



Monocytes of patients with unstable angina express high levels of chemokine and pattern-recognition receptors

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ABSTRACT

Background and aims: Macrophages derived from monocytes play an important role in atherosclerosis progression. Subpopulations of circulating classical, intermediate, and non-classical monocytes possess distinct functions and phenotypes, and participate in the pathogenesis of disease. The aim of this study was to compare the quantity and phenotypes of circulating monocyte subpopulations in patients with established atherosclerosis and healthy control individuals. Additionally, the study aimed to provide insight into the functional activity of monocytes against a heat shock protein (HSP60).

Methods: Chemokine and pattern recognition receptors in monocyte subsets obtained from peripheral blood of acute and chronic coronary artery disease patients and controls were quantified by flow cytometry. Furthermore, monocytes from healthy controls were stimulated *in vitro* with HSP60, and the cytokines produced by them were evaluated by flow cytometry.

Results: Eighteen controls (C), 34 individuals with risk factors for cardiovascular disease (RF), 32 patients with stable angina (SA), and 16 patients with unstable angina (UA) were enrolled in the study. The absolute count of intermediate monocytes was found to be increased in patients of the UA group; high frequencies of the chemokine receptors CCR2, CCR5, and CX3CR1 were also observed in this subpopulation. Moreover, the pattern recognition receptors TLR2 and TLR4 were more frequent in intermediate monocytes from the UA group. Furthermore, the intermediate monocytes from healthy individuals produced IL-12p70 after stimulation with HSP60.

Conclusions: Our results show that intermediate monocytes of UA patients exhibited an enhanced expression of the receptors involved in the recognition of damage-associated molecular patterns (DAMPs) and enhancement of the migratory function. Hence, they might contribute to the propagation and progression of inflammation observed in atherosclerosis, especially in the acute setting.

1. Introduction

Atherosclerosis is a chronic inflammatory process involved in many cardiovascular diseases such as stroke, ischemic heart disease, peripheral arterial disease, and acute coronary syndrome (ACS) [1]. Despite the decline in mortality, these diseases still represent the most frequent cause of death worldwide [2].

The most accepted initial trigger to atherosclerotic lesion

development is low density lipoprotein (LDL) cholesterol infiltration, accumulation, and modification at susceptible sites in the arterial intima [3]. Heat shock protein 60 (HSP60) has also been implicated in the development of atheroma [4]. This endogenous molecule, which is highly released under stress conditions, can act as a DAMP and trigger both innate and adaptive response in the initial stages of the disease [5]. The classical risk factors for atherosclerosis accelerate the generation of modified LDL and HSP60, which promote the activation of

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the endothelium, resulting in the migration of leukocytes to the arterial intima [4,6].

Monocytes and macrophages are not only some of the cell groups involved in the formation and development of atherosclerotic lesions but are also considered to be central drivers for vascular inflammation in atherosclerosis.

Human circulating monocytes represent a heterogeneous population of cells, grouped based on the expression of the surface receptors CD14 and CD16 [7]. Beyond the differences in these surface markers, the classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺), and non-classical (CD14⁺CD16⁺⁺) monocytes differ with regard to the expression and functioning of other receptors, such as chemokine receptors [8,9]. While classical monocytes are involved in phagocytosis and antimicrobial protein production, and express high levels of CCR2, intermediate monocytes are involved in antigen presentation, and they express high levels of CCR5; the non-classical monocytes are involved in endothelial cell patrolling and express high levels of CX3CR1 [10]. It has been shown that the risk factors associated with atherosclerosis development, such as hypercholesterolemia, obesity, and insulin resistance, are associated with an increased level of circulating CD16⁺ monocytes [11–13]. Furthermore, it seems that the elevated CD14⁺⁺CD16⁻ monocyte count was useful for predicting cardiovascular events in individuals with coronary arterial disease independently of the other risk factors [14]. Alterations in monocyte subpopulations were also observed after acute myocardial infarction, along with an initial increase in the classical monocyte population within 3 or 4 days, followed by an elevation in CD16⁺ populations [15]. However, few studies have shown a well-established phenotype of monocyte subsets in different stages of coronary arterial disease (CAD).

The aim of this study was to provide a detailed phenotypic comparison of the different monocyte subsets and their expressed receptors that are involved in inflammation and angiogenesis in patients with established CAD (both stable and unstable angina) and healthy individuals (with or without cardiovascular risk factors). Additionally, we provided an insight into subsets of monocytes showing a functional activity against HSP60, a widely accepted atheroma antigen.

