Classical monocytes, the non-inflammatory phenotype, display the most robust pro-inflammatory response to LPS *in vitro* after immediate cytometric analysis.

Claudio Karsulovic[1,2,3], Fabian Tempio[3,4] , Mercedes Lopez[3,4], Julia Guerrero[1], Annelise Goecke[1,2]

[1] Laboratorio de Inmunomodulación Neuroendocrina, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile.

[2] Sección de Reumatología, Hospital Clínico Universidad de Chile, Universidad de Chile, Santiago, Chile.

[3] Laboratorio de Regulación e Inmunología del Cáncer, Facultad de Medicina, Universidad de Chile, Santiago, Chile.

[4] Instituto Milenio de Inmunologia e Inmunoterapia, Facultad de Medicina, Universidad de Chile

*** Correspondence:**
Correspondence has to be addressed to Claudio Karsulovic at ckarsulovic@gmail.com or Annelise Goecke at igoecke@hcuch.cl

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Abstract

BACKGROUND: Circulating monocytes have been classified according to expression of certain membrane markers. CD14 (Monocyte identifying Toll-Like Receptor) and CD16 (FcyRIII co-receptor, marker of inflammatory monocytes) were used to define 3 subpopulations of circulating monocytes with different attributes in terms of inflammatory and phagocytic behavior. There are contradictory reports regarding response of circulating monocytes to pro-inflammatory or non-inflammatory stimuli *in vitro*. Here we aimed to analyze the phenotypic changes in circulating monocytes when stimulated with pro and non-inflammatory stimuli.

METHODS: Whole blood from 9 healthy donors was extracted and studied within 90 minutes using erythrocyte lysis protocol. Monocyte subpopulations were directly measured using flow cytometry without PBMC Ficoll extraction method. Pro-inflammatory interleukin IL-1β was measured by intracellular cytometry. Whole blood- extracted monocytes were stimulated using LPS and IL-4 as previously described. Changes against non-stimulated (N-S) populations were statistically analyzed.

RESULTS: Compared to N-S, LPS-stimulated monocytes display a singular behavior, peaking intracellular IL-1β in parallel with CD14+CD163-/CD14+CD163+ ratio. CD163 shows positive correlation with levels of IL-1β. In t-SNE (T-distributed Stochastic Neighbor Embedding) analysis, after LPS stimulation, subpopulation CD14+CD16-CD163-, containing classical monocytes, show a higher number of IL-1β+ cells.

CONCLUSION: Whole blood extracted early monocyte analysis avoids previously described artifacts in membrane protein expression seen when using PBMC Ficoll protocol. Using this method, we were able to show that classical monocytes display the most intense response to LPS. Additionally, CD163 appears to be a suitable addition to CD14-CD16 classification to improve its performance.

Introduction

Circulating monocytes have been classified according expression of certain markers on its membrane. In 2010, an international meeting sponsored by the IUIS (International Union of Immunological Societies) and the WHO (World Health Organization), proposed a subset classification for circulating monocytes (1). CD14 (Monocyte identifying Toll-Like Receptor) and CD16 (FcyRIII co-receptor, marker of inflammatory monocyte) were used to define 3 subpopulations

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of circulating monocytes with different attributes in terms of inflammatory protein expression and phagocytic behavior (2). Classical monocytes CD14++CD16-, accounting 80 to 90% percent of the entire population of monocytes, express low levels of pro-inflammatory proteins and show a more phagocytic behavior than intermediate CD14+CD16+ and Non-classical monocytes CD14+CD16++ (3). These last subsets express higher levels of inflammatory cytokines when stimulated with pro inflammatory stimuli and display lower phagocytic abilities (4).

During the last years, multiple studies have been published regarding a certain expansion or contraction of a single subpopulation with diseases like lupus, rheumatoid arthritis, atherosclerosis or membranous glomerulonephritis (5-7), among others. Even when intermediate subpopulation tends to show expansion in some inflammatory conditions, these results are not consistent between different publications and usually are just mildly significant (8).

