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ORIGINAL ARTICLE

Isolation of functional mature peritoneal macrophages from healthy humans

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Abstract

Macrophages play an important role in the inflammatory response. Their various biological functions are induced by different membrane receptors, including Toll-like receptors, which trigger several intracellular signaling cascades and activate the inflammasomes, which in turn elicit the release of inflammatory mediators such as cytokines. In this study, we present a novel method for the isolation of human mature peritoneal macrophages. This method can be easily implemented by gynecologists who routinely perform laparoscopy for sterilization by tubal ligation or surgically intervene in benign gynecological pathologies. Our method confirms that macrophages are the main peritoneal leukocyte subpopulation isolated from the human peritoneum in homeostasis. We showed that primary human peritoneal macrophages present phagocytic and oxidative activities, and respond to activation of the main proinflammatory pathways such as Toll-like receptors and inflammasomes, resulting in the secretion of different proinflammatory cytokines. Therefore, this method provides a useful tool for characterizing primary human macrophages as control cells for studies of molecular inflammatory pathways in steady-state conditions and for comparing them with those obtained from pathologies involving the peritoneal cavity. Furthermore, it will facilitate advances in the screening of anti-inflammatory compounds in the human system.

INTRODUCTION

Macrophages are a heterogeneous cell population that belong to innate immune system capable of initiating the adaptive immune response and have a critical role in the development and maintenance of different metabolic, degenerative and inflammatory diseases. Macrophages are present in virtually all tissues of the organism as resident cells or as migratory cells recruited from peripheral blood monocytes following any challenge, such as inflammation, tissue injury or pathogen invasion.[1] Significant functional and phenotypical heterogeneity has been described in

macrophages, which results from their specialization for each particular environment.[2] Currently, most studies on tissue macrophages have been performed in murine peritoneal macrophages[3] or *in vitro* human blood monocyte-differentiated macrophages.[4]

Peritoneal macrophages play a crucial role in infectious and inflammatory diseases[5] through multiple effector mechanisms and immune regulatory functions.[6] Although murine peritoneal macrophages are one of the most studied types of these cells, little is known about human peritoneal macrophages in homeostasis. This is mainly because of the difficulty in obtaining these cells from healthy donors, which in turn has hampered the study of their intracellular signaling pathways and the development of robust drug screening data and validation in primary human mature macrophages.

In this study, we present an optimized method for obtaining peritoneal macrophages from the peritoneal cavity of healthy women undergoing tubal ligation sterilization by laparoscopy. Our results confirm that macrophages correspond to the largest peritoneal leukocyte subpopulation in steady state. These peritoneal macrophages have functional signaling pathways, such as phagocytosis and activation of main proinflammatory pathways including Toll-like receptors and inflammasome. Therefore, this method provides a useful tool for characterizing primary human macrophages as control cells for studies of molecular inflammatory pathways in steady-state conditions as well as for comparing them with those obtained from pathologies involving the peritoneal cavity.

RESULTS

Macrophages are the main leukocyte subset present in the human peritoneal cavity

A consecutive series of 212 individuals undergoing sterilization by tubal ligation or with benign gynecological pathologies was recruited for this study. Healthy peritoneal cell samples were obtained by laparoscopy (Figure 1) or laparotomy for tubal ligation or benign gynecological pathologies. After ocular inspection during surgery or pathological analysis of biopsies, samples from 15 individuals were discarded as healthy controls because of pathology confirmation, carcinoma ($n = 4$) or endometriosis ($n = 11$). From the healthy individual samples, we discarded 41 samples owing to small cellularity that was insufficient for further analyses, and 10 samples that were contaminated with peripheral blood during surgery. Therefore, 68.9% of samples obtained from individuals who fulfilled all the inclusion criteria were finally validated as healthy control samples with enough cells, which resulted in a sample size of $n = 146$. Clinical and analytical characteristics of the individuals are detailed in Table 1. As Table 1 also shows, the amount of peritoneal fluid present in the peritoneal cavity of healthy individuals was very scarce or unavailable.

We initially used flow cytometry analysis to characterize the different leukocyte subpopulations found in steady-state human peritoneum (see gating strategy in Supplementary figure 1). Results presented in Figure 2 showed that macrophages are the main leukocyte subset present in the peritoneal cavity, with a ratio of 52.95 $\pm$ 26.5% (median of % $\pm$ interquartile range), followed by T lymphocytes (23.5 $\pm$ 27.4%). We could also identify a smaller proportion of dendritic cells (9.25 $\pm$ 11%), polymorphonuclear (PMN) cells (2.45 $\pm$ 5.1%), natural killer cells (0.7 $\pm$ 0.9%) and practically no B lymphocytes (0.09 $\pm$ 0.2%).

The distribution of macrophages and PMN subsets validates the purity of peritoneal lavages

One of the main contaminant cells in the peritoneal lavage is peripheral blood cells, which can alter the analysis of resident peritoneal leukocyte populations. A fast initial test to indirectly determine the samples with high blood contamination is to look for red coloration in the peritoneal lavages. By measuring the absorbance of the cell-free lavages obtained by centrifugation of samples, we were able to measure the level of hemolysis in the lavages as an indirect measurement of blood contamination and set up a threshold to initially discard these samples. We established that samples with a corrected absorbance at 415 nm higher than 0.05 could be directly discarded (Figure 3a) and thus they were not included in the study. Lavage samples with red pellet after centrifugation or with a corrected absorbance smaller than, but close to 0.05 in the cell-free supernatant, were suspected of blood contamination and were further analyzed by flow cytometry.

The differential percentage of mononuclear myeloid and PMN cells in blood and peritoneal lavage was found to be a strong indicator of blood contamination in peritoneal samples (Figure 3b). Uncontaminated peritoneal lavage samples should have a leukocyte content lower than 22% of PMN and higher than 28% of macrophages (Figure 3b); these samples could be considered as free of blood contamination. Hence, the analysis of cell size and complexity could be used to quickly determine blood contamination in peritoneal lavage samples (Figure 3c).