2. Materials and methods

Participants were enrolled at the Cardiology Unit of the Hospital de Clinicas at the State University of Campinas (UNICAMP) and the Samaritano Hospital of Campinas, Sao Paulo, Brazil, from March 2014 to March 2016. The local Ethics Committee approved the study, which followed the current guidelines; each participant gave informed consent. Participants were divided into four groups: 1) healthy individuals without CAD or risk factor(s) for atherosclerosis (control group – C, n = 18), selected from volunteers in the outpatient clinic of Hospital de Clinicas; 2) individuals without established CAD, but having at least one risk factor for atherosclerosis, such as hypertension, diabetes, dyslipidemia, and smoking (risk factor group – RF, n = 34), selected in the primary care outpatient clinic of Hospital de Clinicas; 3) patients with stable angina and chronic CAD, documented by cardiac catheterization (stable angina – SA, n = 32), selected in the cardiology outpatient clinic of Hospital de Clinicas; and 4) patients with acute coronary syndrome, characterized by non-ST-segment elevation, no increase in troponin levels, and initial symptoms within 24 h of blood collection (unstable angina – UA, n = 16), selected from hospitalized patients in the coronary care unit of both hospitals. Assessment of the inclusion criteria was done by one of the authors of the study by reviewing each patient's records prior to consultation and then by an objective interview. For categorization into each group, the following definitions were used: hypertension was defined as a resting blood pressure $\geq 140 \times 90$ mmHg or the use of anti-hypertensive medications; dyslipidemia was characterized by and LDL ≥ 130 mg/dL or the use of cholesterol-lowering medication; diabetes a fasting glucose ≥ 126 mg/dL, glycated hemoglobin $> 6.5\%$ or the use of hypoglycemic agents;

stable angina defined by coronary angiography findings of current or previous coronary lesions $> 50\%$ diameter stenosis; unstable angina symptoms of typical chest pain with high probability of coronary artery disease, with or without accompanying symptoms of sweating, fatigue, dizziness or nausea.

Exclusion criteria included pregnancy, valvular heart disease, trauma, or surgery in the last 4 weeks, a history of acute or chronic inflammatory disease, or the use of immunosuppressants, neoplasia, congestive cardiac failure, and chronic kidney disease. These conditions were actively searched for exclusion during interviews and through review of the past medical records.

The sample size was calculated based on the study of Kashiwagi et al. [16] which showed the proportion of CD14⁺⁺CD16⁺ cells in controls, SA and UA and Myocardial infarction patients as 12%, 18%, 10%, and 8% respectively. We estimated 20 subjects in each group that would allow us to detect a relative difference of 10% in the amount of the cells among the groups with a power of 0.8 and alpha error of 0.05 (Hintze, J 2011. PASS 11. NCSS, LLC. Kaysville, EUA).

2.1. Blood sampling

Blood samples (9 mL) from the participants were collected in heparinized tubes, and red blood cells were lysed after incubation of 500 μ L of blood with 10 mL of lysis buffer (NH₄Cl 155 mM, KHCO₃ 10 mM and EDTA 1 mM) for 10 min at room temperature. This was followed by centrifugation at 2500 rpm for 10 min at 4 °C. After washing twice in PBS (phosphate buffered saline), the leukocytes were resuspended in PBS-BSA-A (PBS-BSA 0.1%; sodium azide 0.2 mM) and submitted to phenotypic characterization by flow cytometry. Plasma samples were stored at -20 °C for C-reactive protein (CRP) measurement.

2.2. Flow cytometry

Surface staining of the leukocytes was performed using the following labeled antibodies: anti-CD14 (PercPCy5.5), anti-CD16 (APC), anti-CX3CR1 (FITC), anti-SLAM (CD150) (FITC), anti-CD36 (FITC), anti-TLR2 (FITC), anti-CCR2 (PE), anti-Tie2 (CD202b) (PE), anti-CCR5 (PE), and anti-TLR4 (PE) (all from Biolegend, San Diego, CA, USA). The antibodies were diluted in PBS-BSA-A and incubated with the leukocytes for 30 min in the dark at 4 °C. The cells were then washed twice with PBS-BSA-A, fixed in 2% formaldehyde, and stored at 4 °C. Before acquisition, AccuCheck Counting Beads (LifeTechnology, Frederick, MD, USA) were added to each tube, according to the manufacturer's instructions. The cells were acquired on a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA), and analysis was performed using FCS Express Software (DeNovo Software, Glendale, CA, USA).

