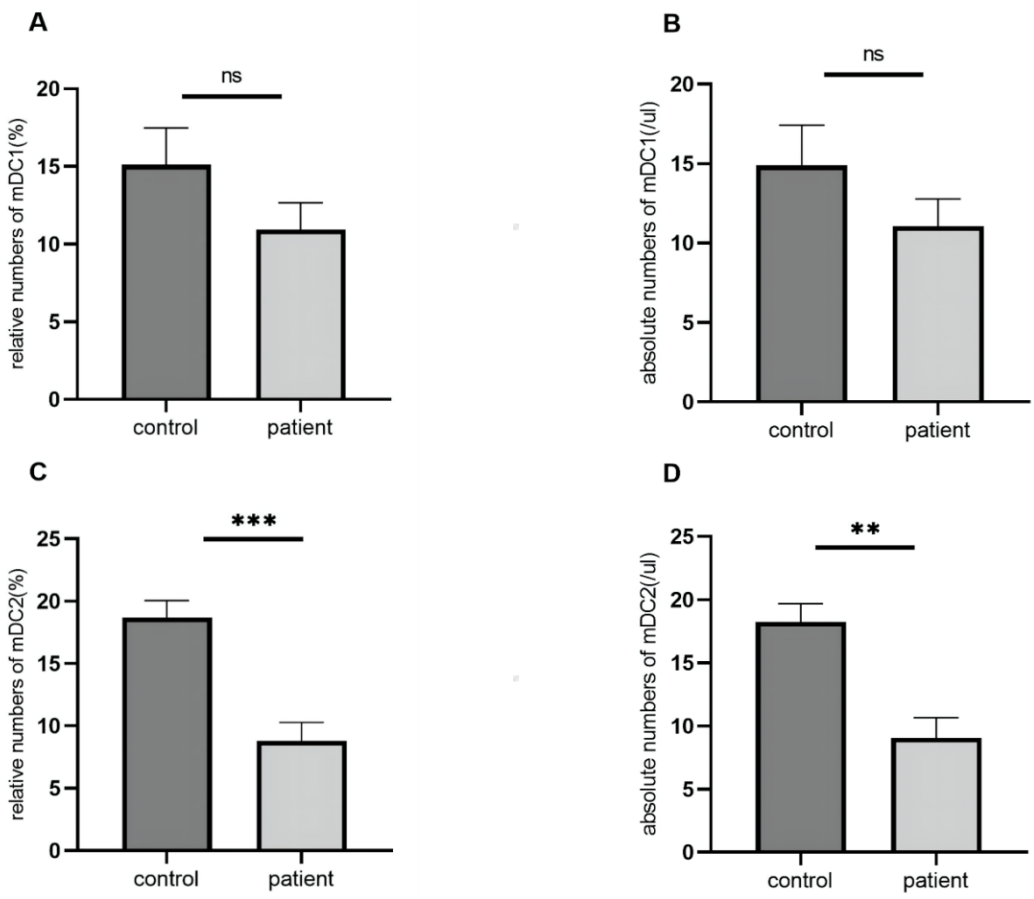

Figure 12. Relative and absolute numbers of circulating mDC1 and mDC2 in control group (n=9) and patients with MR (n=15). Circulating mDC1 is shown as a percentage of leukocytes **(A)** and as cells per milliliter(/µl) **(B)**. Circulating mDC2 is shown in **(C, D)**. Data is presented as mean ± SEM. **P<0.01, ***P<0.001.

3.10. Analysis of DCs surface markers

To phenotype DCs, we detected the expression of costimulatory molecules HLA-DR (MHC class II), adhesion molecule CD11a and CD209 with flow cytometry. A high amount of HLA-DR and CD11a was found constitutionally expressed on both pDCs and mDCs, which was known in the prior studies.[79,80] CD209, also named dendritic cell specific intracellular adhesion molecule 3 (ICAM-3)-grabbing non-integrin (DC-SIGN), was expressed on mDCs and monocyte-derived DCs, but was absent on pDCs.[81,82,83,84,85] Table 8 shows the comparative surface markers on DC subsets. (Attachment 4 shows the raw data of FACS analysis)