In atherosclerosis and its complications like myocardial infarction and stroke, it has been found by some studies that the intermediate subpopulation of monocytes evidently expands (8), but in others only a reduction in classical monocytes without significant changes in intermediate or non-classical monocyte subsets (9). Many studies have covered this point without agreement, showing, until now, conflicting evidence of measurable changes in cell frequencies in clinical settings(10). Given this, in studies using flow cytometry, changes in pro-inflammatory and non-inflammatory protein-ratio have been published as a proxy of a functional switch of circulating monocytes. Trying to emulate the macrophage phenotype classification, pro-inflammatory monocytes were called M1 and non-inflammatory monocytes, M2, according to its singular membrane protein expression (11). Williams et al, showed that circulating monocytes show an augmented M1/M2 ratio in post-stroke patients using CD86 (co-stimulatory T signal, typically described M1 Monocyte/Macrophage phenotype) and CD163 (High-affinity scavenger receptor typically related with M2 Monocyte/Macrophage phenotype) as makers of M1 and M2 respectively (12). In other clinical entities like membranous glomerulopathy, similar results have been found using broader group of markers defining M1 and M2 phenotypes (5).

Until now, many changes have been introduced trying to find which markers are more accurate to define every subpopulation. In a study from Thomas et al using multiparametric analysis, tSNE (t-distributed stochastic neighbor embedding), a certain number of markers were better correlated with a simple flow cytometric definition like CCR2 (CC Chemokine Receptor Type 2), HLA-DR (Human Leukocyte Antigen – DR isotype) and CD163 (10). This last marker could be particularly important because it does not show a clinical correlation with CD16 protein which defines current monocyte classification (13).

In vitro, there are contradictory reports in terms of response of circulating monocytes to pro-inflammatory or non-inflammatory stimuli. Mukerjhee et al. showed that monocytes from healthy donors do not display cell number expansion or contraction in any subpopulation in response to LPS. Also, intermediate and non-classical monocytes express higher levels of IL-1β and TNF-α, but an intermediate group responds with higher levels of IL-10 to the same stimulus (7). Subsequent studies have shown that M1 monocytes express higher levels of IL-6 when stimulated with LPS (11).

Given the lack of a solid answer regarding which classification strategy is better, and the behavior of cultured monocytes to LPS, we analyzed monocytes from nine healthy donors and stimulated them with LPS and IL-4 describing a singular cytokine production and phenotypic pattern. We show that less-reactive-non-inflammatory classical monocyte subpopulation has the most robust response to *in vitro* stimulus with LPS. This subpopulation also could be better classified using other membrane

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markers as CD163. Classical monocytes show also a more "flexible" M1/M2 ratio, compared to intermediate and non-classical monocytes, becoming the subpopulation with the highest functional plasticity.

Material and Methods

2.1. *Healthy donors.* We recruited 9 healthy donors ranging from 26 to 40 years, 5 man and 4 women, from our facility. A medical anamnesis was performed by an internal medicine specialist before whole blood extraction. No medication use nor relevant past medical history were reported. The ethics committee of Facultad de Medicina de la Universidad de Chile approved the study protocol.

2.2. *Whole blood lysis protocol and flow cytometry.* Venous blood (30ml) was obtained by cubital venopunction from all participants. Pre-cytometry whole blood lysis protocol was performed 1-hour maximum post blood extraction. 1% Fc-Block (BD Biosciencies) was first used. 20µl of WB were stained in duplicate with the following antibodies: FITC-A-anti-CD14, APC-H7-anti CD16, PERCP-Cy5.5-anti-CD86, APC-A-anti-CD16, V450-A-anti-IL-1β (Biolegend, US) at room temperature for 30 minutes. 1ml of BD FACS Lysing Solution (BD Biosciences) at 1x concentration was used for erythrocyte lysis. Finally cells we fixed and permeabilized using a fixation/permeabilization BD Kit (BD Biosciences).