The number of macrophages obtained in peritoneal lavages is independent of body mass index, age and washing volume

We then found that the mean number of total leukocytes and macrophages obtained from the peritoneal cavity of

healthy individuals was $29.9 \pm 4.3 \times 10^5$ and $15.6 \pm 2.6 \times 10^5$ cells, respectively (Table 1), with a percentage of macrophages of 59.42 ± 1.7%. In all cases, the samples showed a viability rate above 95% as determined by optical microscopy using the Trypan Blue exclusion technique. The number and percentage of recovered leukocytes and macrophages did not change according to body mass index or age (Figure 4a, b). Therefore, abdominal fat does not hinder the peritoneal lavage and macrophage isolation procedure.

We also studied the effect of the volume of solution used for the peritoneal lavage on the isolation of peritoneal leukocytes and macrophages. The absence of correlation between the number and percentage of recovered

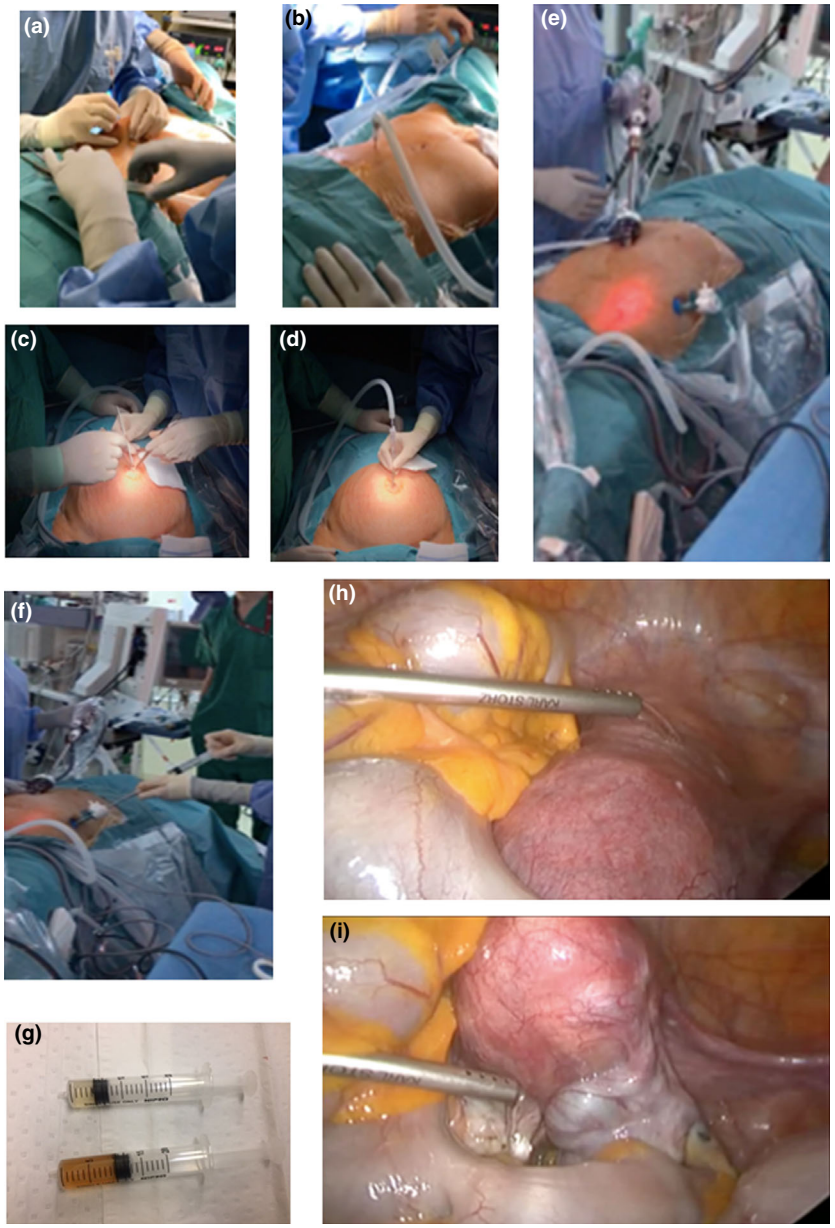

Figure 1. Obtaining human peritoneal cells from the peritoneal cavity of healthy women by laparoscopy. The peritoneal cavity was accessed using a Veress needle at the **(a, b)** Palmer's point or **(c, d)** umbilical point to minimize possible hematic contamination. Once the primary umbilical trocar was established, **(e)** a single 5-mm accessory trocar was inserted in the right or left iliac fossa. **(f, g)** The peritoneal fluid was aspirated by an endoscopic aspirator and a sterile syringe. The peritoneal surfaces were then washed with 10 mL of sterile saline without anticoagulants and the peritoneal lavage with its cellular content was collected in **(h)** the vesicouterine space and **(i)** the pouch of Douglas.

Table 1. Clinical and analytical characteristics of patients included in the study.

Study patients	Healthy controls (n = 212)		
Sample type	Valid control	Blood contaminated	Low cellularity
Method, n (%)	Laparoscopy 141 (96.6) Laparotomy 5 (3.4)	Laparoscopy 8 (80) Laparotomy 2 (20)	Laparoscopy (100) Laparotomy (0)
Female sex, n (%)	146 (100)	10 (100)	41 (100)
Age (years)	39.4 ± 1	42.1 ± 2.9	44.9 ± 1.9*
Etiology, %	Tubal ligation (13.7) Benign cysts (86.3)	Tubal ligation (0) Benign cysts (100)	Tubal ligation (10) Benign cysts (90)
Peritoneal liquid (mL), range	6.85 ± 2.6 (5–8.7)	n.d.	n.d.
Peritoneal washing (mL), range	47.9 ± 2.4 (5–112)	35.9 ± 10.2 (5–85)	38.7 ± 4.1 (6–100)
Body mass index (kg m^{-2})	25.8 ± 0.5	23.7 ± 0.7	27.7 ± 3.5
Leukocytes ($\times 10^5$)	29.9 ± 4.3	32.1 ± 5.2	0.5 ± 1.5
Macrophages ($\times 10^5$)	15.6 ± 2.6	4.04 ± 1.3	0.14 ± 0.04

Data represent the mean ± s.e.m. (Mann–Whitney U-test). n.d., not determined. *P-value < 0.05.

