

Standardized Flow Cytometry Assay for Identification of Human Monocytic Heterogeneity and LRP1 Expression in Monocyte Subpopulations: Decreased Expression of This Receptor in Nonclassical Monocytes

Darío G. Ferrer,^{1†} Javier R. Jaldín-Fincati,^{1†} José L. Amigone,² Raul H. Capra,² César J. Collino,¹ Ricardo A. Albertini,³ Gustavo A. Chiabrando^{1*}

¹Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI-CONICET), Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina

²Laboratorio Central, Hospital Privado Centro Médico de Córdoba, Córdoba, Argentina

³Servicio de Clínica Médica, Hospital Privado Centro Médico de Córdoba, Córdoba, Argentina

Received 8 October 2013; Revised 27 December 2013; Accepted 18 February 2014

Grant sponsor: Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECyT UNC), Grant numbers: 124/13; 162/12; Grant sponsor: Fondo para la Investigación Científica y Tecnológica (FONCyT); Grant sponsor: Préstamo BID Proyecto de Investigación en Ciencia y Tecnología (PICT), Grant number: 2012–2607; Grant sponsor: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Proyecto de Investigación Plurianual (PIP), Grant number: 112–200801-02067.

Additional Supporting Information may be found in the online version of this article.

• Abstract

In this article, we present a flow cytometry assay by which human blood monocyte subpopulations—classical (CD14⁺⁺CD16[−]), intermediate (CD14⁺⁺CD16⁺), and nonclassical (CD14⁺CD16⁺⁺) monocytes—can be determined. Monocytic cells were selected from CD45⁺ leukocyte subsets by differential staining of the low-density lipoprotein receptor-related protein 1 (LRP1), which allows reducing the spill-over of natural killer cells and granulocytes into the CD16⁺ monocyte gate. Percentages of monocyte subpopulations established by this procedure were significantly comparable with those obtained by a well-standardized flow cytometry assay based on the HLA-DR monocyte-gating strategy. We also demonstrated that LRP1 is differentially expressed at cell surface of monocyte subpopulations, being significantly lower in nonclassical monocytes than in classical and intermediate monocytes. Cell surface expression of LRP1 accounts for only 20% of the total cellular content in each monocyte subpopulation. Finally, we established the within-individual biological variation (bCV%) of circulating monocyte subpopulations in healthy donors, obtaining values of 21%, 20%, and 17% for nonclassical, intermediate, and classical monocytes, respectively. Similar values of bCV% for LRP1 measured in each monocyte subpopulation were also obtained, suggesting that its variability is mainly influenced by the intrinsic biological variation of circulating monocytes. Thus, we conclude that LRP1 can be used as a third pan-monocytic marker together with CD14 and CD16 to properly identify monocyte subpopulations. The combined determination of monocyte subpopulations and LRP1 monocytic expression may be relevant for clinical studies of inflammatory processes, with special interest in atherosclerosis and cardiovascular disease. © 2014 International Society for Advancement of Cytometry

• Key terms

monocyte heterogeneity; inflammation; biological variation; atherosclerosis

INTRODUCTION

MONOCYTES are cornerstones of the immune system linking innate and adaptive immunity and are critical drivers in many inflammatory diseases (1). Three monocytic subpopulations have been defined in humans, based on the differential expression of the lipopolysaccharide (LPS) receptor CD14 and the FcγIII receptor CD16, as classical [CD14⁺⁺CD16[−]], intermediate [CD14⁺⁺CD16⁺], and nonclassical [CD14⁺CD16⁺⁺] monocytes (2). The measurement of monocyte subpopulations

[†]Darío G. Ferrer and Javier R. Jaldín-Fincati contributed equally to this work.

*Correspondence to: Gustavo A. Chiabrand, Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI-CONICET),

Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina. E-mail: gustavo@fcq.unc.edu.ar

Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com)

represents an attractive tool for the diagnosis of several inflammatory diseases, such as cardiovascular diseases (3). Although flow cytometric differentiation of monocyte subpopulations is based on quantification of surface CD14 and CD16 expression, the correct identification of monocytes is performed through a third pan-monocytic marker. Staining protocols which only analyze CD14 and CD16 expression fail to correctly distinguish monocytes from other leukocyte subsets. These protocols usually rely on cellular physical characteristics for defining monocytes by measuring forward-angle scattered light [FSC] and orthogonal side scatter [SSC] (2). Following such approach, CD16⁺ monocytes cannot be properly separated from other CD16-expressing leukocytes, namely, neutrophil granulocytes (NGs) and natural killer (NK) cells, leading to an inaccurate assessment of monocyte subpopulations. As a consequence, different approaches using either CD86 or HLA-DR as third pan-monocytic marker have been recently developed (4–6). When using the CD86 monocyte-gating strategy, monocytic cells are gated in a SSC versus CD86⁺ plot, identifying monocytes as CD86⁺ cells with monocytic scatter properties (5, 6). The HLA-DR monocyte-gating strategy allows to exclude CD16⁺ NK cells from the HLA-DR versus CD14 plot, whereas CD16⁺ NG cells are separated from monocytes from the FSC versus SSC plot (4).

Low-density lipoprotein receptor-related protein 1 (LRP1), also known as CD91, is a cell surface glycoprotein synthesized as a precursor protein of 600 kDa. It is proteolytically cleaved by furin into two subunits: a large subunit of 515 kDa (LRP1- α), containing the extracellular binding domain, and a subunit of 85 kDa (LRP1- β) comprising the membrane spanning and cytoplasmic domains. These subunits are associated through noncovalent interactions (7). LRP1 is an endocytic receptor which binds and internalizes multiple structurally and functionally diverse ligands, including activated α_2 -macroglobulin (α_2 M*), *Pseudomonas* exotoxin A, lipoprotein lipase, and apolipoprotein E-enriched lipoproteins (8). Although LRP1 is considered to be an endocytic receptor, it has been reported to promote intracellular signaling, which mediates proliferation, migration, and differentiation of different types of cells, including macrophages, vascular smooth muscle cells (VSMCs), and neurons (9–13). These cellular events have been associated with different inflammatory and pathological processes such as atherosclerosis and Alzheimer's disease (14–17). LRP1 is expressed in a variety of cell types, including hepatocytes, fibroblasts, neurons, and smooth muscle cells (18). Among peripheral blood leukocytes, there are several studies reporting that LRP1 is mainly expressed in

monocytes (19–22). However, LRP1 expression in each monocyte subpopulation has not been established. This could unveil specific functions of the receptor in each monocyte subset during inflammatory processes, in particular in the development of atherosclerosis.

In this study, we have developed a flow cytometry assay for the determination of monocyte heterogeneity and LRP1 monocytic expression in human whole peripheral blood. Based on this LRP1 monocyte-gating strategy, we have established the within-individual biological variation of the monocyte heterogeneity and LRP1 expression in monocyte subpopulations for healthy subjects. These analytical and biological features become the combined analysis of both parameters in an attractive diagnostic tool to be used in the characterization of different inflammatory processes.

MATERIALS AND METHODS

Groups of Subjects

Groups of subjects were created for different studies; each individual was enrolled in the Hospital Privado Centro Médico de Córdoba (Córdoba, Argentina), after each individual had signed the corresponding informed consent. These studies were approved by Hospital Privado's Ethics Committee.

Group A: Analysis of method comparison. Twenty-three subjects (14 females and nine males; aged between 26 and 59 years) were included. They were nondiabetic and had had no fever or infections in the week prior to the blood draw.