2.3. *LPS and IL-4 Stimulation and flow cytometry.* To phenotype monocyte subsets, 1×10^6/cells per tube were stimulated in duplicate with lipopolysaccharide, (1 µg/mL) (BD Biosciences, NY) and IL-4 (200 U/ml) (BD Biosciences, NY) in RPMI 1640 (BD Biosciences, NY) medium mixed with 10% fetal bovine serum (BD Biosciences, NY) for 2 h and 4 h respectively at 37°C in 5% CO2 and exposed to brefeldin A (BD Biosciences) as described in previous studies. Then, the cells were washed, followed by staining with V710-anti-CD3 (Biolegend, US), V650-anti-CD19, (Biolegend, US), FITC-A-anti-CD14, APC-H7-anti CD16, PERCP-Cy5.5-anti-CD86, APC-A-anti-CD16, (Biolegend, US). Subsequently, they were fixed and permeabilized and stained with V450-A-anti-IL-1β (Biolegend, US). The frequencies monocyte subsets were assessed on a FACS Verse instrument (BD, Franklin Lakes, US), and the data was analyzed with FlowJo software (v7.6.1; TreeStar; Ashland, US).

2.4. *Statistical Analysis.* Variables were shown as median and range values. We employed the Mann–Whitney U nonparametric test to evaluate the differences among groups. The relationship between variables was analyzed by the Spearman rank correlation test. All of the data were carried out with the GraphPad Prism version 6.01 software. P value of <0.05 represented statistical significance.

Results

CD163 marker and no CD16, select cells that respond to LPS. Monocytes where gated using CD14 and CD16 for usual three-subsets classification (Fig1, C.1.). Also, monocytes where classified with CD14 and CD163, given that the last one, has been associated with different phenotypes. Monocytes show an almost 50-50% distribution regarding CD163 positivity in N-S condition (Fig1, C.2). When recently extracted monocytes are stimulated with 1ug/ml LPS and 200UI IL-4, no differences in cell count are seen (Fig1, D). When these same monocytes are separated in 3 subsets, relative percentages do not change with inflammatory or non-inflammatory stimulation (Fig1, E). If these monocytes where initially classified using CD163

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marker, after LPS stimulation, CD163- monocytes reach approx. 99% of cells, phenomenon not seen on non-stimulated or IL-4 stimulated monocytes (Fig1. F).

All monocytes raise IL-1b after LPS stimulation, but only classical monocytes show changes in CD163 marker. Classical, intermediate and non-classical monocytes show higher levels of IL-1b after LPS stimulation, but in classical subset is significantly higher (Fig2, A, B). As we already mentioned, CD163- cells raise after stimulation with LPS. When every subset is analyzed in CD14+CD163-/CD14+CD163+ ratio, only classical monocytes show this raise, showing that changes we see in CD14+ monocytes depends entirely on classical monocytes response (Fig2. C-F).

Only CD163 and no CD16, show correlation with IL-1b after LPS stimulation. When we analyzed separately positivity to either CD163 or CD16 and levels of IL-1b after LPS stimulation, only CD163 negativity adjust to peaks of IL-1b in all subsets (Fig3, A). After correlation tests, again only CD163 show positive and significant correlation with MFI IL-1b after LPS stimulation (Fig3, B, C). No correlation is seen between CD16 and IL-1b (Fig3, D, E).

CD14+CD163- inflammatory subset rather CD14+CD16+ subset, respond to LPS stimulation raising intracellular IL-1b. Using t-SNE multiparametric reduction (of CD3, CD19, CD14, CD16, CD163, CD86 markers), we were able to reduce al monocytes to two clouds of cells, CD14+CD16+ and CD14+ CD163- which interestingly, where mutually exclusive (Fig4, A). When stimulated with LPS, IL-1b+ monocytes invade CD163- cells without significantly grow on CD16+ cloud, showing that CD163- monocytes are especially responsive (Fig4, B, C).

Discussion

During the last years, numerous studies have tried to find an association between certain monocyte subpopulations and prevalent inflammatory and metabolic diseases. Autoimmune entities and atherosclerosis as a metabolic dysregulation are the most frequently studied, nevertheless, their correlation with expansion or contraction of these subpopulations has been difficult to study and not completely reproducible. Many reasons for the latter have been proposed. One of the most frequently argued explanation is how difficult is to separate 3 “different” subpopulations using just a small group of markers. Many times, depending on the inflammatory status of the patient, the first selection criteria in the gating process, as is the FSC (granularity) in flow cytometry, could represent a challenge trying to gate only monocytes out of a “universe” of neutrophils.