Table 8 The expression of surface markers on DC subsets

	MFI of HLA-DR ($x10^3$)		MFI of CD11a ($x10^3$)		MFI of CD209 ($x10^3$)
	pDCs	mDCs	pDCs	mDCs	mDCs
Controls	41.31±7.63	69.30±5.95	42.77±8.54	91.0±9.16	1.80±0.55
Patients	44.58±5.58	46.50±3.92	62.48±14.88	203.1±31.67	12.62±7.60
P	0.77	0.002	0.41	<0.001	0.014

The expressions of surface markers on pDCs and mDCs are presented as mean fluorescence intensity (MFI). controls, n=9; patients, n=15. Data is presented as mean ± SEM. $P<0.05$ is considered statistically significant.

We detected no significant changes in both costimulatory molecules HLA-DR and adhesion molecule CD11a for pDCs in patients compared to controls. (MFI: 44.58x10^3 vs. 41.31x10^3, P=0.77 for HLA-DR, Figure 13A; MFI: 62.48x10^3 vs. 42.77x10^3, P=0.41 for CD11a, Figure 13D). However, we observed a significantly decreased expression of HLA-DR and increased expression of adhesion molecule CD11a on mDCs in patients compared to controls, (MFI: 46.50x10^3 vs. 69.30x10^3, P=0.002 for HLA-DR, Figure 13B; MFI: 203.1x10^3 vs. 91.0x10^3, P<0.001 for CD11a, Figure 13E). The expression of CD209 on mDCs was increased in patients with MR compared to healthy controls. (MFI: 12.62x10^3 vs. 1.80x10^3, P=0.014) (Figure 13C).

Figure 13

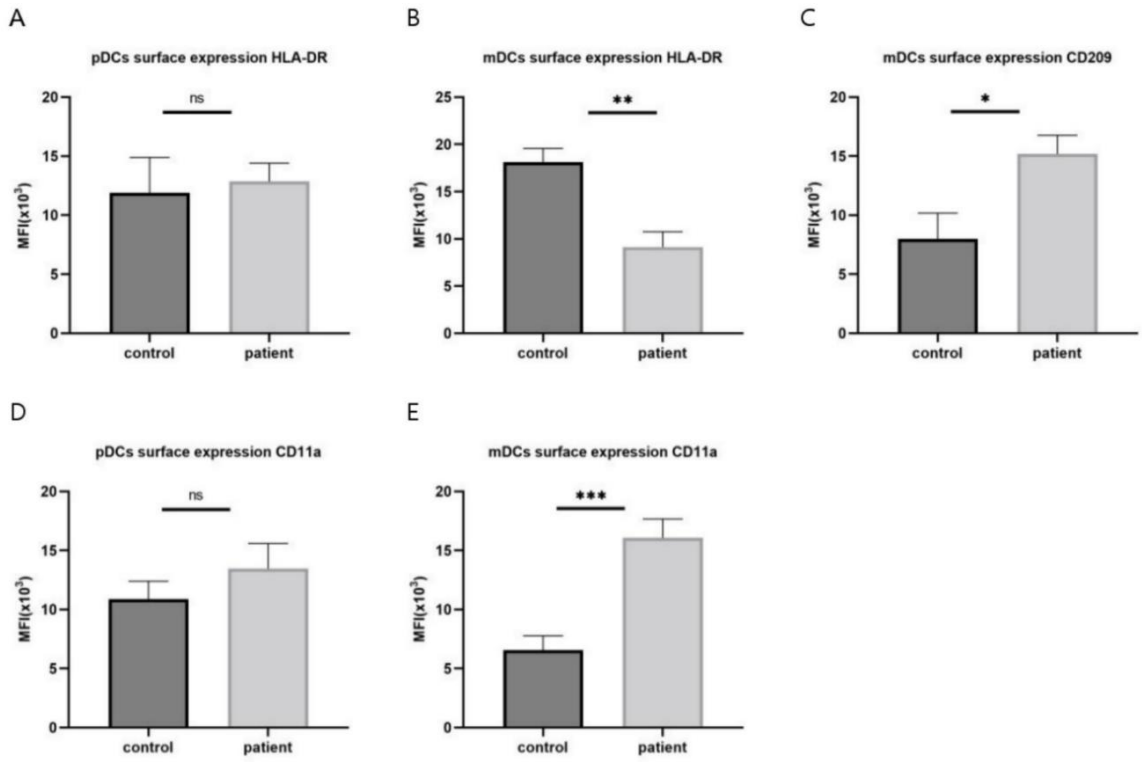

Figure 13. Expression of costimulatory molecules HLA-DR **(A, B)** and adhesion molecule CD11a **(D, E)** on pDCs and mDCs, respectively. Expression of CD209 on mDCs is shown in **(C)**. control, n=9; patient, n=15. Data is presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