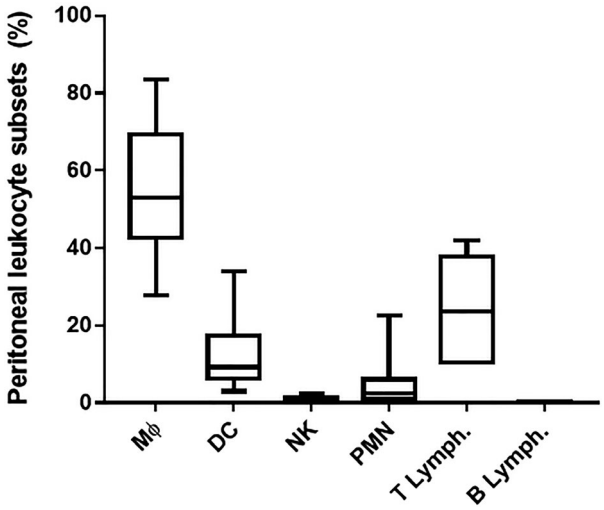

Figure 2. Identification of leukocyte subsets in healthy human peritoneal samples. Flow cytometry was used to analyze both the morphology and CD receptor expression in the leukocyte subpopulations. The figure shows the percentage of macrophages (Mφ) ($CD33^+$), dendritic cells (DCs; $CD1c^+$), natural killer cells ($CD3^-CD14^-CD19^-CD16^+$), polymorphonuclear (PMN), T lymphocytes ($CD3^+$) and B lymphocytes ($CD19^+$) present in peritoneal samples from healthy participants (30 000–200 000 cells analyzed/sample, n = 51). Lymph., lymphocytes; NK, natural killer.

leukocytes and macrophages with the volume of solution used (Figure 4c) indicated that the yield of leukocytes or macrophages is not directly related to the peritoneal washing volume. Then, we selected 10 mL as the recommended volume for the washings. This result suggests that this technical procedure is able to recover low-adherent or nonadherent human leukocytes from the peritoneum.

Microscopic determination of human primary mature macrophages

We routinely analyzed the cells obtained from peritoneal lavages with a phase contrast microscope followed by flow cytometry analysis. Microscopic observation allows us to distinguish the erythrocytes from the leukocytes and to estimate the percentage of macrophages from the leukocyte population. Comparison of further cytometry analyses and several microscopic counts performed by four different researchers revealed that microscopic macrophage estimation yields similar results to cytometric analysis; however, the former is a faster, easier and cheaper method (Figure 5a). Furthermore, we confirmed with fluorescent microscopy that the cells previously considered to be macrophages according to phase contrast microscopy were indeed stained with myeloid-specific markers (Figure 5b and Supplementary figure 2), with a variation from 0.29 to 82.3 × 10^5 macrophage per peritoneal lavage. Samples with macrophage counts under this minimal value were considered to be "low cellularity samples," and thus further experimental analysis was not performed.

Isolated human peritoneal macrophages are functional

Macrophages ($CD33^+$ cells) present in peritoneal lavages were able to phagocytize bacteria, showing 65.9 ± 8.7% of phagocytic cells after 10 min incubation with *Escherichia coli* at 37°C, and an intracellular bacterial load of phagocytic cells of 13.2 ± 3.2 measured as median fluorescence intensity, whereas the remaining cells ($CD33^-$ cells) showed no phagocytic activity (Figure 6a). Peritoneal macrophages were also able to increase the production of reactive oxygen species; thus, 75.5 ± 2.8% of macrophages showed oxidative potential and the median fluorescence

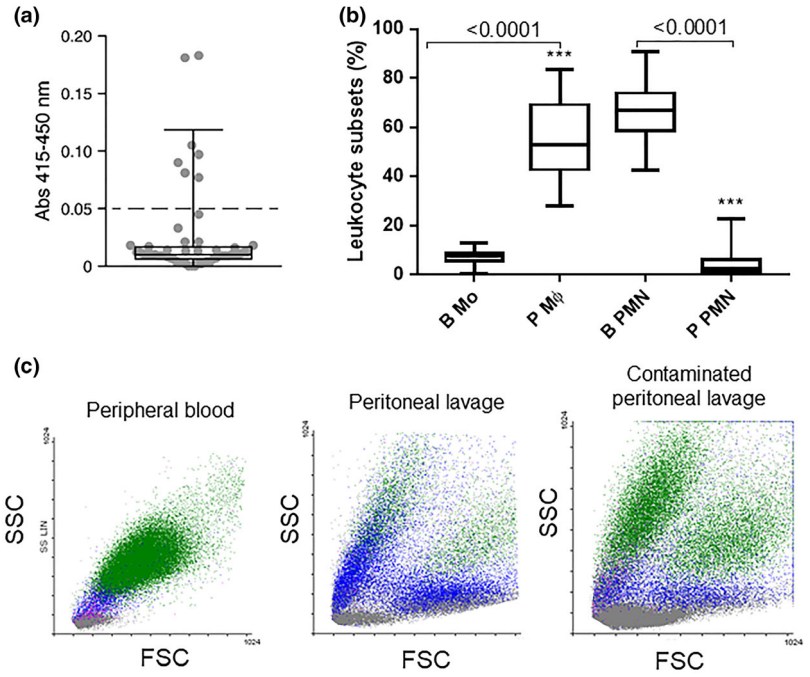

Figure 3. Identification of peripheral blood-contaminated peritoneal lavages. **(a)** Absorbance of cell-free peritoneal lavage samples (performed in triplicate, $n = 56$). **(b)** Flow cytometry was used to analyze both the morphology and CD receptor expression in leukocyte subpopulations of peripheral blood or peritoneal lavage samples from healthy controls (30 000–200 000 cells analyzed/sample, $n = 97$). The percentages of myeloid mononuclear and polymorphonuclear (PMN) cells present in both anatomical locations are shown. (B, blood; P, peritoneum; Mo, monocyte; Mφ, macrophage). **(c)** Representative fluorescence-activated cell sorting dot plots show the forward/side scatter distribution (FSC/SSC) of cells in peripheral blood, noncontaminated peritoneal lavages or blood-contaminated peritoneal lavages. Blue colored events represent the myeloid mononuclear cells and green events represent the PMN cells. Mann–Whitney *U*-test between the anatomical locations for the same leukocyte subset. ***P-value < 0.001. Abs, absorbance.