2.3. Monocyte isolation and stimulation in culture

Venous blood (90–100 mL) was collected from healthy donors into heparinized tubes. Peripheral blood mononuclear cells (PBMCs) were separated using the Ficoll-Hypaque density gradient according to the manufacturer's instructions (GE Healthcare Piscataway, NJ, USA). The monocytes were isolated from PBMCs using a positive isolation kit (MACs; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Monocytes were cultured at a concentration of 1×10^6 /mL in 6-well plates in RPMI 1640 medium (Gibco, Frederick, MD, USA) supplemented with 2 mM glutamine (Sigma Aldrich, St Louis, MO, USA), 5 μ g/mL gentamycin (Sigma Aldrich), and 10% fetal bovine serum (Gibco). Monocytes were stimulated with 5 μ g/ml of HSP60 (Sigma Aldrich) and positive control (100 ng/mL LPS, Sigma Aldrich), and incubated for 18 h at 37 °C in 5% CO₂. Cells were incubated with 10 μ g/ml of Brefeldin A (Sigma Aldrich) for another 6 h.

After stimulation, monocytes were collected and subjected to surface staining with the specific labeled antibodies anti-CD14 (PercPCy5.5) and anti-CD16 (APC) (All from Biolegend). After surface

staining, cells were fixed in 2% formaldehyde, permeabilized with saponin (0.5% in PBS) and stained for detecting IL-1 β (FITC), IL-8 (FITC), IL-10 (Alexa 488), IFN- γ (FITC), IL-6 (PE), MCP-1 (PE), TNF- α (PE), and IL-12p70 (PE) (all from Biolegend) and fixed in 2% formaldehyde. Cells were acquired on a FACSCalibur flow cytometer (BD Bioscience) and analysis was performed with FCS Express Software (DeNovo Software).

2.4. Measurement of C-reactive protein (CRP)

Quantification of plasma CRP levels was done using a highly sensitive latex-enhanced immunonephelometric assay (BN ProSpec System, Siemens, Erlanger, Germany). The limit of detection of the test was 0–175 mg/L and the coefficient of variation was < 5%.

2.5. Statistical analysis

Continuous data are presented as mean (standard deviation) for normal data or median [interquartile range] for non-normal data. One-way ANOVA was followed by Bonferroni *post hoc* test or Kruskal–Wallis test, which was followed by the Mann-Whitney *post hoc* test for the analyses of normally and non-normally distributed variables. Chi-square was used for the analyses of categorical variables. All p-values for intergroup comparisons between non-normal and categorical variables were Bonferroni-corrected. For the *in vitro* experiments, the normal data were analyzed by the one-way ANOVA test for repeated measurements, followed by the Tukey post-test for multiple comparisons. The statistical analyses were performed using Stata version 14.1 (Stata Corp, College Station, TX). A p-value of < 0.05 was considered statistically significant.

3. Results

Table 1. shows the demographic and clinical characteristics of the study group. While age and gender did not differ significantly, the body mass index (BMI) in the RF group was higher than that in the C group.

Table 1
Demographics and clinical characteristics of study groups.

	C	RF	SA	UA	p value
Patients n	18	34	32	16	
Age (years, mean \pm SD)	56.4 \pm 9.3	59.1 \pm 8.3	61.1 \pm 8.9	58.7 \pm 11.6	0.41
Sex (M/F)	7/11	20/14	23/9	11/5	0.12
BMI	25.6 \pm 4.1	29.7 \pm 5.8 ^a	28.8 \pm 4.5	26.5 \pm 6.1	0.034
<i>Cardiovascular risk factors n (%)</i>					
Hypertension	0	21 (62) ^a	27 (87) ^a	11 (69) ^a	< 0.001
Diabetes	0	6 (18)	14 (45) ^a	5 (31)	0.003
Smoking	0	4 (12)	5 (16)	10 (63) ^{a,b,c}	< 0.001
Dyslipidemia	0	32 (94) ^a	29 (91) ^a	15 (94) ^a	< 0.001
<i>Medication n (%)</i>					
Antihypertensive	0	18 (60) ^a	31 (97) ^{a,b}	11 (73) ^a	< 0.001
Cholesterol-lowering	0	17 (57) ^a	26 (81) ^a	9 (60) ^a	< 0.001
<i>Biochemical parameters</i>					
LDL (mg/dL)	105.8 \pm 20.1	107.2 \pm 36.6	92.4 \pm 34.0	103.9 \pm 31.8	0.29
Triglycerides (mg/dL)	100 [75, 126]	141 [107, 185] ^a	129 [99, 185]	150 [90, 242]	0.036
HDL (mg/dL)	58.0 \pm 10.1	52.0 \pm 17.0	51.3 \pm 26.8	37.1 \pm 9.9 ^a	0.015
Triglycerides/HDL	1.78 [1.21, 2.42]	3.00 [1.82, 4.36] ^a	2.80 [1.65, 4.13]	4.15 [2.52, 7.10] ^a	0.003
Glucose (mg/dL)	85 [82, 87]	102 [93, 117] ^a	114 [96, 140] ^a	87 [77, 138]	< 0.001
CRP (mg/L)	1.30 [0.90, 2.30]	1.95 [0.90, 4.35]	0.94 [0.50, 2.58]	8.75 [2.58, 15.55] ^{a,c}	< 0.001