4. Discussion

Dendritic cells (DCs) are essential mediators of the innate and adaptive immune systems. They function to induce primary immune responses and facilitate the development of immunological memory. These cells are primarily responsible for antigen capture, migration and T cell stimulation and are therefore referred to as professional antigen presenting cells (APCs).[65] Such immunological responses are involved in the psychophysiology mechanisms of cardiovascular diseases, with DCs shown to play a role in atherosclerosis, hypertension, and heart failure. A better understanding of the interplay between the immune system and cardiovascular diseases will therefore be critical for developing novel therapeutic treatments as well as innovative monitoring tools for disease progression.

Multi-parametric flow cytometry is widely used for phenotyping immune cell populations in human blood samples. However, it takes a lot of efforts to generate an optimal multi-color panel. In this study, we detail the steps that were taken to establish a robust protocol for immunophenotyping from fresh whole blood, using a 11-color cytometry panel. We present the design of cytometry panel used for phenotyping and quantifying major subsets of human blood dendritic cells.

In our study, whole blood staining was preferred to peripheral blood mono-nuclear cells (PBMC) to reduce technical manipulations and the blood sampling needed to perform the tests. Moreover, studies have demonstrated that the Ficoll purification can alter the composition and frequency of leucocyte population and the expression of certain surface markers. Working on whole blood avoids the loss or

reduction or phenotype modifications of some cell subsets (granulocytes,[66,67] dendritic cells,[67] monocyte,[67] lymphocytes[68,69,70]) that happen during PBMC isolation or freezing. These cells can be of major importance and ultimately poorly studied because of their short lifespan after blood sampling (granulocytes) or their absence or very low proportion in PBMCs (granulocytes and dendritic cells, respectively).

Antibody titration is crucial to optimize resolution and to obtain robust results for population identification and expression level measurements for multi-color panels. Titration allows for calculation of SI values and to optimize the concentration of antibodies for the best separation of leukocyte sub-populations in whole blood samples and, where possible, to use less antibody than recommended by the supplier[71] while identifying populations where sub-saturating antibody concentrations do not change the result and can improve population resolution by reducing non-primary detector spillover. In this study, all the antibodies were titrated to determine the best antibody concentration.

Compensation is a necessary component of multi-color flow cytometry experiments. The spillover of emitted fluorescence of individual fluorophores into channels that are not being used to detect that particular fluorophore is necessary in order to properly compensate and thus analyze the flow cytometry data. Antibody capture beads offer many advantages for performing compensation. However, for those antibodies which cannot be proper compensated by beads, we used cells. In our study, we performed the compensation with cells for FITC-Lineage,

PerCP/Cy5.5-HLA-DR, Alexa700-CD45, BV510-CD16 and BV605-CD123 to get proper compensations. We have tested this for each antibody in order to determine whether bead-based compensation is sufficient or whether cell-based compensation needs to be applied.

Usually, it is recommended that a fluorescent viability marker be added to most cell preparations before performing flow cytometry. In our study, we tested the Fixable Viability Dye Zombie Aqua (BioLegend) for the live/dead cells staining. Only a very small number of dead cells (<1%) were detected, as we can exclude most of the dead cells, debris by our gating strategy. Moreover, this viability dye had a lot of spillover into the other channels. Therefore, it was not included in our panel.

Here we developed a 11-color flow cytometry panel for human specimens, capable of characterizing and measuring precisely major blood DC populations. To optimally study DCs in cardiovascular diseases individuals, a panel for simultaneous detection of all cell types is advantageous. Furthermore, a multi-color panel allows the simultaneous phenotypic analysis of different cell types that express the same antigen. This leads to more accurate data and a better understanding of the immune responses during cardiovascular diseases.