intensity values for the macrophage population with oxidative capacity were 10.4 ± 1.6% (Figure 6b). Next, we found that peritoneal macrophages were able to release interleukin-6 (IL-6) and tumor necrosis factor-α after lipopolysaccharide (LPS) stimulation (Figure 7a), and also IL-1β in response to LPS and adenosine triphosphate (ATP) treatment (Figure 7c), a specific trigger for the NLRP3 inflammasome.[7,8] Then, in addition to the release of IL-1β, the activation of the inflammasome was further assessed using the flow cytometry technique time-of-flight inflammasome evaluation.[9] After NLRP3 activation by LPS and ATP stimulation, the $CD33^+$ cells presented oligomeric ASC specks, whereas the $CD33^-$ population did not (Figure 7b, c and Supplementary figure 3). Hence, peritoneal macrophages were able to oligomerize ASC (Figure 7b, c) and release IL-1β to the media (Figure 7c) upon specific NLRP3 activation by LPS and ATP.

DISCUSSION

Studies of macrophages from humans are commonly performed with cell lines or *in vitro*-differentiated blood monocytes, whose phenotypes and functional abilities do not necessarily reflect the physiological responses of mature primary human macrophages. In addition, extended culture of macrophage cell lines can induce gene loss and diminished immune functions in these cell lines.[10] Thus, isolating human primary macrophages is critical to advancing our knowledge of human macrophages. The difficulties that are inherent in routinely obtaining human primary macrophage samples from healthy individuals have impaired the study of biological functions of tissue-resident macrophages, functions that include intracellular activation pathways, cell differentiation patterns, effector functions and *in vitro* drug testing. In this study we described a method for obtaining primary mature human peritoneal macrophages isolated from healthy individuals. We show that these healthy human tissue-resident macrophages actively respond to stimuli as they are able to phagocytize bacteria, induce respiratory oxidation to kill them, activate the NLRP3 inflammasome and secrete proinflammatory cytokines.

Macrophages are tissue-resident cells that in homeostatic conditions are maintained by self-renewal

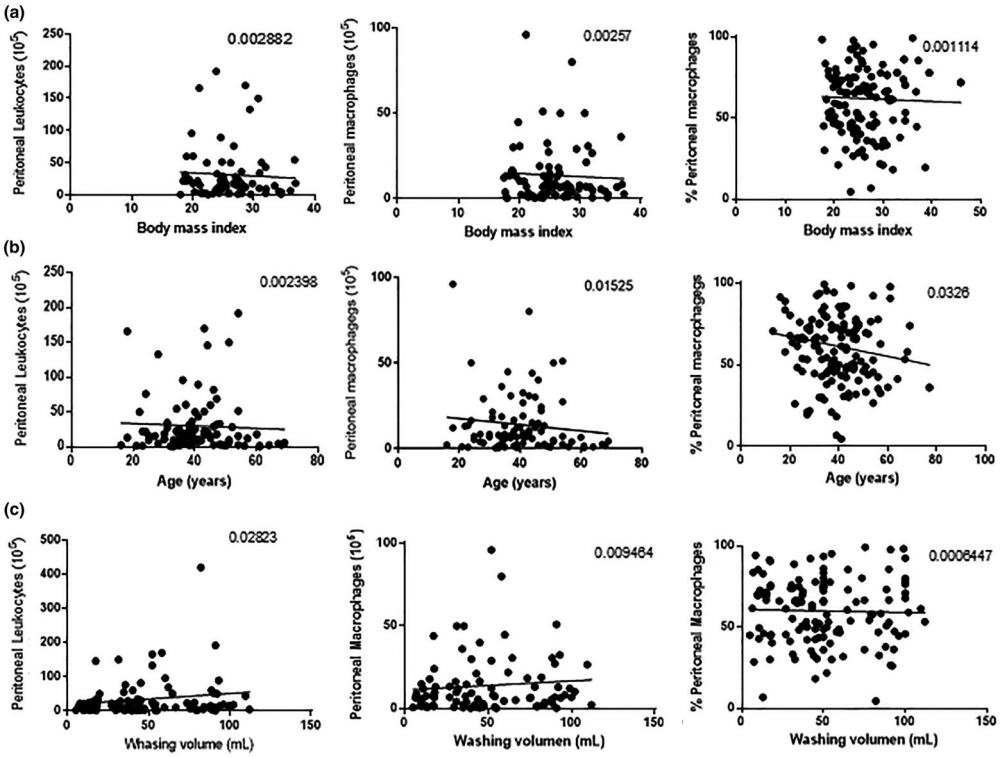

Figure 4. Peritoneal leukocyte and macrophage isolation are not related to body mass index, age or washing volume. Correlation analysis between the number of peritoneal leukocytes, macrophages (n = 97) or the percentage of macrophages (n = 146) obtained by flow cytometry (30 000–200 000 cells analyzed/sample) and **(a)** the body mass index and **(b)** age of patients, or **(c)** the washing volume used in the isolation protocol. *P*-values are indicated in the graphs.

and by the infiltration of blood monocytes in response to infection or tissue injury.[11] To study the biology of macrophages, mature macrophages from mice have been extensively used, especially murine peritoneal macrophages.[12] To date, the most common murine macrophage sources are bone marrow, the spleen, and the peritoneal cavity. Compared with bone marrow-derived macrophages and splenic macrophages, peritoneal macrophages appear to be more mature with a higher expression of inducible cytokines and are more stable in their functionality and phenotype.[10,13] Therefore, peritoneal macrophages isolated from the peritoneal cavity of mice are the most common source of macrophages for different *in vitro* assays, including phagocytosis, stimulation with Toll-like receptor ligands, cytokine and chemokine production, cell signaling assay and toxicology study.[14] Murine peritoneal macrophages are among the best studied tissue macrophages and play significant roles in clearing apoptotic cells[15] and coordinating inflammatory responses.[16–18] However, there are striking differences in the innate immune function and in particular macrophage biology between human and mouse species, as they present different surface antigen proteins and differences in the activation of major proinflammatory pathways and AIM2, NLRP3 or NLRP1 inflammasomes.[19–23] Therefore, we optimized a method to obtain human macrophages from the peritoneal cavity of healthy women. Our results confirm the existence of several peritoneal leukocyte subsets in steady state, of which the macrophages are the most abundant, followed by T lymphocytes. This is similar to mice, as mouse peritoneal macrophages are also the most abundant leukocyte subpopulation, although mice present an elevated subpopulation of B lymphocytes,[3,24] which our results indicate are practically not present in the human peritoneal cavity. The number of leukocytes obtained from the peritoneal cavity of healthy individuals in our study was low and varied depending on the donor, although all samples showed a high viability rate. Similarly, the number of macrophages in mouse peritoneum has been reported to be low and insufficient for extensive studies. Therefore, the use of sterile eliciting agents such as thioglycolate has increased the yield of macrophages obtained from the mouse peritoneum.[10] Studies from mouse peritoneal lavages reported minimal contamination with red blood cells; this is because of the