Continuous data are presented as mean (standard deviation) for normal data or as median [interquartile range] for non-normal data. One-way ANOVA was followed by Bonferroni *post hoc* test or Kruskal–Wallis test, which was followed by Mann-Whitney *post hoc* test for the analyses of normally and non-normally distributed variables. Chi-square was used for the analyses of categorical variables. All p-values for intergroup comparisons of non-normal and categorical variables have been Bonferroni-corrected.

C = controls; RF = risk factor; SA = stable angina; UA = unstable angina; BMI = body mass index; HDL = high density lipoprotein; LDL = low density lipoprotein; CRP = C reactive protein.

^a p < 0.05 versus C.

^b p < 0.05 versus RF.

^c p < 0.05 versus SA.

Hypertension and dyslipidemia were significantly higher in the RF, SA, and UA groups than that in the C group. There were a predominantly higher number of smokers in UA group than in the others, while the number of individuals with diabetes was higher in the SA group than that in the C group.

The use of antihypertensive drugs was higher in RF, SA, and UA patients when compared to the C group, and it was higher in the SA group when compared to the RF group. The number of individuals using cholesterol-lowering medication was higher in the RF, SA, and UA groups than that in the C group, which might explain the similarity in LDL levels among these groups.

The individuals in the UA group had lower levels of HDL than those in the C group. The levels of C reactive protein (CRP) were higher in individuals in the UA when compared to those in the C and SA groups (Table 1).

3.1. Total monocyte count

The absolute number of intermediate monocytes was higher in patients with UA than that in individuals in the RF and SA groups. The UA group also presented higher absolute counts of classical and total monocytes compared to those of the SA group. On the other hand, the absolute number of classical, intermediate, and total monocytes was lower in the SA group as compared to that in the C group. The frequency of monocyte subsets did not differ among the groups. Surprisingly, the frequency and absolute number of all monocyte subsets did not differ between the UA and C groups (Table 2).

3.2. Chemokine receptors

CCR2 was more frequently expressed in classical and intermediate monocytes, in which almost 100% of positive cells. However, even with this high frequency, it was possible to detect a significant difference between CCR2⁺ cells of the intermediate population of UA patients and those in the RF and SA groups. The non-classical monocytes exhibited a

Table 2
Characteristics of monocyte subsets in study groups.

	C	RF	SA	UA	p value
Patients n	18	34	32	16	
Total monocytes/ μL #	365 [320, 586]	311 [163, 397]	195 [137, 269] ^a	394 [309, 501] ^c	< 0.001
Classical/μL #	297 [240, 468]	264 [137, 328]	151 [102, 217] ^a	315 [207, 401] ^c	< 0.001
Intermediate/μL #	19 [14, 27]	13 [9, 23]	10 [6, 16] ^a	24 [16, 29] ^{b,c}	< 0.001
Non-Classical/μL #	44 [29, 68]	41 [19, 63]	32 [21, 46]	56 [31, 76]	0.08
Classical – %	84 [82, 87]	80 [76, 85]	78 [74, 83]	78 [76, 84]	0.08
Intermediate – %	4 [4, 6]	5 [4, 7]	5 [4, 6]	6 [5, 10]	0.19
Non-Classical – %	11 [9, 13]	15 [11, 19]	17 [13, 19]	14 [10, 18]	0.07

Data are presented in terms of the median [interquartile range]. The Kruskal–Wallis test was followed by the Mann-Whitney *post hoc* test for all analyses. All p-values for intergroup comparisons have been Bonferroni-corrected.

#: absolute number; %: Frequency; C: controls; RF: risk factor; SA: stable angina patient; UA: unstable angina patient.

^a p < 0.05 versus C.

^b p < 0.05 versus RF.

^c p < 0.05 versus SA.

lower frequency of CCR2⁺ cells in all groups (median: 34–83%), but again, the UA patients exhibited a higher frequency of CCR2⁺ cells when compared to the C, RF, and SA groups (Table 3).

The CCR5 receptor was more frequently expressed in classical and intermediate monocytes of UA patients than in C, RF, and SA group

Table 3
Characteristics of monocyte markers in the study groups.