Group B: Determination of monocyte heterogeneity and LRP1 expression in monocyte subpopulations. Twenty-three healthy subjects (12 females and 11 males; aged between 20 and 47 years) were included.

Group C: Establishment of within-individual biological variation. Ten healthy subjects (five females and five males; aged between 25 and 35 years) were included. During the study period, they took no medication and did not change their daily habits of life.

The criteria for healthy subjects were as follows: total cholesterol, <200 mg/dl; HDL-cholesterol, \geq 50 mg/dl (females) and \geq 40 mg/dl (males); fasting plasma glucose, <100 mg/dl; plasma creatinine, \leq 1.2 mg/dl; nonsmoking; and without arterial hypertension.

Calculation of Within-Individual Biological Variation

Six specimens of venous blood were collected from each subject of Group C, during 6 weeks at intervals of one per

week. Specimens of each individual were analyzed in duplicate. To minimize preanalytical variance as much as possible, the same phlebotomist collected the specimens at approximately the same time of the day (between 08:00 and 09:30 a.m.). To calculate the within-individual biological variation, we applied the procedure described by Fraser and Harris (23). Briefly, the within-run analytical variance ($aS(2)$) of duplicate patients was calculated as follows:

$$aS^2 = \sum (d)^2 / 2 \times n,$$

where $\sum(d)$ is the sum of the differences of duplicates and n is the total number. The within-individual biological variance ($bS(2)$) was calculated as follows:

$$bS^2 = tS^2 - aS^2,$$

where $tS(2)$ is the total intraindividual variance. The Cochran test was used to detect the presence of outliers. To evaluate whether the individual mean value differed significantly between subjects, we used Reed's criterion. Then, the mean values of analytical and biological variances were obtained from teen subjects. Finally, the results of each variability were expressed as relative coefficient of variation for analytical imprecision (aCV%) and within-individual biological variation (bCV%), calculated from the same mean value for LRP1 expression in each monocyte subpopulation. In the same experiment, aCV% and bCV% were calculated for monocyte subpopulations. These variables were calculated from the measurement of monocyte subpopulation counts (cells per microliter), which were established by referring the percentage of each monocyte subpopulation determined by flow cytometry with the automated white blood cell count.

The analytical performance of the flow cytometry assay for monocytic heterogeneity based on the LRP1 monocyte-gating strategy was compared with the well-established method based on the HLA-DR monocyte-gating strategy (4).

Monoclonal Antibodies

To detect LRP1 in monocyte subpopulations by flow cytometry, PE-conjugated anti-LRP1 monoclonal antibody (clone MCA1955PE; isotype: mouse IgG1; AbD Serotec, UK) was used. All antibodies are detailed in Supporting Information Table S1. All isotype controls were purchased from their respective manufacturers.

Blood Sampling and Staining Procedure

Fresh whole blood was drawn into EDTA-K3 collection tubes (DVS, No. 1213752.5; Buenos Aires, Argentina), and specimens were prepared for flow cytometry within 30 min. About 50 μ l of whole blood was added to a 5-ml polystyrene round-bottomed tube (No. 352008; BD Biosciences), and 1 μ l of each antigen-specific fluorochrome-labeled antibody (dilution 1:50) was added. The sample was then incubated for 20 min at 4°C in the dark. Lysis of erythrocytes was performed using a lysing buffer (No. 555899; BD Pharm Lyse) for 15

min. To ensure maximum viability, stained cells were analyzed promptly.

Flow Cytometry Gating Strategy

First, CD45-positive leucocytes were visualized in a SSC versus CD45 plot showing all fluorescence-3 (PC5-positive) events. An acquisition threshold was set such that any unwanted events like CD45-negative platelets, dead cells, and debris were not recorded. The monocytes were then defined by sequential gating on all CD45-positive leukocytes using the SSC versus LRP1-staining plot, whereas monocyte subpopulations were identified from CD14 versus CD16 plot following the criteria previously defined (2). Using isotype controls, voltage, and compensation, the instrument was set such that the cells were adequately positioned in the dot plots. The mean fluorescence intensity (MFI) for LRP1 in classical, intermediate, and nonclassical monocytes was determined from the cell distribution pattern obtained in the CD14 versus CD16 plot. MIFlowCyt standard is available in Supporting Information S1.

Confocal Microscopy

To visualize LRP1 cellular localization, each monocyte subpopulation was isolated first from monocytes selected from the SSC versus CD45 plot using the BD FACSAriaTMIlu cell sorter then from the CD14 versus CD16 plot. Isolated cells were incubated and processed for fluorescence microscopy (details in Supporting Information S2). Fluorescent images (0.25- μ m z-axis optical sections) were obtained with an Olympus FluoView FV300 Confocal Laser Scanning Biological Microscope (Olympus, New York, NY) and processed using FV10-ASW Viewer 3.1 software (Olympus). The quantification of fluorescence intensity corresponding to LRP1 detection at intracellular (permeabilized condition) and plasma membrane (nonpermeabilized condition) levels was carried out using the ImageJ software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, <http://imagej.nih.gov/ij/>, 1997–2012), where at least 50 cells per condition were analyzed.

Western Blot and Reverse Transcriptase-PCR Assays

To evaluate LRP1 expression in leukocyte subsets, lymphocytes, granulocytes, and monocytes were isolated from the SSC versus CD45 plot using the BD FACSAriaTMIlu cell sorter, and Western blot analysis was performed as described previously (24). To evaluate the specific mRNA for LRP1, each isolated leukocyte subset was treated with TRIzol® Reagent (Invitrogen, Buenos Aires, Argentina). Specific primers for LRP1 and GADPH were used. More details for both assays are available in Supporting Information S2.

Statistics

For statistical analysis, the parametric paired single-sided Student's *t*-test was used. For method comparison, linear regression analysis, Pearson's correlation coefficient (*r*), and Bland-Altman analysis were used. For statistical significance, the threshold below the *P* value calculated was chosen at the 0.05 level. Other statistical tests were calculated using the InfoStat software (version 2011; Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

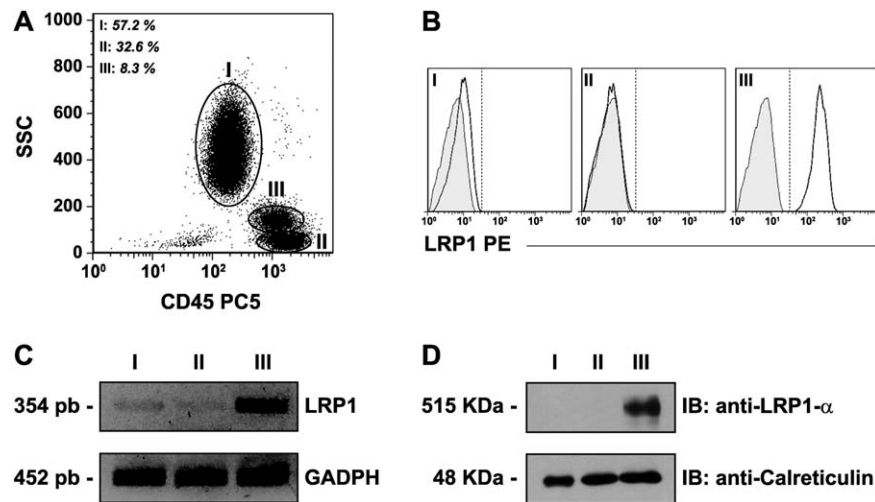


Figure 1. Characterization of LRP1 expression in monocytes from whole peripheral blood. **A:** SSC versus CD45 plot for granulocytes (I), lymphocytes (II), and monocytes (III) selection. Percentages of cells in Regions I, II, and III are indicated. **B:** Representative flow cytometry analysis showing the expression of LRP1 in granulocytes (I), lymphocytes (II), and monocytes (III). The LRP1 expression level (open histogram) is compared with isotype control (gray histogram). **C:** RT-PCR analysis for specific transcript of LRP1 in cell lysates of granulocytes (I), lymphocytes (II), and monocytes (III) gated and isolated by cell sorting from SSC versus CD45 plot. GAPDH is shown as loading control. **D:** Western blot analysis for LRP1 in cell lysates of granulocytes (I), lymphocytes (II), and monocytes (III) gated and isolated as indicated before. Calreticulin is shown as protein loading control. In Panels A and B, whole blood was stained with fluorochrome-conjugated antibodies to CD45 and LRP1, and samples were analyzed by flow cytometry after red cell lysis. All results shown are representative of at least three independent experiments.