Thomas et al. were able to demonstrate using tSNE with more than 20 different parameters, that a group of at least 12 markers commonly available, must be used to perform positive and negative selection in order to obtain a proper separation (14). Even when these markers help to better classify circulating monocytes, it is yet expensive and time consuming to process it.

When these monocyte subpopulations are analyzed *in vitro*, its response to stimuli turns more erratic because PBMC Ficoll separation technique has been reported as a possible source of artifacts in membrane protein expression. Even considering this, most of the studies available, are prone to point to intermediate monocyte subpopulation as the most “plastic” and responsive to LPS.

In our experience, with using monocytes from whole blood and analyzing them immediately, we were able to show that non-inflammatory classical monocytes constitute the most responsive subpopulation. Classical monocytes uniquely raise levels of intracellular IL-1b. Also, when these monocytes are reclassified using CD163, CD163- cells are the most responsive. When compared

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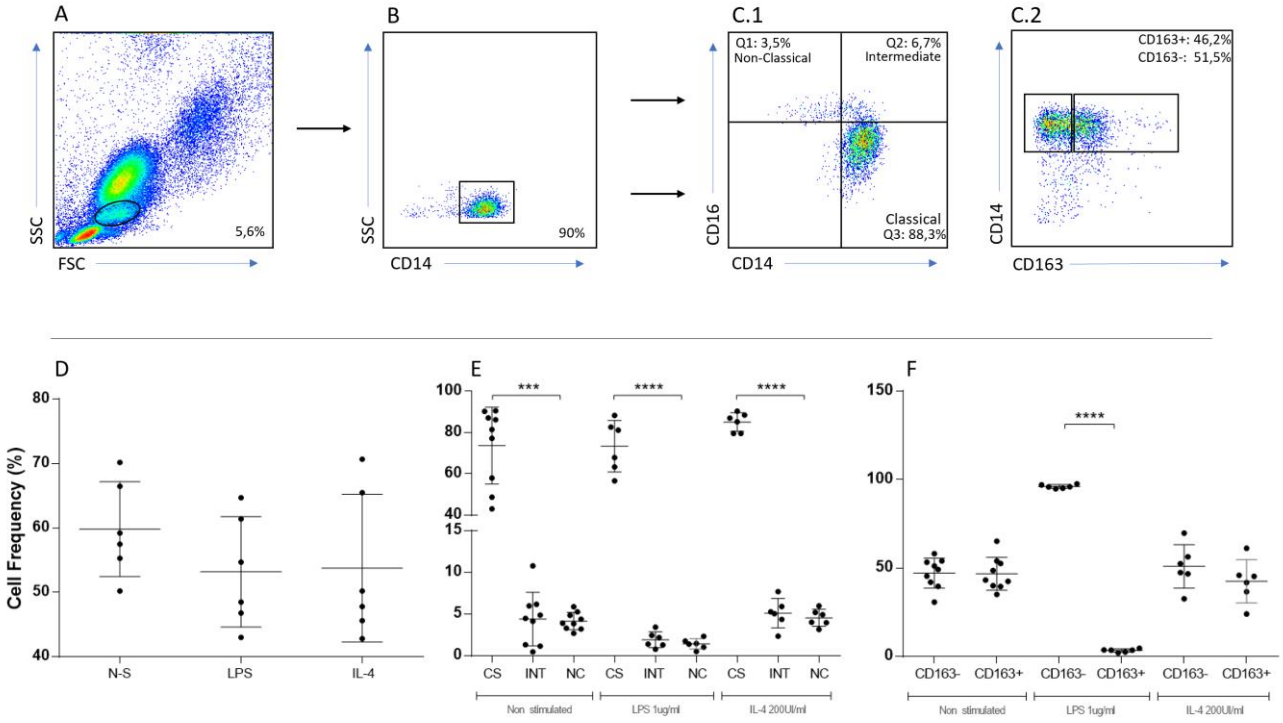
CD16 marker and CD163 marker, the last is the one who better correlate with intracellular levels of IL-1b. Classical monocytes subset, a priori classified as less-responsive/non-inflammatory subset, contains almost 100% of high responsive CD163- cells.