	C	RF	SA	UA	P value
Patients n	18	34	32	16	
Chemokine receptors					
CCR2 Classical	100	100	100	100	0.20
CCR2 Intermediate	100 [99, 100]	99 [98, 100]	99 [98, 100]	100 ^{b,c}	0.001
CCR2 Non-classical	44 [30, 53]	43 [26, 58]	34 [23, 45]	83 [54, 94] ^{a,b,c}	< 0.001
CCR5 Classical	28 [4, 100]	80 [10, 100]	62 [18, 96]	100 ^{a,b,c}	< 0.001
CCR5 Intermediate	68 [14, 100]	94 [28, 100]	84 [51, 99]	100 ^{a,b,c}	< 0.001
CCR5 Non-classical	7 [1, 73]	50 [4, 74]	40 [17, 64]	93 [75, 98] ^{a,b,c}	< 0.001
CX3CR1 Classical	25 [17, 96]	37 [21, 86]	26 [9, 41]	90 [58, 96] ^c	0.002
CX3CR1 Intermediate	72 [52, 100]	93 [62, 100]	86 [69, 96]	99 [99, 100] ^{b,c}	0.005
CX3CR1 Non-classical	45 [28, 97]	93 [32, 99]	59 [30, 95]	99 [97, 100] ^{a,c}	0.001
Activation and phagocytic receptors					
TLR2 Classical	11 [3, 5]	17 [2, 79]	24 [4, 70]	92 [68, 97] ^{a,b,c}	< 0.001
TLR2 Intermediate	31 [10, 92]	44 [19, 99]	42 [19, 91]	99 [97, 100] ^{a,b,c}	< 0.001
TLR2 Non-classical	1 [0, 52]	15 [0, 81]	6 [1, 32]	85 [75, 91] ^{a,b,c}	< 0.001
TLR4 Classical	81 [75, 98]	56 [34, 92]	68 [24, 82] ^a	99 [93, 100] ^{b,c}	< 0.001
TLR4 Intermediate	90 [76, 100]	70 [37, 95]	72 [26, 90]	100 [98, 100] ^{b,c}	< 0.001
TLR4 Non-classical	52 [27, 63]	21 [11, 57]	23 [5, 52]	94 [79, 100] ^{a,b,c}	< 0.001
CD36 Classical	99 [96, 100]	100 [98, 100]	100 [99, 100]	100 [99, 100] ^a	< 0.001
CD36 Intermediate	97 [89, 99]	100 [96, 100]	99 [97, 100]	100 ^a	0.010
CD36 Non-classical	27 [11, 48]	50 [25, 71]	35 [23, 55]	69 [60, 83] ^{a,b,c}	0.002
Angiogenic receptors					
Tie2 Classical	34 [30, 39]	25 [7, 37]	15 [7, 33] ^a	92 [54, 100] ^{a,b,c}	< 0.001
Tie2 Intermediate	57 [50, 79]	36 [10, 59]	27 [13, 63]	97 [63, 100] ^{b,c}	< 0.001
Tie2 Non-classical	40 [32, 56]	15 [4, 37] ^a	8 [4, 24] ^a	83 [45, 98] ^{b,c}	< 0.001
SLAM Classical	13 [3, 20]	6 [3, 18]	4 [1, 10]	40 [9, 52] ^{b,c}	0.003
SLAM Intermediate	39 [16, 47]	12 [5, 38]	9 [4, 41]	50 [18, 64] ^{b,c}	0.005
SLAM Non-classical	12 [4, 20]	3 [1, 10]	2 [1, 7] ^a	21 [8, 32] ^{b,c}	< 0.001

Data are presented in terms of the median [interquartile range]. The Kruskal–Wallis test, followed by Mann-Whitney *post hoc* test was used in all analyses. All p-values for intergroup comparisons have been Bonferroni-corrected.

C: controls; RF: risk factor; SA: stable angina patient; UA: unstable angina patient.

^a p < 0.05 versus C.

^b p < 0.05 versus RF.

^c p < 0.05 versus SA.

individuals. As expected, out of the three populations of monocytes analyzed, the frequency of CCR5⁺ cells was the lowest in the control group. The frequency of CCR5⁺ monocytes was also elevated in the non-classical monocyte population of the UA patients, and their values were significantly higher than values exhibited by the C, RF, and SA groups (Table 3).

Patients of the UA group showed a high level of homogeneously distributed intermediate and non-classical monocytes expressing the fractalkine receptor CX3CR1. This receptor was also highly expressed in the intermediate and non-classical monocyte population of patients in the RF group. The frequency of CX3CR1⁺ cells was lower in the classical monocyte population in the C, RF, and SA groups, while the frequency of CX3CR1⁺ cells was higher in the non-classical monocyte population of the UA group than that in the C and SA groups (Table 3).