The power of multi-parameter flow cytometry lies in the ability to study rare subsets of cells like DCs. DCs are important professional antigen-presenting cells.[38,39,40,41] DCs comprise <1% of total leukocytes and are heterogeneous, thus requiring multi-parameter flow cytometry to correctly identify the DC subsets. Classically, two major different types of DCs and their migratory route have been

described in humans.[58,72] Both types of DCs appear to be in transit in the peripheral circulation as precursor cells before they migrate into peripheral tissues.[41] Significant alteration of circulating DC precursors have been reported in several immune-mediate diseases,[28,73,74] and it is assumed that their reduced presence is mediated by enhanced recruitment into inflamed tissue.[28] In the cardiovascular system in mice, it was demonstrated that DCs are present within the aortic wall and heart valves, and the localization of DCs is mainly in areas with turbulent flow.[45]

More recently, MacDonald et al. distinguished five cell subsets within HLA-DR$^+$ Lin$^-$ cells: HLA-DR$^+$ Lin$^-$ CD123$^+$ pDCs, CD34$^+$ hematopoietic stem cells, and three subsets of CD11c$^+$ mDCs expressing CD16, CD1c (BDCA-1), or CD141 (BDCA-3) similar to what we describe here.[53] However, the authors had to use a cell purification technique to remove all the unwanted cells, prior to the staining. Another technique to ‘‘remove’’ the unwanted cells from the analysis is the use of a lineage cocktail used as exclusion markers for DC (CD3, CD14, CD20, CD56). Often, these exclusion markers are all grouped in one single channel, referred to as a dump channel, and, thus, it is impossible to also identify lymphocyte and monocyte populations.[75,76]

Using a channel for ‘‘removing’’ all the unwanted cells and/or dead cells from the analysis is commonly employed. However, gating out these unwanted cells is difficult and there is usually no clear separation between the unwanted cells and the cells of interest. Furthermore, when the cells of interest are rare populations like DCs, it is important to clearly remove cells that can lead to contamination. With the

availability of measuring more parameters on flow cytometers, it is now possible to use exclusion markers in individual channels and this should be used whenever possible.

Another important advantage of a multi-parameter flow panel over four- or six-color flow panels is the decreased level of contamination among populations. An example for this is a well characterized, both phenotypically and functionally, $CD11c^+$ $CD16^+$ mDC subset.[52] The CD16 molecule is expressed on many cells such as NK cells, monocyte, macrophages, granulocytes, and DCs. It is impossible to gate DCs only based on their size and granularity, as DC overlap with the lymphocyte and monocyte gates. Therefore, it is very easy to misidentify these cells as being NK cells, unless one includes HLA-DR or CD11c in the flow cytometry panel as we have done, or even DC4, as all DCs express low levels of CD4, in contrast to NK cells. Finally, one last advantage of a 11-color flow cytometry panel is the ability to use small volumes of blood to monitor changes in the phenotyping of DC subsets. During longitudinal studies, the volume of blood from patients can be limited. Using one flow cytometry panel with markers for all major blood cell populations is of interest and can save material for other analysis such as cell sorting or in vitro assays.

The 11-color flow cytometry panel has already been implemented in our lab for the clinical studies, and preliminary analyses have already yielded interesting results, confirming the value of our approach.

The multi-color panel designed in our study also has minor limitations. A single cocktail of mAb-fluorochrome conjugates to identify all the DCs allows the identification of all DC sub-populations in a single tube decreasing staining variability due to different cells per tube or mAb-fluorochrome conjugates per tube. However, using a single tube of cells and elimination gates decreases the possibility of counting cells as multiple populations due to expression of multiple antigens. In this study, we used a flow cytometry with, 13 fluorescent parameters. If on a device with more colors, we could probably include a live-dead cells marker as well as individual markers for each lineage antibody to get even better purification of DCs. And we could also include additional markers (such as costimulatory molecule CD86) to assess the activation status of the DC subsets.