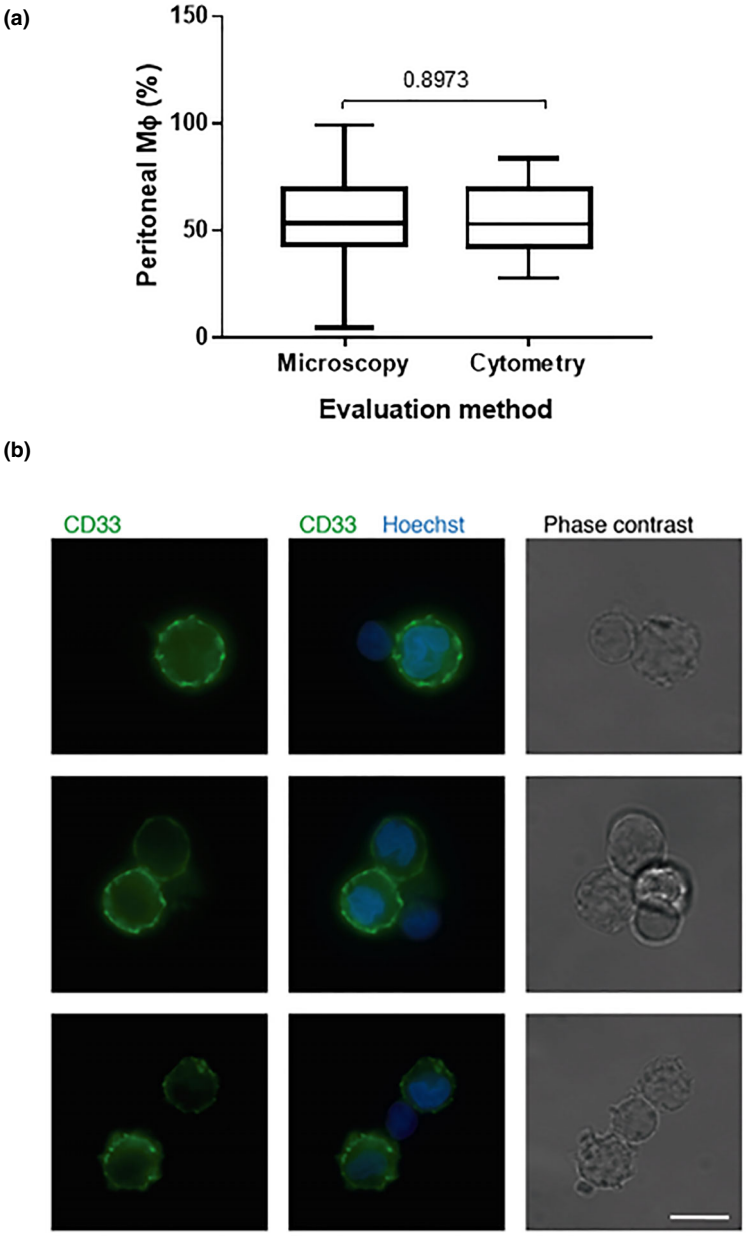

Figure 5. Microscopic determination of macrophages renders similar results to cytometric determination. **(a)** The percentage of macrophage subsets from the leukocyte population was determined by both microscopic cell counting (performed in duplicate and analyzed by four different researchers) and cytometry (30 000–200 000 cells analyzed/sample) in peritoneal lavage samples from healthy controls (n = 97). P-value between microscopy and cytometry is indicated in the graph. **(b)** Representative images of peritoneal cell suspension labeled with FITC-CD33 (green) and nuclei stained with Hoechst (blue); scale bar represents 10 μm. FITC, fluorescein isothiocyanate; Mφ, macrophage.

minimum tissue damage produced.[12] However, during human laparoscopy, and even more during laparotomy, we found that the presence of peripheral blood contamination could be a common phenomenon. This could be easily identified by routine inspection of the cell pellet color, reading the absorbance of the cell-free samples and by assessing the different proportions of PMN and myeloid mononuclear leukocyte subsets in human peritoneal lavage samples. This is an important issue because blood monocytes respond differently to stimuli than mature macrophages. In this sense, the commonly used *in vitro* model for studying human macrophages are monocytes-derived macrophages from blood, in which the addition of different differentiation