3.3. Pattern recognition receptors

The expression of pattern recognition receptors and CD36 was evaluated in monocyte subpopulations.

TLR2⁺ cells were more frequent in intermediate subset of monocytes of UA patients when compared to those of other groups. The same pattern of expression was observed in classical and non-classical monocytes (Table 3).

Intermediate monocytes also presented a high frequency of TLR4. Once again, the UA group showed a higher frequency of TLR4⁺ cells in all monocyte subsets when compared to that in the RF and SA groups. The frequency of classical and non-classical TLR4⁺ monocytes was lower in the control group than in the SA and UA groups (Table 3).

The highest frequency of CD36⁺ cells was seen in classical and intermediate monocytes. The frequency of classical and intermediate

CD36⁺ monocytes was higher in the UA group than that in the C group. Non-classical monocytes showed a lower expression of CD36⁺ cells in all studied groups. However, CD36⁺ cells were observed most frequently in the non-classical monocytes of the UA group (Table 3).

3.4. SLAM and Tie2

Finally, we evaluated the expression of receptors related to angiogenesis. The frequency of Tie2⁺ cells was higher in all monocyte subsets in UA patients, when compared to those in the RF and SA groups (Table 3). The frequency of Tie2⁺ cells among the classical and non-classical monocyte populations of the control group was higher than that in the SA group. Slam⁺ monocytes showed a pattern of expression that was similar to that observed in Tie2⁺ cells; they occurred at a higher frequency in all monocyte subsets in the UA group when compared to those in the RF and SA groups (Table 3).

3.5. HSP60 monocyte stimulation

To examine the pattern of cytokine expression by monocyte subsets against proteins released under stress conditions, as observed in unstable angina, monocytes obtained from control subjects were stimulated with HSP60 and LPS (positive control). HSP60 induced a high frequency of intermediate monocytes expressing IL-12p70 (Fig. 1D). Moreover, we also observed a slight increase in the frequency of IFN- γ ⁺ monocytes in this subpopulation (Fig. 1E). On the other hand, LPS stimulation induced an increase in the percentage of intermediate

monocytes IL-1 β ⁺ and IL-8⁺ (Fig. 1A. and G).

The expression of the examined cytokines in classical and non-classical monocytes was not affected by stimulation with HSP60 (data not shown).

4. Discussion

The aim of this study was to evaluate the circulating monocyte subpopulations in individuals with acute and chronic CAD, and in healthy individuals with or without cardiovascular risk factors. Our results showed that the expression of the analyzed markers was higher in all monocyte subsets in the UA group, and especially in the intermediate subpopulation. Furthermore, HSP60 stimulation induced intermediate monocytes to produce IL12p70.

Chapman et al showed that the monocyte count was an independent predictor of carotid atherosclerosis, and high monocyte numbers were associated with increased plaque formation and carotid intima-medial thickness [17]. Results from other studies also revealed that the elevated CD16⁺ monocyte level in CAD patients correlated with increased levels of serum TNF- α [18] and coronary plaque vulnerability [19]. In contrast, Shantsila et al reported that there were no significant differences in the monocyte count between patients with CAD and healthy controls [20]. Our results showed a lower absolute number of total, classical and intermediate monocytes in SA group in relation to the other ones. These findings were somewhat unexpected, but maybe they were a consequence of the large number (81%) of patients under statin therapy in the SA group. These agents' properties may be responsible