RESULTS

LRP1 Expression in Human Monocytes from Leukocyte (CD45⁺) Subsets

To evaluate whether LRP1 can be used as a third marker for monocyte identification, we examined LRP1 expression in each leukocyte subset as identified from a SSC versus CD45 plot (Fig. 1A). With this graphical representation, three leukocyte subsets were identified: granulocytes (I), lymphocytes (II), and monocytes (III). To evaluate LRP1 expression in each region, a PE-conjugated antibody against LRP1- α was used and analyzed by flow cytometry. Figure 1B shows that all events gated in Region III were LRP1 positive, whereas all events gated in Regions I and II did not show a significant staining for this receptor when compared with fluorescence control signals. In addition, all events of the three regions represented in the SSC versus CD45 plot were isolated by cell sorting to evaluate LRP1 expression by RT-PCR and Western blot analysis. Figure 1C shows that the specific RNA transcript for LRP1 was clearly amplified in monocytes (III), whereas in granulocytes (I) and lymphocytes (II), either a weak or no amplification was detected. By Western blot analysis, LRP1 was only immunodetected in monocyte lysates with a mouse monoclonal antibody anti-LRP1- α (515 kDa; Fig. 1D). Similar results were obtained when Western blot assays were performed using a mouse monoclonal antibody anti-LRP1- β (85 kDa; data not shown).

LRP1 Monocyte-Gating Strategy for the Measurement of Monocyte Subpopulations

To evaluate whether LRP1 can be used as a marker for monocyte-gating strategy, whole peripheral blood was treated with the following fluorescence-conjugated antibodies: anti-CD14-FITC, anti-LRP1- α -PE, anti-CD45-PC5, and anti-

CD16-APC-Cy7. Figure 2A shows a SSC versus LRP1 plot from the leukocyte CD45⁺ separation (Fig. 1A), where the monocyte region (III) is LRP1-positive and clearly separated of granulocyte (I) and lymphocyte (II) regions. Then, all events gated in LRP1-positive monocytes (III) were represented in a CD16 versus CD14 plot (Fig. 2B). In this plot, three monocyte subpopulations can be observed: CD14⁺⁺CD16⁻, CD14⁺⁺CD16⁺, and CD14⁺CD16⁺⁺, which are in accordance with classical, intermediate, and non-classical monocytes (5, 25, 26). May-Grünwald-Giemsa staining of each subpopulation isolated by cell sorting (Supporting Information Fig. S1) clearly shows that these cells are monocytes (Fig. 2C). Figure 2D shows that MFI of LRP1 in non-classical monocytes is approximately twofold lower than in classical and intermediate monocytes (Fig. 2D).

To evaluate whether NK and NG are included in monocyte subpopulations selected using the LRP1 gating strategy, whole peripheral blood were stained with anti-CD15-FITC or anti-CD56-FITC, together with anti-CD45-PC5 and anti-LRP1-PE antibodies. Based on the SSC versus LRP1 plot (Fig. 2A), none of the events gated in Region III showed a positive staining for CD15 or CD56 when compared with events gated in Regions I and II, respectively (Fig. 2E). In addition, and as expected for NG, CD15⁺ cells were identified in all events gated in Region I but not in Region II, which is in agreement with the NG identification. CD56⁺ cells were partially detected in events gated in Region II but not in Region I, which is in concordance with the NK detection in the region of lymphocyte subset. Finally, all events gated in Regions I, II, and III showed some level of positive staining for CD16 expressed in NG, NK, and in monocytes corresponding to intermediate and nonclassical subpopulations.