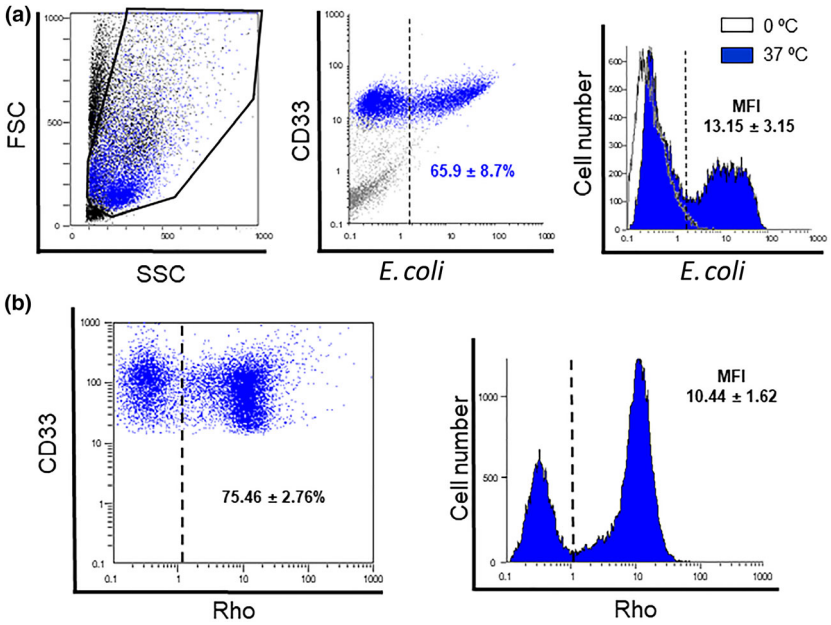

Figure 6. Phagocytic activity and oxidative potential of human peritoneal macrophages. **(a)** Phagocytosis analyzed by intracellular fluorescein isothiocyanate (FITC)-labeled *Escherichia coli* fluorescence (30 000–200 000 cells analyzed/sample, $n = 5$). Representative fluorescence-activated cell sorting (FACS) dot plots show morphological distribution of peritoneal cells (forward/side scatter). Phagocytosis of the gated population selected from morphological analysis was analyzed after incubation with the FITC-labeled *E. coli* for 10 min. Blue events represent $CD33^+$ macrophages and gray events represent $CD33^-$ cells. Empty histogram showing median fluorescence intensity (MFI) of peritoneal macrophages corresponds to the control-FITC signal obtained at 0°C and the blue filled histogram to FITC signal at 37°C. MFI values are indicated on the right panel. **(b)** Oxidative potential measured by dihydrorhodamine (DHR) 123-based assay (30 000–200 000 cells analyzed/sample, $n = 5$). Positive oxidative cells were detected following reactive oxygen species oxidation of DHR to green fluorescent rhodamine 123 (Rho). Representative FACS dot plots and histograms show Rho production of unstimulated peritoneal $CD33^+$ macrophages incubated with DHR. FSC, forward scatter; SSC, side scatter.

cytokines, protocols or culture conditions results in different macrophage phenotypes,[25] and thus complicates the interpretation of those results as representative of mature tissue-resident macrophage.

To date, the isolation of viable human tissue-resident macrophages has been restricted to samples isolated from different pathological scenarios, including lung macrophages from patients with chronic obstructive pulmonary disease,[26] tumor-associated macrophages from surgery recessions in oncology patients[27] and from peritoneal dialysis in patients with renal failure[28] or peritoneal ascites from cirrhotic patients.[29] During pathology, macrophages change their phenotype and activate different intracellular pathways. The use of disease-derived macrophages to study their biology and signaling pathways clearly means that functional tissue-resident controls obtained from healthy individuals are also needed. Studies in mice showed that macrophages of different origins, existing in the same milieu, are able to acquire different phenotypes that change during the inflammatory response.[30,31] These macrophage phenotypes are especially important to peritoneal immune response in individuals with acute and chronic peritoneal inflammation, as these cells are constantly exposed to inflammatory stimuli from translocating bacteria caused by intestinal permeability alterations. Furthermore, two mouse peritoneal macrophages subpopulations, large ($F4/80^{high}MHC\text{-}II^{low}$) and small ($F4/80^{low}MHC\text{-}II^{high}$), with different phenotypes and functions have recently been described as residing in the mouse peritoneal cavity.[32] Large peritoneal macrophages appear not to have originated from hematopoietic precursors and retain the capability to proliferate *in situ* to maintain physiological numbers, under steady-state conditions.[33,34] By contrast, small peritoneal macrophages, whose numbers increase noticeably under inflammatory conditions, appear to have originated from circulating monocytes.[6,32,34] Similarly, in patients with decompensated cirrhosis the presence of three or two functionally and phenotypically distinct macrophage subpopulations has been described based on the expression of CD14 and CD16[29] or the C–C chemokine receptor type 2 and the complement receptor of the immunoglobulin superfamily,[35] respectively, that share