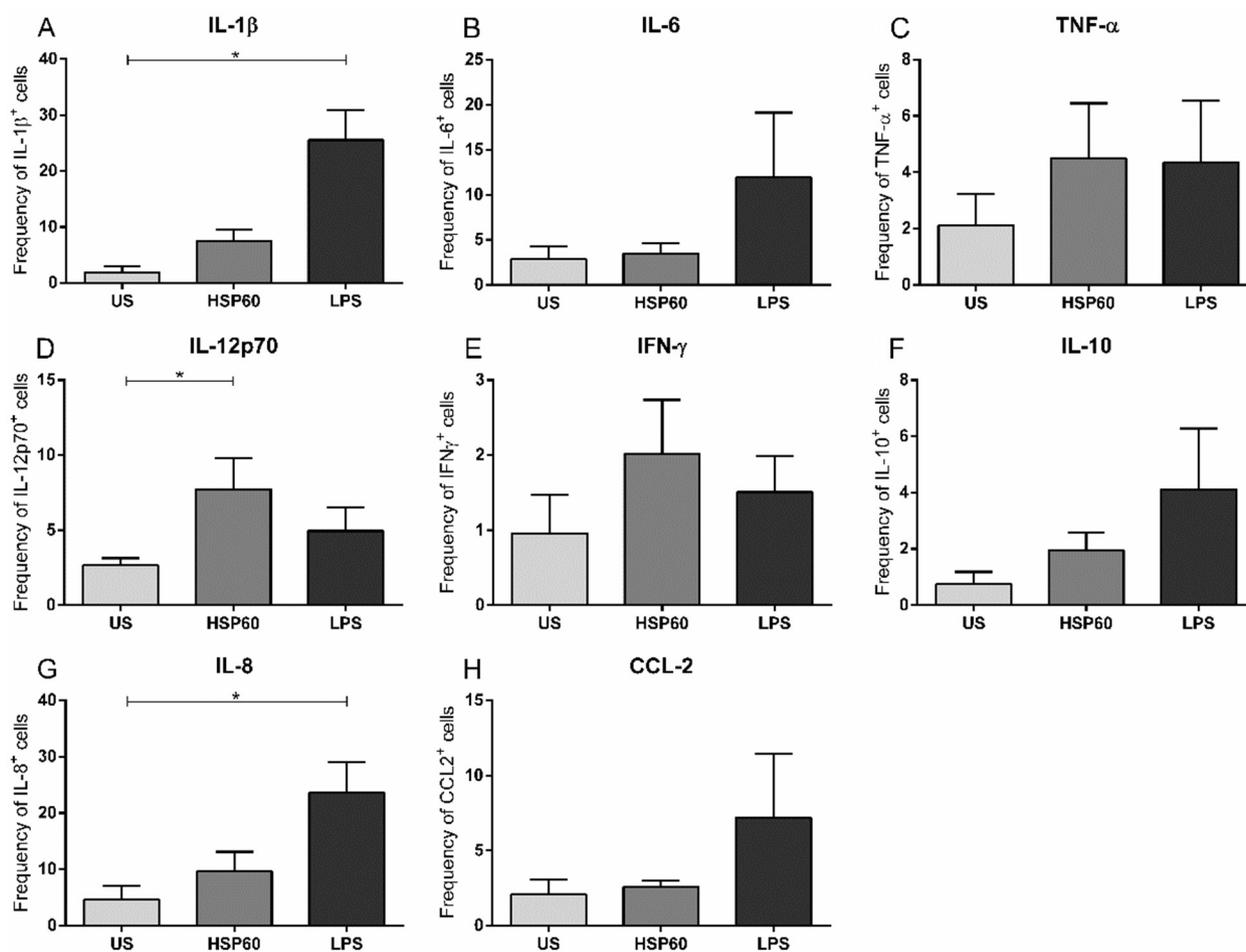


Fig. 1. Frequency of intracellular cytokines in intermediate monocytes after *in vitro* stimulation with HSP60. Monocytes from control individuals were stimulated with 5 μ g/mL of HSP60 or 100 ng/mL of LPS or maintained unstimulated (US) for 24 h. The frequencies of IL-1 β (A), IL-6 (B), TNF- α (C), IL-12p70 (D), IFN- γ (E), IL-10 (F), IL-8 (G), and CCL-2 (H) positive cells were evaluated by flow cytometry. * $p < 0.05$.

for decreased monocyte count as well as CRP in the SA group. However, in the UA group, given that baseline inflammation would be more prominent, the anti-inflammatory effects of statins would not produce the same results as in the SA group. Many studies have shown the pleiotropic immunomodulatory properties of statins that include reduction of CD16⁺ monocytes [21] and down regulation of adhesion molecules, IL-6, IL-8 and MCP-1 expression in circulating monocytes [22]. Another study performed by our group evaluating frequency of CD4⁺CD28^{null} T cells, found somewhat similar results among the study groups, corroborating our hypothesis. We compared the CD4⁺CD28^{null} subsets from patients with CAD (SA and UA) as well in controls with (RF) and without (C) risk factors for CAD. We found a lower frequency of CD4⁺CD28^{null} expressing granzymes A and B, perforin and TNF- α , as well as plasma levels of CRP in SA patients compared to UA and RF group [23].

Another important aspect to be mentioned is the percentage of cigarette smokers in the UA group, statistically higher than in the other groups. Smoking is a major risk factor for the pathogenesis and development of atherosclerosis [24]. It has been shown that it affects the adhesion of monocytes to endothelial cells, a critical step in the atherogenesis process [25]. Moreover, animal studies also demonstrate cigarette smoking can alter the monocyte phenotype in blood and bone marrow [26]. In agreement with these findings, we hypothesize that the striking alterations observed in monocyte phenotype in UA patients may be related, among other factors, to cigarette smoking.