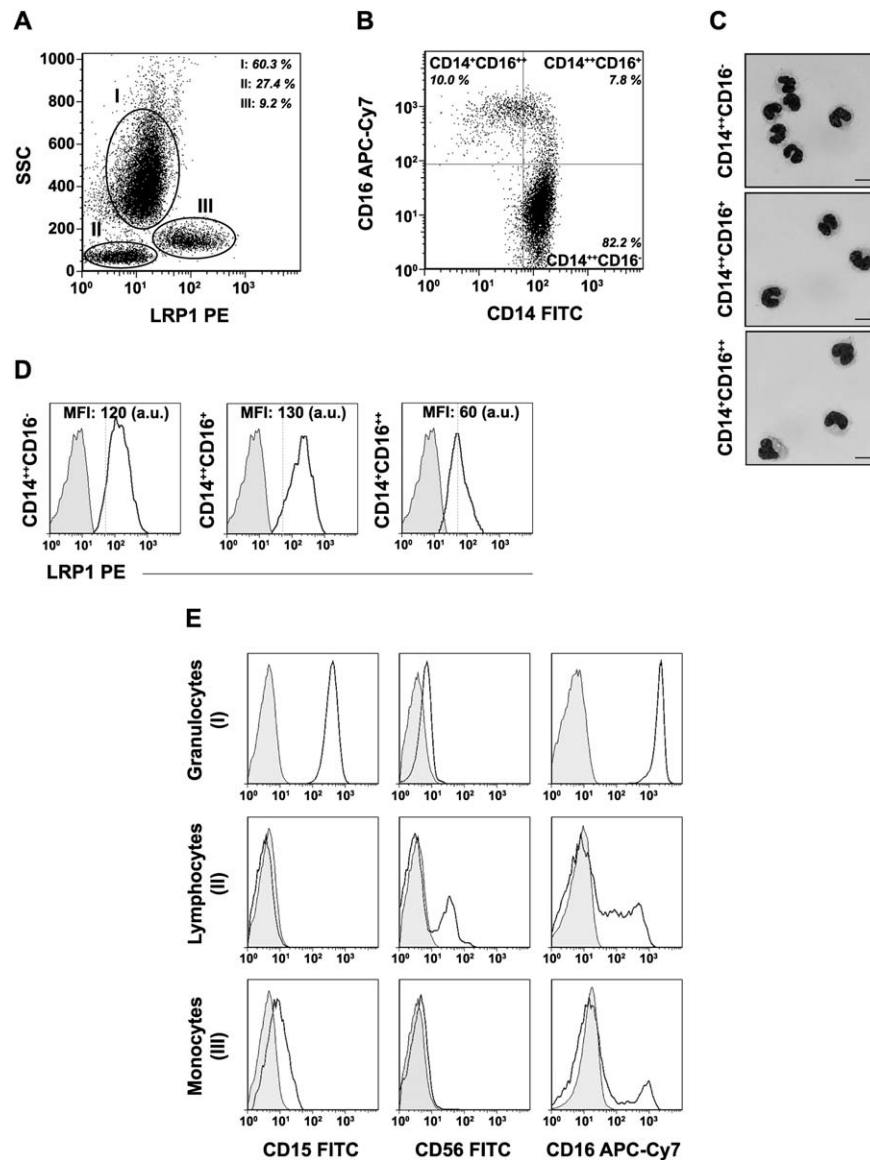


Figure 2. LRP1 monocytic gating strategy for identification of monocyte subpopulations. **A:** SSC versus LRP1 plot, where LRP1⁺ monocytes (III) are selected with respect to LRP1-negative cells and scatter properties as granulocytes (I) and lymphocytes (II). Percentages of cells in Regions I, II, and III are indicated. **B:** CD14 versus CD16 plot, where CD14⁺CD16⁻ or classical, CD14⁺CD16⁺ or intermediate, and CD14⁺CD16⁺⁺ or nonclassical monocytes are identified. Percentages of cells gated in classical, intermediate, and nonclassical monocytes are indicated. **C:** Representative May-Grünwald-Giemsa (MG-G) staining of classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺), and nonclassical (CD14⁺CD16⁺⁺) monocytes gated and isolated by cell sorting from CD14 versus CD16 plot. Scale bars represent 10 μ m. **D:** Representative flow cytometry analysis showing the LRP1 expression in classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺), and nonclassical (CD14⁺CD16⁺⁺) monocytes, where MFI values are indicated. The LRP1 expression level (open histogram) is compared with isotype control (gray histogram). In Panels A, B, and D, whole blood was stained with fluorochrome-conjugated antibodies to CD45, CD14, CD16, and LRP1, and samples were analyzed by flow cytometry after red cell lysis. **E:** Representative flow cytometry analysis showing the expression of CD15, CD56, and CD16 in leukocytes separated by SSC versus LRP1 gating strategy of granulocytes (I), lymphocytes (II), and monocytes (III). The expression level of each marker (open histogram) is compared with its respective isotype control (gray histogram). Whole blood was stained with fluorochrome-conjugated antibodies to CD15, CD56, CD16, and LRP1, and samples were analyzed by flow cytometry after red cell lysis. All results shown are representative of at least three independent experiments.

Considering that the LRP1 monocyte-gating strategy identifies monocyte subpopulations by flow cytometry, we compared our LRP1-based versus the classical HLA-DR monocyte-gating strategy, by determining the percentage of monocytic subpopulations from whole peripheral blood of 22

donors (defined in Group A of subjects in the Materials and Methods section). The HLA-DR strategy was performed by the flow cytometry assay described by Heimbeck et al. (4), following the procedure detailed in Supporting Information Figure S2. No differences were observed between the mean of

Table 1. Percentages of monocyte subpopulations determined by flow cytometry assays with LRP1 strategy (LRP1) and HLA-DR strategy (HLA-DR) in whole peripheral blood of 23 donors^a

MONOCYTES STRATEGY	CLASSICAL		INTERMEDIATE		NONCLASSICAL	
	LRP1	HLA-DR	LRP1	HLA-DR	LRP1	HLA-DR
Mean (%)	84.9 ^b	84.7 ^b	5.9 ^b	6.8 ^b	9.2 ^b	8.4 ^b
SD (%)	4.3	4.5	2.3	2.0	3.1	3.3
Min (%)	76.4	77.3	2.4	3.5	4.4	3.6
Max (%)	93.1	92.8	11.1	11.4	14.0	15.6

SD, standard deviation; Min, minimal value; Max, maximal value.

^aFourteen females and nine males.

^bNonsignificant differences between means ($P > 0.05$).

percentages in classical, intermediate, and nonclassical monocytes measured by both flow cytometry assays ($P > 0.05$), which demonstrates that both approaches have the same ability for detecting a broad interval of percentages in each monocyte subpopulation (Table 1). Moreover, the method comparison analysis demonstrated that both flow cytometry assays are comparable for the determination of monocyte subpopulations, showing significant values of the Pearson's correlation coefficient ($r \sim 0.9$ and $P < 0.0001$; Fig. 3). Finally, Bland-Altman analysis shows that the majority of the differences (22 of 23) are symmetrically distributed around the mean bias within 95% of confidence interval (± 1.96 SD) in each monocyte subpopulation (Supporting Information Fig. S3), which indicates that both flow cytometry assays may be considered identical methods for the determination of monocyte subpopulations in peripheral blood. However, the apparent differences observed in intermediate and nonclassical monocytes may be attributed to technical aspects, mainly related to the analytical and instrumental setting of the HLA-DR strategy used as reference method.

Differential Expression of LRP1 in Monocyte Subpopulations

It is known that the subcellular distribution of LRP1 is predominantly intracellular ($\sim 70\%$, against $\sim 30\%$ at the plasma membrane) in different types of cells (27). As shown above, by using flow cytometry, we determined that LRP1 surface expression levels are higher in classical and intermediate than in nonclassical monocytes (Fig. 2D). However, the subcellular distribution of LRP1 in monocytes is unknown. Therefore, here we used fluorescence confocal microscopy to evaluate the LRP1 expression at intracellular and plasma membrane levels in each monocyte subpopulation isolated by cell sorting. Then, LRP1 expression was examined with a mouse monoclonal antibody against LRP1- β (clone 5A6) under permeabilized and nonpermeabilized cell conditions in classical, intermediate, and nonclassical monocytes. Figure 4 shows that under nonpermeabilized conditions, LRP1 surface expression levels are higher in classical than in nonclassical monocytes, which is in agreement with flow cytometry results presented before. Under permeabilized conditions, intracellular LRP1 expression presented a characteristic of perinuclear punctuate in both monocyte subpopulations, which is also visualized in other types of cells (24). The image quantifica-

tion demonstrated that the intracellular LRP1 expression is also greater in classical than nonclassical monocytes (Fig. 4). In addition, the cellular distribution of LRP1 in intermediate monocytes showed a similar pattern of expression to classical monocytes (data not shown). Finally, from the analysis of fluorescence intensity between nonpermeabilized and permeabilized conditions, we estimate that $\sim 80\%$ of LRP1 is expressed at the intracellular level in both classical and nonclassical monocytes.

To verify that LRP1 is highly expressed in classical/intermediate monocytes when compared with nonclassical monocytes, we used the flow cytometry assay based on LRP1 monocyte-gating strategy and measured LRP1 in monocyte subpopulations in whole peripheral blood of healthy donors (defined in Group B of subjects in the Materials and Methods section). Figure 5A shows that mean values of MFI for LRP1 were significantly lower in nonclassical than in classical and intermediate monocytes, whereas there were no significant differences between the latter subpopulations. Moreover, no significant difference of MFI was observed between female and male healthy donors (data not shown).