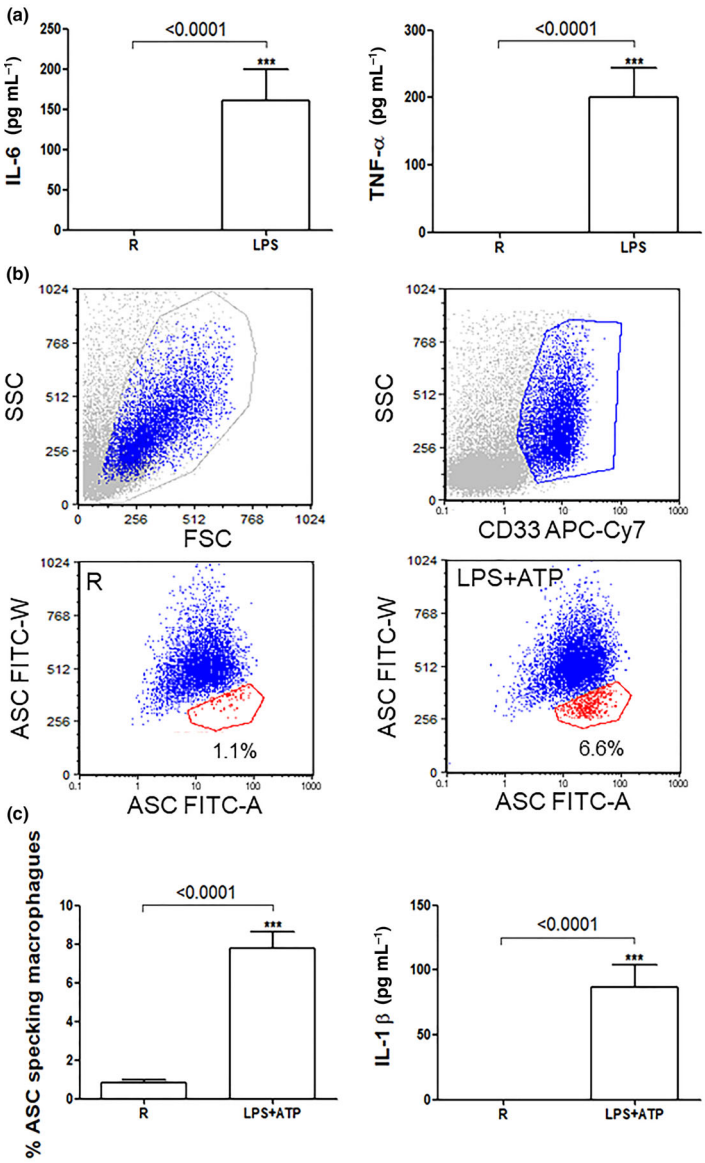

Figure 7. Inflammasome activation and production of proinflammatory cytokines by human peritoneal macrophages. **(a)** Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in supernatants from peritoneal macrophages stimulated or not with 1 μg mL^{-1} of LPS for 2 h (performed in triplicate, $n = 3$). **(b)** Representative flow cytometric plots showing the presence of intracellular ASC specks measured by time-of-flight inflammasome evaluation analysis in resting (R) and LPS + ATP-treated peritoneal $CD33^{+}$ macrophages (30 000–200 000 cells analyzed/sample, $n = 3$). **(c)** Percentage of ASC-specking peritoneal macrophages detected as in **b** and associated IL-1β release (performed in triplicate, $n = 3$). The *P*-value between R and LPS or LPS + ATP is indicated in the histograms. ****P*-value < 0.001. ATP, adenosine triphosphate; FSC, forward scatter; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; SSC, side scatter.

similarities with large and small peritoneal macrophages from mice. However, in contrast to mice, there is a lack of studies analyzing the distribution and function of peritoneal macrophage subpopulations under steady-state conditions in humans. This has hampered our understanding of this key innate immune cell in human pathology. With the method presented in this study, we are providing a tool that presents a reliable source to obtain mature tissue-resident macrophages in steady-state conditions[36] isolated from healthy individuals that could be used to compare the biology, phenotype and the role played by macrophages in different pathologies. Thus, we have demonstrated that the peritoneal macrophages isolated following the described protocol displayed functional pathways such as phagocytic and oxidative activities and activated major proinflammatory pathways

such as the NLRP3 inflammasome and the secretion of inflammatory cytokines. This corroborates previous studies from our group which described how human peritoneal macrophages were able to induce the production of prostaglandins or sense hypotonic-induced volume changes to activate the NLRP3 inflammasome[37–39] and expressed both proinflammatory and anti-inflammatory (M1 and M2) polarization markers, suggesting a constitutive preactivated state that allows to quickly respond to any stimulus and to maintain homeostasis in the peritoneal cavity.[36]

CONCLUSIONS

We have optimized a new method to isolate functional mature peritoneal macrophages, the most abundant human peritoneal leukocyte subpopulation in healthy individuals. As proof of principle, we present viability and functional data for these macrophages, importantly reporting the assembly of the NLRP3 inflammasome by detecting intracellular inflammasome oligomers using flow cytometry. We also present strong data supporting that these macrophages are distinct from blood monocytes and we provide several indications in our method on how to distinguish peripheral blood monocytes in a peritoneal lavage sample. By performing this methodology in samples from healthy individuals undergoing sterilization, we present previously undescribed material on human mature macrophage population that advances our general understanding of this cell type, that will facilitate advances in the screening of anti-inflammatory compounds in the human system and that will also open up the way to isolating macrophages from individuals affected with different diseases in which peritoneal macrophages could play an important role, such as endometriosis, hepatic cirrhosis or obesity.

METHODS

Study patients

Gynecological patients were admitted to the Gynaecology Units of the Hospital General Universitario Reina Sofía and the Hospital Clínico Universitario Virgen de la Arrixaca, both located in Murcia, Spain. The ethics committees (Comité Ético de Investigación Clínica del Hospital Clínico Universitario Virgen de la Arrixaca, Comité Ético de Investigación Clínica del Hospital Clínico Universitario Reina Sofía and Comité de Bioética de la Universidad de Murcia) approved the study protocol and all patients enrolled signed an informed consent form so they could be included in this study. Healthy peritoneal cell samples were obtained during laparoscopies for tubal ligation. We also included patients undergoing therapeutic laparoscopies or laparotomies for benign gynecological pathologies such as simple ovarian cysts. Exclusion criteria included immune diseases, inflammatory diseases, anti-inflammatory drugs intake, antibiotics other than routine surgical prophylaxis, age older than 80 or younger than 18 and refusal to participate in the study. A cohort of 212 patients who fulfilled all inclusion/exclusion criteria and signed the informed consent were included in the study.

Collection of human peritoneal lavage

Human peritoneal lavage was obtained from the peritoneal cavity of healthy women by clinical surgery (laparoscopy or laparotomy). By laparoscopy, the peritoneum was accessed with a Veress needle at the Palmer's point (Figure 1a, b) or umbilical point (Figure 1c, d) to minimize possible hematic contamination. CO_2 gas was insufflated to a maximum pressure of 18–20 mmHg to reduce the risk of lesion to intestinal loops and large vessels during blind insertion of the primary trocar. Then, the main umbilical trocar (11 mm, Optiview conical, Johnson & Johnson, New Brunswick, NJ, USA) was introduced using a lens of 0° with fixed light intensity at 180 W (Storz SCB Xenon 300 W at 60% intensity, Karl Storz, Tuttingen, Germany). Once the primary umbilical port was established, a single 5-mm accessory trocar was inserted in the right or left iliac fossa (Figure 1e). Then, we inspected the abdominal cavity, reduced the intra-abdominal pressure to 10–12 mmHg and placed the patient in the Trendelenburg position (30°). Any peritoneum fluid was aspirated by an endoscopic aspirator and a sterile syringe (Figure 1f, g). The peritoneal surfaces were then washed with 10 mL of sterile saline without anticoagulants and the peritoneal lavage was collected in the vesicouterine space (Figure 1h) and the pouch of Douglas (Figure 1i). It should be noticed that the time between the inoculation of the buffered solution to perform the lavage and its extraction should be over 1 min, and that both manual external massage and pressure on the abdominal wall to spread the inoculated solution were important to avoid low cellularity of the samples. Regardless of whether hysteroscopy and laparoscopy are necessary in the same procedure, the laparoscopic technique should be performed first to avoid hysteroscopic fluid contamination. The peritoneal lavage (Figure 1g) was transferred to sterile 100-mL flasks (Deltalab, Barcelona, Spain) and stored at 4°C. The maximum time between collection and placement under cold conditions should not exceed 20 min. Pathological examination of biopsies confirmed the inclusion criteria as valid healthy controls.