We were also able to show that, in the way of a Russian *matryoshka*, classical monocytes display an intern behavior with different grades of responsiveness depending at least in part, on CD163- cells number. These findings rise the question about if we are properly classifying monocytes subsets using a bidimensional analysis. More studies are needed to understand if the most responsive subpopulation CD14+CD163- displays different sub-phenotypes with also different grades of response to LPS and other stimuli using more markers or multiparametric analysis. How these sub-phenotypes behave in clinical scenarios remain a question to be looked into.

Figures

FIGURE 1.



Gating strategy, flow cytometry analysis of different monocytes subpopulations and cell count on each condition. Whole blood from healthy donors was stained with anti-CD14, anti-CD16, anti-CD86 and anti-CD163. The cells were gated firstly on mononuclear cells (A) and secondly on CD14+ monocytes (B). Afterwards, the count of CD14++CD16-, CD14+CD16+ and CD14+CD16++ were determined by flow cytometry. In C.1, Widely used quadrant separation. In C.2, CD163+ and CD163 – cell count. Percentage of monocytes are the same in non-stimulated and stimulated conditions (D). Classical monocytes are significantly higher than intermediate and non-classical monocytes (E) ****p≤0,0001; ***p≤0,001. Using CD163 as marker, LPS-stimulated monocytes drastically separate in two different subpopulations (F).

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FIGURE 2.