Individual Biological Variation of LRP1 Expression in Monocyte Subpopulations

To evaluate whether LRP1 expression may be affected by monocyte variations, we calculated the within-individual biological variation of both monocyte heterogeneity and LRP1 expression in monocyte subpopulations using an experimental design previously proposed by Fraser and Harris (23). This procedure allows determining the total within-individual variability, which includes analytical imprecision (aCV%) and within-individual biological variation (bCV%), from a continuous analysis of blood samples of normal subjects during an established period of time. Thus, both absolute monocyte subpopulations (cells per microliter of whole blood) and LRP1 levels (MFI values) were measured in 10 donors, and then subsequent variability was calculated. A summary of data obtained from this study is shown in Supporting Information Figure S4 and Tables S2 and S3. Figure 5B shows that the mean percentage of bCV% for LRP1 in monocytes was significantly comparable in nonclassical (18.9%), intermediate (16.5%), and classical (16.0%) subpopulations ($P > 0.05$). Analytical imprecision (aCV%) was also similar in the three monocyte subpopulations analyzed, with aCV% $< 5\%$. Values

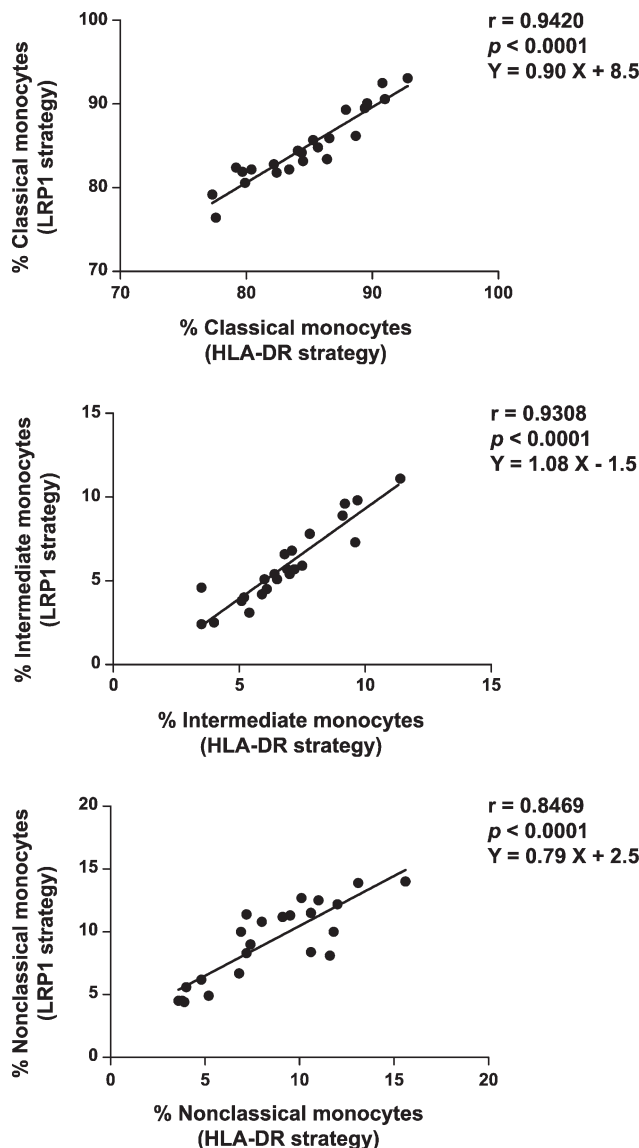


Figure 3. Analysis of method comparison for monocyte heterogeneity by flow cytometry assays using LRP1 and HLA-DR mono-cyte-gating strategy. Each panel represents the comparative analysis for percentages of classical, intermediate, and nonclassical monocytes. Data of regression analysis, Pearson's correlation coefficient (r), and P significance are shown. Samples of whole blood extracted from 23 subjects (14 females and nine males) were divided into two specimens and stained with fluorochrome-conjugated antibodies to CD45, CD14, CD16, and LRP1 or CD45, CD14, CD16, and HLA-DR and then analyzed by flow cytometry after red cell lysis.

of bCV% for monocyte subpopulations calculated from monocyte counting (nonclassical, 21.1%; intermediate, 19.6%; classical, 17.5%) were not different ($P > 0.05$) from those calculated for LRP1 expression in monocyte subpopulations. In addition, the analytical imprecision was $<5\%$ for the determination of monocyte subpopulation counts, which is comparable with the analytical variation (4.1%) reported with the HLA-DR monocyte-gating strategy (4). The within-individual biological variation calculated for

monocyte subpopulations was comparable with the variation previously reported for total monocyte (17.8% in <http://www.westgard.com/biodatabase1.htm>). No significant difference of bCV% for monocytic heterogeneity and LRP1 expression was observed between female and male donors (data not shown).

DISCUSSION

Both monocyte heterogeneity and LRP1 expression have been linked to the development of atherosclerosis and cardiovascular diseases (6, 14, 15, 20, 25, 28). LRP1 is highly expressed in macrophages, VSMCs, and foam cells during atherosclerotic development in human and animal models of atherosclerosis (15). In addition, although it is known that LRP1 is principally expressed in monocytes of peripheral blood cells (19, 21, 22), its expression in relation to monocyte heterogeneity remains to be established. In the current study, we have demonstrated that LRP1 is mainly expressed in monocytes, which allows a clear separation of these cells by flow cytometry from other leukocyte subsets using a SSC versus LRP1 plot. All the monocytes gated from this plot were negative for CD15 and CD56, which are cell markers for NG and NK, respectively. Both NG and NK express high levels of CD16 and can produce an important bias in the determination of nonclassical and intermediate monocytes if these cells are incorrectly included in the monocyte-gating strategy (4, 25). We have also demonstrated that all events gated in granulocyte and lymphocyte regions of SSC versus LRP1 plot showed a high level of expression of CD16; however, these gated regions did not detect the expression of LRP1, which indicates that monocytes were not included in these leukocyte subsets. Thus, we conclude that, from SSC versus LRP1 plot, monocytes can be clearly separated and that this marker may be conveniently used as a third pan-marker for monocyte selection by flow cytometry. Our findings confirm the previous characterization of LRP1 as a monocyte differentiation antigen (22), which is expressed in the total monocytic subpopulations. In addition, CD300e (IREM-2) is another antigen that is selectively expressed by monocytes and myeloid dendritic cells, which is positive in at least 80% of monocytes in normal peripheral blood (29). Although CD300e is proposed to be a marker to classify myeloid leukemias (especially those with monocytic component), the combined detection with LRP1 may constitute a potential tool for the diagnostic of this type of leukemias.

Two different flow cytometry assays for the determination of monocyte subpopulations have been proposed. These approaches, using either CD86 (5, 6, 30) or HLA-DR (4) as a third pan-monocytic marker, may yield identical results. We determined that percentages of monocyte subpopulations measured by our LRP1 monocytic strategy were significantly comparable with those obtained with the HLA-DR procedure, which may be considered as an alternative procedure for the measurement of monocyte subpopulations by flow cytometry. In addition, Heimbeck et al. (4) demonstrated that women have lower absolute number of nonclassical monocytes than men, which is attributed to an estrogenic effect. In our study,