Chemokines and their receptors are necessary for the migration of immune cells to inflammatory sites. The chemokine/receptor axes CCL2/CCR2, CCL5/CCR5, and CX3CL1/CX3CR1 are responsible for the recruitment of monocytes and are strongly associated with plaque progression in CAD patients [27]. CCR2 is highly expressed in classical monocytes, while the expression of CCR5 is greater in intermediate subsets and CX3CR1 in non-classical monocytes [28,29]. Although the phenotypes of monocyte subpopulations are well established, there are no studies that show how these subpopulations behave in patients with different degrees of atherosclerosis [30]. In our study, we found that the UA group exhibited the highest frequency of CCR2⁺, CCR5⁺, and CX3CR1⁺ monocytes in all subpopulations. The marked expression of chemokine receptors and high monocyte count together suggest that besides being produced and released in large quantities into the circulation, these cells can migrate to atherosclerotic lesions in patients with acute disease. It was observed in an animal model that the chemokine receptors, and mainly CCR5, were related to both development and disease progression [31].

In addition, recognition receptors such as toll-like receptors (TLRs), especially TLR2 and TLR4, play an essential role in the development of atherosclerosis. These surface molecules are involved in the recognition of altered particles, such as oxidized LDL (oxLDL), and danger signals, such as HSP60, largely present in atherosclerotic lesions [32,33]. The recognition of these ligands by TLR leads to the activation of NF- κ B, and consequently, to the production of inflammatory cytokines [34]. In contrast to the data presented by Kashiwagi et al, who did not observe any difference in the TLR4 expression between SA patients and healthy controls [16], our data showed that monocytes in the UA group exhibited a higher level of TLR4⁺ expression when compared to those of other groups. Furthermore, TLR2 was more frequent in the intermediate and non-classical monocytes of the UA group.

Similarly, the scavenger receptor CD36 involved in oxLDL recognition and foam cell formation [35,36] was more greatly expressed in classical and intermediate monocytes from the UA group. These findings are in line with those of previous studies reporting the upregulation of CD36 expression in the monocytes of patients with ACS [37]. Moreover, it is reported that the activation of CD36 by oxLDL results in the inhibition of migration of monocytes and macrophages, thus representing a mechanism of cell retention in atherosclerotic plaques, allowing for disease progression [36]. Altogether, our data suggested that in addition to the rise in the quantity of circulating

monocytes accompanying an increased migration capacity, the elevated expression of the pattern recognition receptors TLR2, TLR4, and CD36 might enable these cells to recognize and respond to DAMPs, such as oxLDL and HSP60, which are generated in the acute phase of the disease.

It is reported that HSPs are involved in causing the inflammation observed in atherosclerosis. Hypertension and the presence of circulating oxidized lipids increase the production of these molecules, especially HSP60, by endothelial cells, smooth muscle cells, and macrophages present in atherosclerotic plaques [38]. Though the presence of HSP60 and the antibodies against it were associated with atherosclerosis progression and intima-media thickness in studies involving humans [39,40], in animal models, this protein strongly induced a TLR4-dependent production of TNF- α by macrophages [33]. Our study showed for the first time that HSP60 induced IL-12p70 production mainly in intermediate monocytes, a subset that is associated with antigen presentation [28]. This cytokine induces the differentiation of Th1 lymphocytes, which have an inflammatory profile due to their elevated levels of production and secretion of IFN- γ , which promotes the activation of macrophages, endothelial cells, and smooth muscle cells, thereby enhancing inflammation and contributing to rupture mechanisms in unstable lesions [41].

The production of IL-12p70 by intermediate monocytes stimulated with HSP60, in addition to the increased expression of chemokine and pattern recognition receptors, as seen in the UA group, could account for the worsened prognosis associated with this condition. In these patients, the CD14⁺CD16⁺ intermediate monocytes exhibited an enhanced expression of CCR2, CCR5, and CX3CR1, which may direct them towards inflammatory sites. Moreover, these cells are more responsive to altered molecules, such as oxLDL and HSP60; thus, they exhibited an augmented expression of TLR2, TLR4, and CD36. Furthermore, in addition to a previously reported role in antigen presentation [28], the production of IL12p70, which is induced by HSP60, might contribute to Th1 lymphocyte polarization [41].

In conclusion, our results showed that UA patients have elevated intermediate monocytes, and that these cells have an enhanced DAMP recognition and migratory capacity, which may contribute to the propagation and progression of inflammation that is observed in atherosclerosis.

5. Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.cyto.2018.06.008>.

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