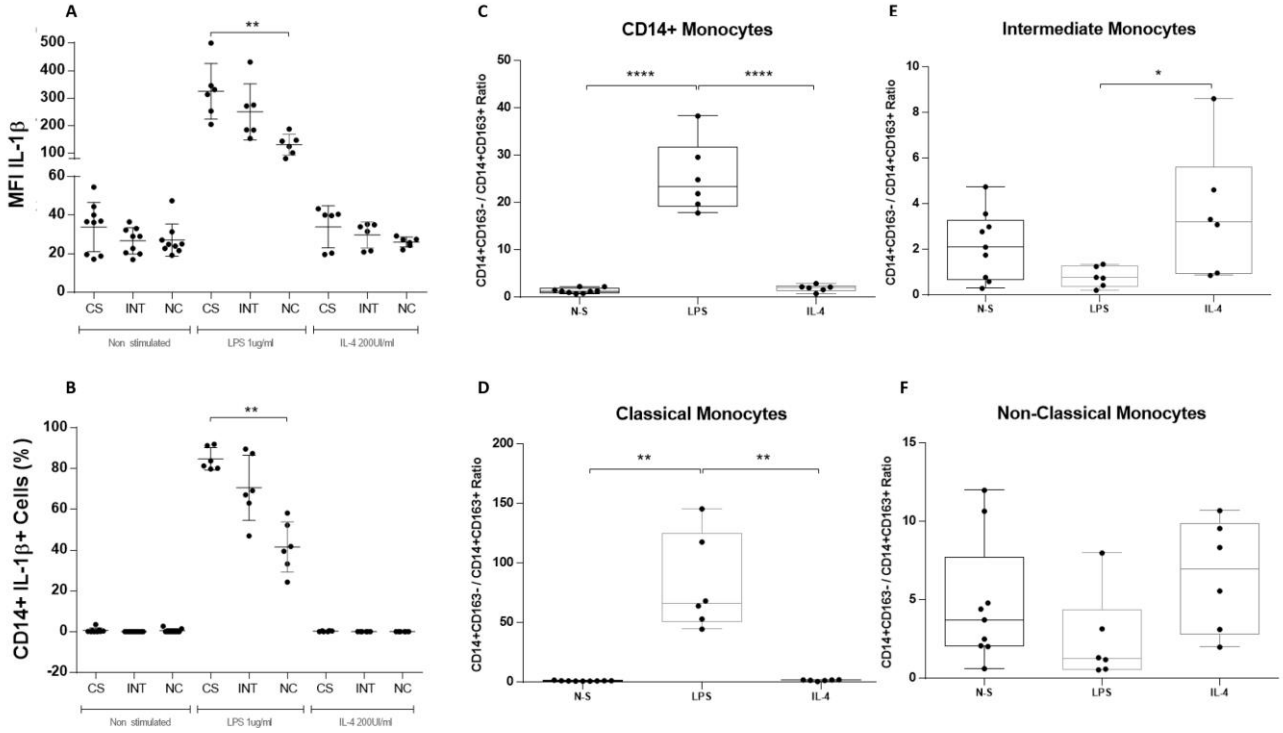

Figure 2: IL-1b and CD63 in N-S, LPS and IL-4 conditions. MFI and IL-1b+ cells are significantly higher in LPS stimulated classical monocytes (A, B). When CD163-/CD163+ ratio is analyzed, CD14+ total monocytes show significantly higher ration when stimulated with LPS (C) ****p≤0,0001. When separated in classical, intermediate and non-classical monocytes, are only the first which respond to LPS (D, E, F) **p≤0,008.

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FIGURE 3.