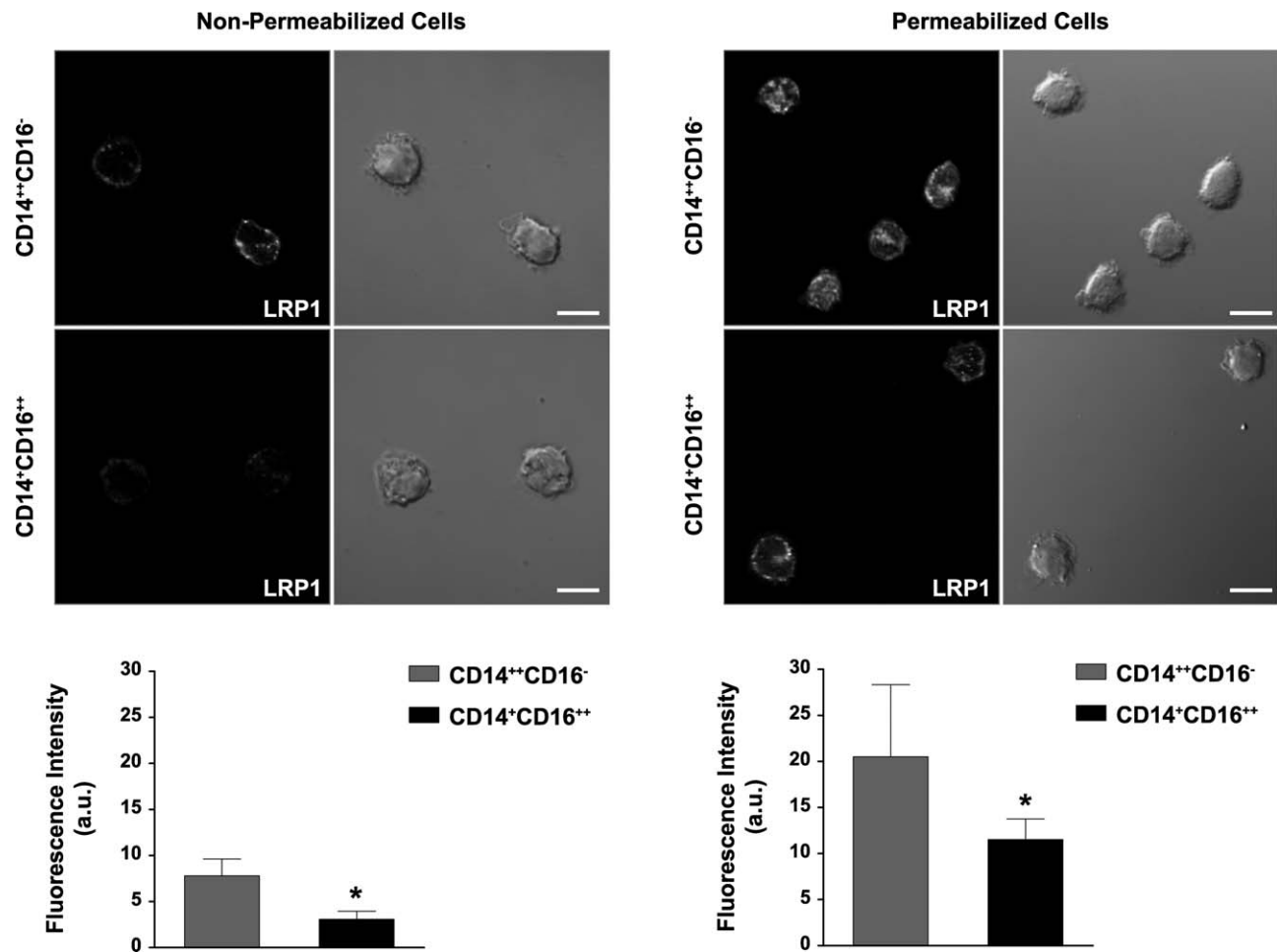


Figure 4. Representative confocal microscopy for the analysis of LRP1 cellular distribution in classical (CD14⁺⁺CD16⁻) and nonclassical (CD14⁺CD16⁺⁺) monocytes. Left panel: Cells were treated under nonpermeabilized conditions. Right panel: Cells were treated under permeabilized conditions. In each condition, LRP1 was stained with an anti-LRP1 (β -subunit) monoclonal antibody and revealed with an Alexa Fluor⁴⁸⁸-conjugated secondary antibody. DIC images of monocytes are shown. The graphs represent the quantitative analysis of fluorescence intensity. Bars are the mean value \pm 1 SEM (standard error of the mean) of fluorescence intensity for at least 50 cells per condition. Three independent experiments were performed in triplicate. * $P < 0.05$ indicates statistical significance of the fluorescence intensity in nonclassical (CD14⁺CD16⁺⁺) with respect to classical (CD14⁺⁺CD16⁻) monocytes both in nonpermeabilized and in permeabilized conditions. White scale bars represent 10 μ m.

using both HLA-DR and LRP1 monocyte-gating strategies, we also observed that there was a trend toward lower values in females ($n = 14$) with respect to males ($n = 9$); however, in our case, this was not significant (8.1% [$\pm 3.0\%$] vs. 8.9% [$\pm 3.9\%$] for HLA-DR strategy; and 8.9% [$\pm 3.0\%$] vs. 9.6% [$\pm 3.3\%$] for LRP1 strategy). Finally, one possible advantage of our assay, when compared with the HLA-DR monocyte-gating strategy, is that a low proportion of NK subset expresses HLA-DR, which can be expanded in response to IL-2 during certain immune responses (31). Therefore, under these conditions, these HLA-DR⁺ NK cells could be computed as CD16⁺ monocytes. As we demonstrated that LRP1 is not expressed in NK, HLA-DR⁺ NK would be completely excluded from monocyte events selected through our LRP1 monocyte-gating strategy. Nevertheless, additional studies are necessary to determine whether LRP1 can be expressed by NK subsets under certain immune responses.

LRP1 plays a key role during different cellular events, including adhesion and cell motility of nonmalignant and malignant cells (12, 24, 32, 33). The molecular function of LRP1 would be dependent on its expression at the cell surface level, where LRP1 can interact with and regulate other membrane proteins such as urokinase receptor, platelet-derived growth factor receptor β , and Type I membrane metalloproteinase (MT1-MMP; Refs. 24, 34, and 35). However, LRP1 is expressed in low proportion at the plasma membrane, being mostly detected at the intracellular level (24, 27). In our study, we demonstrate that LRP1 is principally expressed at the intracellular level in three monocyte subpopulations and that $\sim 20\%$ of total receptor is detected at the plasma membrane. In addition, we show that cell surface LRP1 is differentially detected by flow cytometry in monocyte subpopulations, being higher in CD14⁺⁺ monocytes (classical and intermediate) than in CD14⁺ monocytes (nonclassical) of healthy

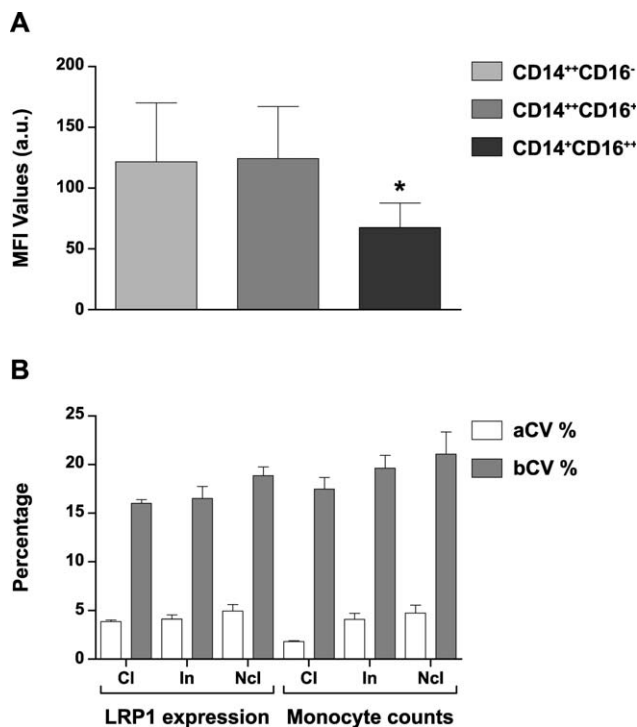


Figure 5. A: Levels of LRP1 expression in monocyte subpopulations of healthy subjects. Bars represent the mean values of MFI (± 1 standard deviation) for LRP1 expression in classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺), and nonclassical (CD14⁺CD16⁺⁺) monocytes by flow cytometry assay using the LRP1 monocyte-gating strategy measured in 23 healthy subjects (12 females and 11 males). * $P < 0.05$ indicates statistical significance of nonclassical (CD14⁺CD16⁺⁺) monocytes with respect to intermediate (CD14⁺⁺CD16⁺) and classical (CD14⁺⁺CD16⁻) monocytes. Whole blood was stained with fluorochrome-conjugated antibodies to CD45, CD14, CD16, and LRP1, and samples were analyzed by flow cytometry after red cell lysis. **B:** Analytical (aCV%) and within-individual biological (bCV%) variation of LRP1 expression in monocyte subpopulations and in monocyte counts of healthy subjects. Bars represent the mean value (%) (± 1 standard error of the mean) of aCV% (white bars) and bCV% (gray bars) of LRP1 expression (left line) and monocyte heterogeneity (right line) in classical (Cl), intermediate (In), and nonclassical (Ncl) monocytes. The variability of LRP1 expression and monocyte heterogeneity were established using the flow cytometry assay based on the LRP1 monocyte-gating strategy. Ten healthy subjects (five females and five males) were studied during 6 weeks with intervals of one by week.