By this panel we investigate the numbers of circulating DC subsets, and their surface expressions of molecule markers in patients with severe mitral regurgitation and in healthy adult controls, and we were able to show that, 1) compared to healthy controls, the numbers, both relative numbers and absolute numbers of total circulating DCs, pDCs, mDCs, as well as mDC2 are reduced in MR patients; 2) the phenotype of circulating DCs changed in MR patients, which showed for example the expression of adhesion molecule CD11a and CD209 on mDCs was increased, the expression of HLA-DR on mDC was decreased in MR patients.

Significant alteration of circulating DC precursors has been reported in several immune-mediate diseases,[28,73,74] and it is assumed that their reduced presence is mediated by enhanced recruitment into inflamed tissue.[28] Immune cells take part

in the progression of heart valve dysfunction.[45] In clinical studies, inflammatory response correlates negatively with LV parameters in valve dysfunction related HF.[46,47] Here, we found that the relative number and phenotypes of peripheral circulating DCs precursors changed in MR patients, which provided that DCs might be a vital participator in valvular disease related HF. However, our limitation is the study subjects are too less (only 15 patients). For further studies, we should include more patients, and include different time points (before PMVR, short-term follow up, long-term follow up) to better understand how DCs participate in MR related HF.

In summary, we show in this study that the main players of the immune system, DCs, can be precisely measured using a 11-color multi-parameter flow cytometry approach. This assay is rapid, does not involve a cell isolation technique, and requires a minimum amount of blood. Moreover, this assay is precise with minimal contamination between populations. Most importantly, the 11-color panel is an important tool to study the interactions between different immune cell populations during cardiovascular diseases and potentially other diseases, and to better understand the roles that DCs play in cardiovascular diseases progression. By this panel, we showed that the relative number and phenotypes of peripheral circulating DCs precursors changed in MR patients, which suggests that the change of circulating DC precursors may be involved in MR related HF.

5. Summary

Aim: Dendritic cells (DCs) play an important role during cardiovascular diseases progression; their functions highly depend on the heterogeneous subsets. However, to identify rare cell populations is difficult. Here, we set up a 11-color flow cytometry panel to investigate the number and phenotype of circulating DC subsets in patients with severe mitral regurgitation (MR), to uncover the potential crosstalk of heart failure with DCs. **Methods:** We detailed the steps to set up a multi-color flow cytometry panel. With this, we further investigate circulating DCs in healthy controls (n=9) and MR patients (n=15). **Results:** We clearly distinguished 6 DC subsets and their frequency in leukocytes: pDCs(0.08%), mDCs(0.22%), mDC1(0.01%), mDC2(0.17%), $CD16^{+}$DC(0.12%) and $CD11b^{high}$DC(0.02%). Compared to controls, MR patients had significantly decreased total DCs, pDCs, mDCs and mDC2, both in relative (0.149 vs. 0.352%, P<0.001; 0.017 vs. 0.075%, P<0.001; 0.084 vs. 0.22%, P<0.001; 0.049 vs. 0.173%, P=0.004, respectively) and absolute number (6.037 vs. 11.41/µl, P=0.012 for DCs, 0.747 vs. 2.454/µl, P=0.002 for pDCs, 3.359 vs. 7.097/ul, P=0.001 for mDCs, 1.993 vs. 4.632/ul, P=0.001 for mDC2). Whereas no significant difference regarding mDC1 (0.003 vs. 0.006%, P=0.165 for relative number, 0.108 vs. 0.213/ul, P=0.209 for absolute number). An increase expression of CD209 and CD11a on mDC was detected in patients (mean fluorescence intensity (MFI) $12.62x10^3$ vs. $1.80x10^3$, P=0.014 for CD209; MFI: $203.1x10^3$ vs. $91.0x10^3$, P<0.001 for CD11a). The expression of HLA-DR on mDCs was reduced (MFI: $46.50x10^3$ vs. $69.30x10^3$, P=0.002). No significant differences for CD11a and HLA-DR on pDCs. **Conclusions:** This robust multicolor panel set up here can be used in our further researches, to better understand the roles DCs play in cardiovascular diseases progression. Our finding suggests that the change of circulating DCs may be involved in the pathophysiology of MR relevant HF.

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7. Attachments

Attachment 1 Basic characteristics

basic clinical characteristics

Attachment 2 Antibody titration

antibody titration

Attachment 3 FACS data for healthy controls

FACS data-healthy cont

Attachment 4 FACS data for patients

FACS data-patients

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