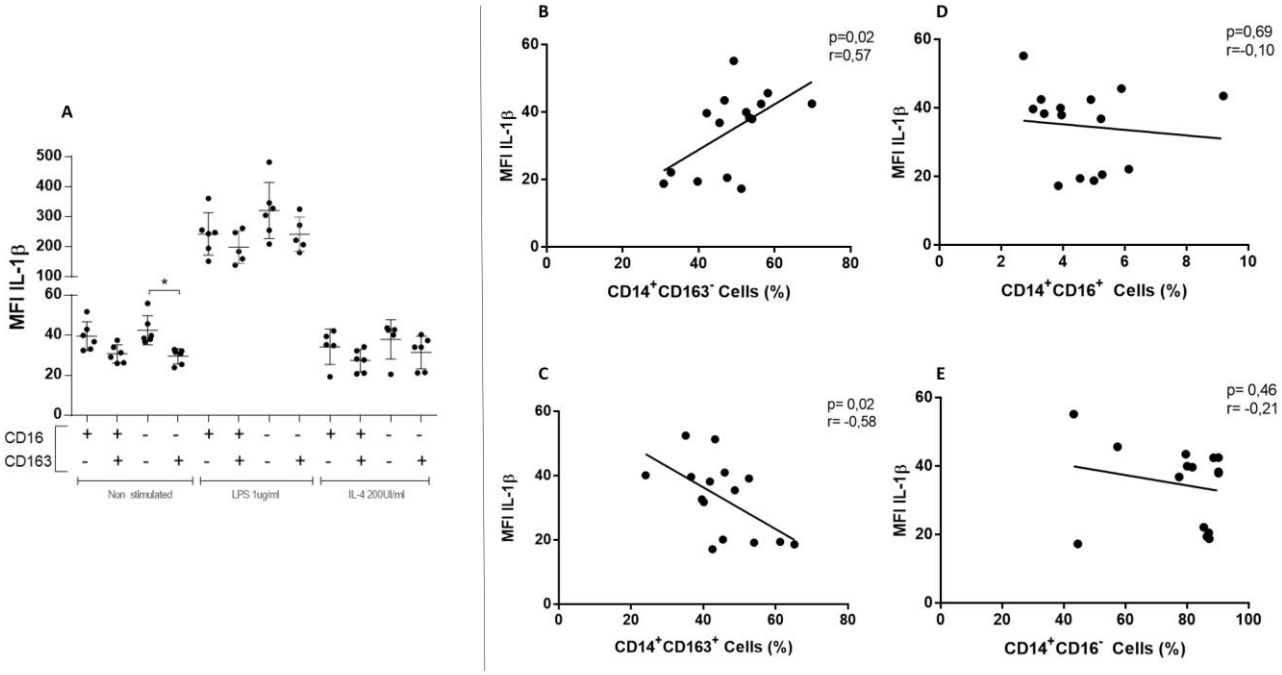

Figure 3: IL-1b MFI correlation with CD16 and CD163 markers. Relation between levels of IL-1b and CD16-CD163 positivity/negativity in each stimulation condition. In A, positivity for CD16 marker, did not show relation with levels of IL-1b. On the contrary, negativity for CD163 is present every time IL-1b peaks, been significant in N-S condition *p≤0,02. Correlation between CD163 and CD16 cells and levels of intracellular IL-1b. CD14+CD163- cells correlate positive and significantly with levels of IL-1b, on the contrary CD163+ have negative correlation (B,C). CD16+ o CD16- cells did not correlate significantly with levels of intracellular IL-1b (D,E).

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FIGURE 4.

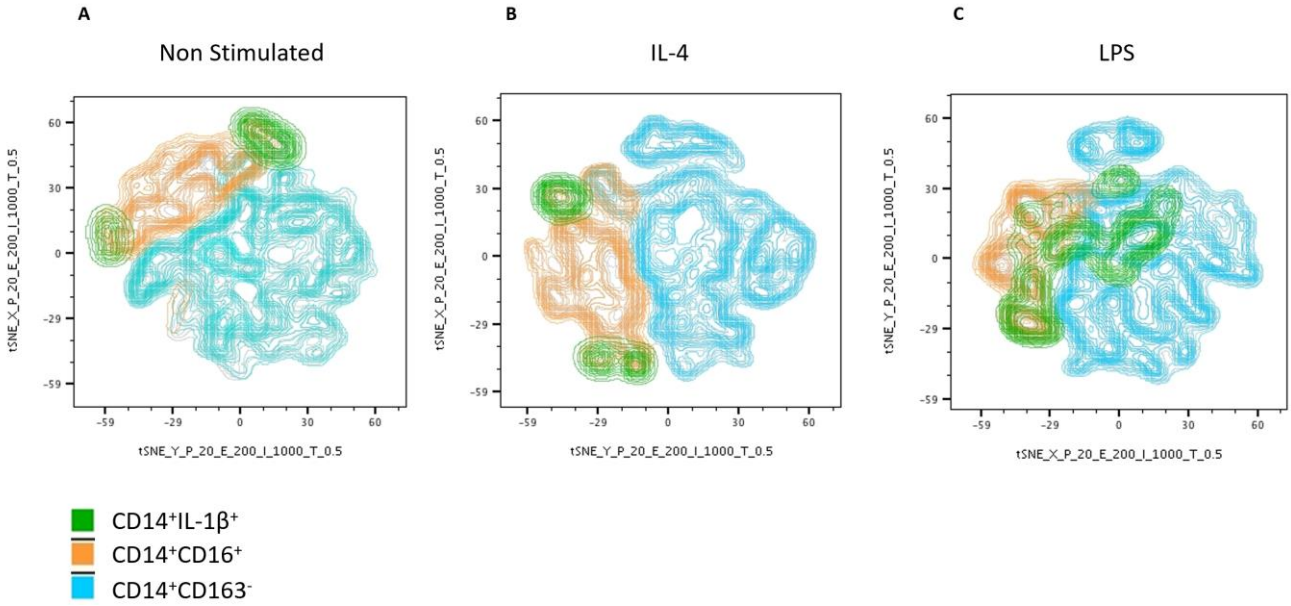

Figure 4: t-SNE analysis for CD14+ monocytes using multiple markers. Orange colored cloud of cells represents CD16+ cells, light blue cloud CD163- cells and green cloud IL-1b+ cells, being the first two, "inflammatory" subpopulation according most used classifications. As is easily seen, CD16+ and CD163- cells are completely different subpopulations (A). In IL-4 stimulation condition, no changes are seen in green cloud, which is comparable to N-S condition (B). In C, after LPS stimulation, green cloud "invades" CD16-CD163- conforming a new subpopulation CD14+CD16-CD163-IL-1b+ (C).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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