subjects. Thus, LRP1 is highly expressed in the monocyte subpopulation that possesses a high inflammatory potential, as they are the main producers of reactive oxygen species in unstimulated conditions and selectively produce IL1 β and TNF α on LPS stimulation (5, 36). On the other hand, LRP1 is expressed at low levels in nonclassical monocytes, which are induced to secrete IL1 β and TNF α by viruses and nucleic acids via the TLR7-TLR8-MyD88-MEK pathway rather than by LPS stimulation (36). Interestingly, it has been reported that LRP1 can mediate the induction of inflammatory cytokines in Schwann cells (37), as well as MMP-9 production in macrophages (12), when these cells are stimulated by the LRP1

ligand, α_2 -macroglobulin. However, it is unknown whether inflammatory factors can modify the LRP1 expression in monocyte subpopulations. In this way, it has been reported that LRP1 is upregulated in total monocytes of human immunodeficiency virus 1-infected long-term nonprogressor patients, suggesting that this receptor may maintain CD8⁺ responses in these patients (19). Thus, further studies are required to establish the functional roles and stability of LRP1 in each monocyte subpopulation during inflammatory processes.

Early studies have shown that bone marrow precursors give rise to monocytes in blood, which circulate for a few days (2–3 days) before they migrate into the tissue where they develop into different types of macrophages (38). At present, scarce data are available on biological factors affecting levels of monocyte subpopulations. Recently, it was reported that intermediate monocytes undergo diurnal variation, which were highest at 6 p.m. and lowest at 6 a.m. (39). In addition, the cellular regulation of LRP1 expression is very complex and can be mediated at the gene level by transcriptional regulation as well as by intracellular traffic through protein sorting to the plasma membrane (40–42). The *lrp1* gene expression is upregulated by intracellular cholesterol ester accumulation and hypoxia in cardiomyocytes and VSMCs in patients with ischemic cardiomyopathy (43). Thus, it can be hypothesized that LRP1 levels in monocytes depend on regulatory factors of LRP1 expression as well as on the biological variations of monocytes. In this way, both LRP1 expression and monocyte subpopulations in peripheral blood undergo natural fluctuations around a homeostatic setting point of each subject, which is known as within-individual biological variation (23). Here, we established the within-individual biological variation (bCV%) for both parameters and, on the basis of the variability data, concluded that the bCV% of LRP1 in monocytes and in each monocyte subpopulation were very comparable. Finally, the total variation yield by the measurement of LRP1 and monocyte subpopulations $[(aCV\%^2 + bCV\%^2)^{1/2}]$ was not higher than 20%. These results obtained from healthy subjects will allow us to evaluate the clinical significance of relative changes in the value of LRP1 expression and monocyte counts in different inflammatory processes, such as atherosclerosis. A priori, we conclude that changes in LRP1 expression and monocyte subpopulation counts lower than 20% will not be clinically relevant because this value is influenced by natural fluctuations in peripheral blood. However, further clinical studies are necessary to verify this possibility with special interest in atherosclerosis and cardiovascular disease.

In conclusion, we propose that the flow cytometry assay based on the LRP1 monocyte-gating strategy may be used to determine, in a unique procedure, monocyte heterogeneity and LRP1 expression in monocytes. These parameters have clinical diagnostic interest in inflammatory diseases, particularly in atherosclerosis. Interestingly, our assay may also be used with other monocyte markers involved in the development of atherosclerosis, such as CD36, CCR2, CCR5, CX3CR1, and CD11a, and thus constitute an attractive

diagnostic tool for the clinical study of subjects with high risk of cardiovascular disease.

ACKNOWLEDGMENTS

The authors thank Dr. Macarena Sahores for her technical contribution to this work and Gabriela Díaz Cortez for her language assistance. D.F. and J.J.-F. are doctoral fellows of CONICET. G.A.C. is a member of the Research Career of CONICET.

LITERATURE CITED

- Hume DA. The mononuclear phagocyte system. *Curr Opin Immunol* 2006;18:49–53.
- Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, Leenen PJ, Liu YJ, MacPherson G, Randolph GJ, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood* 2010;116:e74–e80.
- Gratchev A, Sobenin I, Orekhov A, Kzyshkowska J. Monocytes as a diagnostic marker of cardiovascular diseases. *Immunobiology* 2012;217:476–482.
- Heimbeck I, Hofer TP, Eder C, Wright AK, Frankenberger M, Marei A, Boghdadi G, Scherberich J, Ziegler-Heitbrock L. Standardized single-platform assay for human monocyte subpopulations: Lower CD14+CD16++ monocytes in females. *Cytometry Part A* 2010;77A:823–830.
- Zawada AM, Rogacev KS, Rotter B, Winter P, Marell RR, Fliser D, Heine GH. SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. *Blood* 2011;118:e50–e61.
- Rogacev KS, Seiler S, Zawada AM, Reichart B, Herath E, Roth D, Ulrich C, Fliser D, Heine GH. CD14++CD16+ monocytes and cardiovascular outcome in patients with chronic kidney disease. *Eur Heart J* 2011;32:84–92.
- Herz J, Strickland DK. LRP: A multifunctional scavenger and signaling receptor. *J Clin Invest* 2001;108:779–784.
- Gotthardt M, Trommsdorff M, Nevitt MF, Shelton J, Richardson JA, Stockinger W, Nimpf J, Herz J. Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. *J Biol Chem* 2000;275:25616–25624.
- Barnes H, Ackermann EJ, van der Geer P. v-Src induces Shc binding to tyrosine 63 in the cytoplasmic domain of the LDL receptor-related protein 1. *Oncogene* 2003;22:3589–3597.
- Strickland DK, Ranganathan S. Diverse role of LDL receptor-related protein in the clearance of proteases and in signaling. *J Thromb Haemost* 2003;1:1663–1670.
- Hu K, Yang J, Tanaka S, Gonias SL, Mars WM, Liu Y. Tissue-type plasminogen activator acts as a cytokine that triggers intracellular signal transduction and induces matrix metalloproteinase-9 gene expression. *J Biol Chem* 2006;281:2120–2127.
- Caceres LC, Bonacci GR, Sanchez MC, Chiabrandi GA. Activated $\alpha(2)$ macroglobulin induces matrix metalloproteinase 9 expression by low-density lipoprotein receptor-related protein 1 through MAPK-ERK1/2 and NF- κ B activation in macrophage-derived cell lines. *J Cell Biochem* 2010;111:607–617.
- Bonacci GR, Caceres LC, Sanchez MC, Chiabrandi GA. Activated $\alpha(2)$ -macroglobulin induces cell proliferation and mitogen-activated protein kinase activation by LRP-1 in the J774 macrophage-derived cell line. *Arch Biochem Biophys* 2007;460:100–106.
- Llorente-Cortes V, Badimon L. LDL receptor-related protein and the vascular wall: Implications for atherothrombosis. *Arterioscler Thromb Vasc Biol* 2005;25:497–504.
- Boucher P, Herz J. Signaling through LRP1: Protection from atherosclerosis and beyond. *Biochem Pharmacol* 2011;81:1–5.
- Carter CJ. Convergence of genes implicated in Alzheimer's disease on the cerebral cholesterol shuttle: APP, cholesterol, lipoproteins, and atherosclerosis. *Neurochem Int* 2007;50:12–38.
- Liu Q, Zerbiniatti CV, Zhang J, Hoe HS, Wang B, Cole SL, Herz J, Muglia L, Bu G. Amyloid precursor protein regulates brain apolipoprotein E and cholesterol metabolism through lipoprotein receptor LRP1. *Neuron* 2007;56:66–78.
- Moestrup SK, Gliemann J, Pallesen G. Distribution of the $\alpha 2$ -macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res* 1992;269:375–382.
- Stebbing J, Gazzard B, Kim L, Portsmouth S, Wildfire A, Teo I, Nelson M, Bower M, Gotch F, Shaunak S, et al. The heat-shock protein receptor CD91 is up-regulated in monocytes of HIV-1-infected “true” long-term nonprogressors. *Blood* 2003;101:4000–4004.
- Llorente-Cortes V, Estruch R, Mena MP, Ros E, Gonzalez MA, Fito M, Lamuela-Raventos RM, Badimon L. Effect of Mediterranean diet on the expression of pro-atherogenic genes in a population at high cardiovascular risk. *Atherosclerosis* 2010;208:442–450.
- Mosig S, Rennert K, Krause S, Kzyshkowska J, Neunubel K, Heller R, Funke H. Different functions of monocyte subsets in familial hypercholesterolemia: Potential function of CD14+ CD16+ monocytes in detoxification of oxidized LDL. *FASEB J* 2009;23:866–874.
- Moestrup SK, Kalltoft K, Petersen CM, Pedersen S, Gliemann J, Christensen EI. Immunocytochemical identification of the human $\alpha 2$ -macroglobulin receptor in monocytes and fibroblasts: Monoclonal antibodies define the receptor as a monocyte differentiation antigen. *Exp Cell Res* 1990;190:195–203.
- Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. *Crit Rev Clin Lab Sci* 1989;27:409–437.
- Barcelona PF, Jaldin-Fincati JR, Sanchez MC, Chiabrandi GA. Activated $\alpha 2$ -macroglobulin induces Muller glial cell migration by regulating MT1-MMP activity through LRP1. *FASEB J* 2013;27:3181–3197.
- Zawada AM, Rogacev KS, Schirmer SH, Sester M, Bohm M, Fliser D, Heine GH. Monocyte heterogeneity in human cardiovascular disease. *Immunobiology* 2012;217:1273–1284.
- Rogacev KS, Ulrich C, Blomer L, Hornof F, Oster K, Ziegler M, Cremers B, Grenner Y, Geisel J, Schlitt A, et al. Monocyte heterogeneity in obesity and subclinical atherosclerosis. *Eur Heart J* 2010;31:369–376.
- Bu G, Maksymovitch EA, Geuze H, Schwartz AL. Subcellular localization and endocytic function of low density lipoprotein receptor-related protein in human glioblastoma cells. *J Biol Chem* 1994;269:29874–29882.
- Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: Role in vascular wall integrity and protection from atherosclerosis. *Science* 2003;300:329–332.
- Aguilar H, Alvarez-Errico D, Garcia-Montero AC, Orfao A, Sayos J, Lopez-Botet M. Molecular characterization of a novel immune receptor restricted to the monocytic lineage. *J Immunol* 2004;173:6703–6711.
- Spijkers PP, da Costa Martins P, Westein E, Gahmberg CG, Zwaginga JJ, Lenting PJ. LDL-receptor-related protein regulates $\beta 2$ -integrin-mediated leukocyte adhesion. *Blood* 2005;105:170–177.
- Evans JH, Horowitz A, Mehrabi M, Wise EL, Pease JE, Riley EM, Davis DM. A distinct subset of human NK cells expressing HLA-DR expand in response to IL-2 and can aid immune responses to BCG. *Eur J Immunol* 2011;41:1924–1933.
- Barcelona PF, Ortiz SG, Chiabrandi GA, Sanchez MC. $\alpha 2$ -Macroglobulin induces glial fibrillary acidic protein expression mediated by low-density lipoprotein receptor-related protein 1 in Muller cells. *Invest Ophthalmol Vis Sci* 2011;52:778–786.
- Song H, Li Y, Lee J, Schwartz AL, Bu G. Low-density lipoprotein receptor-related protein 1 promotes cancer cell migration and invasion by inducing the expression of matrix metalloproteinases 2 and 9. *Cancer Res* 2009;69:879–886.
- Gonias SL, Gaultier A, Jo M. Regulation of the urokinase receptor (uPAR) by LDL receptor-related protein-1 (LRP1). *Curr Pharm Des* 2011;17:1962–1969.
- Boucher P, Li WP, Matz RL, Takayama Y, Auwerx J, Anderson RG, Herz J. LRP1 functions as an atheroprotective integrator of TGF β and PDGF signals in the vascular wall: Implications for Marfan syndrome. *PLoS One* 2007;2:e448.
- Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, Puel A, Biswas SK, Moshous D, Picard C, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* 2010;33:375–386.
- Shi Y, Yamauchi T, Gaultier A, Takimoto S, Campana WM, Gonias SL. Regulation of cytokine expression by Schwann cells in response to $\alpha 2$ -macroglobulin binding to LRP1. *J Neurosci Res* 2011;89:544–551.
- Liu K, Waskow C, Liu X, Yao K, Hoh J, Nussenzweig M. Origin of dendritic cells in peripheral lymphoid organs of mice. *Nat Immunol* 2007;8:578–583.
- Shantsila E, Tapp LD, Wrigley BJ, Montoro-Garcia S, Ghattas A, Jaipersad A, Lip GY. The effects of exercise and diurnal variation on monocyte subsets and monocyte-platelet aggregates. *Eur J Clin Invest* 2012;42:832–839.
- Castellano J, Aledo R, Sendra J, Costales P, Juan-Babot O, Badimon L, Llorente-Cortes V. Hypoxia stimulates low-density lipoprotein receptor-related protein-1 expression through hypoxia-inducible factor-1 α in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2011;31:1411–1420.
- Llorente-Cortes V, Casani L, Cal R, Llenas A, Juan-Babot O, Camino-Lopez S, Sendra J, Badimon L. Cholesterol-lowering strategies reduce vascular LRP1 overexpression induced by hypercholesterolemia. *Eur J Clin Invest* 2011;41:1087–1097.
- Laatsch A, Merkel M, Talmud PJ, Grewal T, Beisiegel U, Heeren J. Insulin stimulates hepatic low density lipoprotein receptor-related protein 1 (LRP1) to increase post-prandial lipoprotein clearance. *Atherosclerosis* 2009;204:105–111.
- Cal R, Castellano J, Revuelta-Lopez E, Aledo R, Barriga M, Farre J, Vilahur G, Nasarre L, Hove-Madsen L, Badimon L, et al. Low-density lipoprotein receptor-related protein 1 mediates hypoxia-induced very low density lipoprotein-cholesteryl ester uptake and accumulation in cardiomyocytes. *Cardiovasc Res* 2012;94:469–479.