Determination of hemolysis in peritoneal lavage samples

The absorbance of the cell-free peritoneal lavage was measured at 380 415, and 450 nm using a Synergy Mx plate reader (BioTek, Wiltshire, UK) to quickly determine the degree of hemolysis as an indirect measurement of the amount of blood contamination in the samples.

Peritoneal macrophage isolation procedure

Peritoneal lavages were centrifuged at 500*g* and supernatants were frozen in aliquots at −80°C to determine the different biochemical parameters. Cellular pellets were washed in phosphate-buffered saline (PBS) and finally resuspended in Dulbecco's Modified Eagle Medium (GIBCO Invitrogen, Paisley, UK). Samples were then maintained at 4°C to prevent the cells from adhering to the plastic. The viability and the amount of recovered peritoneal cells were analyzed by optical microscopy using the Trypan Blue exclusion technique and counting in a Neubauer chamber.

Flow cytometry analysis

Peritoneal cell suspensions were stained with monoclonal antibodies and analyzed by flow cytometry to determine cell types. Antibodies were mouse antihuman CD14-FITC (eBioscience, San Diego, CA, USA), CD3-FITC, CD1c-FITC, CD19-PE Cy5, CD33-PE, CD14-PE and CD16-PE Cy5 (BD-Pharmingen, San Diego, CA, USA). The mouse IgG1-PE, mouse IgG1-FITC, mouse IgG1-PE Cy5, mouse IgG2b-FITC and mouse IgG2b-PE antibodies used as isotype controls were from BD-Pharmingen. In brief, peritoneal cells were washed once with PBS and twice with Roswell Park Memorial Institute-1640 medium (RPMI-1640 medium; GIBCO Invitrogen) before being resuspended at a ratio of 0.2×10^6 total white cells in a volume of 100 μL RPMI-1640. Next, cells were stained with 5 μL of the corresponding monoclonal antibody and incubated in the dark on ice, fixed with a fixing-lysing solution (Becton Dickinson, San José, CA, USA), washed twice with PBS and kept at 4°C in the dark until data acquisition. Absence of cell aggregates after resuspension was also determined by microscopy observation. In the case of peripheral blood, 50 μL samples were directly used, with no prior treatment, and then stained as previously explained.

Flow cytometry analyses were performed on a three-color fluorescence FACSCanto II (Becton Dickinson), Epics XL or FC500 (Beckman Coulter, Brea, CA, USA) using FACSCanto Software and Cytomics RXP Analysis Software, respectively, or version 2.5.1 of Flowing Software. A total of 30 000–200 000 gated events were acquired and analyzed. Leukocytes were gated based on forward *versus* side scatter on a linear scale. Then, leukocyte subpopulations were gated on the basis of morphology and $CD33^+$ expression for mononuclear myeloid cells, $CD1c^+$ for dendritic cells, $CD3^+$ expression for T lymphocytes, $CD19^+$ expression for B lymphocytes and $CD3^-CD14^-CD19^-CD16^+$ expression for natural killer cells. Cell aggregates were excluded on the basis of forward scatter-width *versus* forward scatter-area on a linear scale. Besides isotype controls, fluorescence minus one control assays were carried out in order to establish fluorescence control limits to ensure no spread of fluorescence between channels (Supplementary figure 1).

Microscope images

Peritoneal cell suspensions were stained with monoclonal antibody antihuman CD33-FITC as described earlier. Then, the cells were incubated with 1:2000 dilution of Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) in PBS to stain nuclei. Cells were placed in coverslips and imaged with a Nikon Eclipse Ti microscope equipped with a 20× S Plan Fluor objective (numerical aperture 0.45) and a digital Sight DS-QiMc camera (Nikon, Tokyo, Japan) and 387/447-nm and 482/536-nm filter sets (Semrock, Rochester, NY, USA).

Phagocytosis assays

Peritoneal cells were washed twice with RPMI-1640, and resuspended in RPMI-1640 at a ratio of 0.4×10^6 total cells in 100 μL. Phagocytic activity of macrophages was tested with the PHAGOTEST kit (BD-Pharmingen), which contains fluorescein [fluorescein isothiocyanate (FITC)]-labeled opsonized *E. coli*, following the manufacturer's instructions, allowing phagocytosis at 37°C for a short (10 min) and long (1 h) time assay. Cells were then also stained with the correspondent monoclonal mouse antihuman antibodies to characterize macrophage phagocytic activity. Flow cytometry analyses were performed using a FACSCanto II and Epics XL or FC500 (Beckman Coulter) with Cytomics RXP Analysis Software or version 2.5.1 of Flowing Software. A total of 30 000–200 000 gated events were acquired and analyzed. The percentage of positive phagocytic cells (positive for the FITC channel) and the median fluorescence intensity of the macrophage subpopulation were analyzed.

Reactive oxygen species production analysis

Peritoneal cells were washed with Dulbecco's Modified Eagle Medium and resuspended in Dulbecco's Modified Eagle Medium at a ratio of 0.4×10^6 total cells in 50 μL. For each sample, two tests were performed in parallel, one with the addition of phorbol myristate acetate (Sigma Chemical Co., St. Louis, MO, USA) and another without phorbol myristate acetate. Anti-CD33-PE antibody was added to the sample, together with 2.5 μL of dihydrorhodamine 123 (Merck, Darmstadt, Germany) and 10 μL of 2 mg mL^{-1} phorbol myristate acetate. Samples were then incubated for 15 min at 37°C and the oxidative reaction was interrupted by placing the sample tubes on ice and fixing the cells with 1 mL of fixing-lysing solution (Becton Dickinson). Cells were washed twice with PBS and kept at 4°C in the dark until data acquisition. Flow cytometry analyses were then performed, as explained earlier. Phorbol myristate acetate-induced reactive oxygen species oxidize dihydrorhodamine 123 to rhodamine 123, which possesses green fluorescence.

Inflammasome activation assay

Peritoneal cells (1×10^5) were resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum and treated with *E. coli* LPS serotype 055:B5 (Sigma-Aldrich; 1 μg mL^{-1}, 2 h at 37°C). LPS-primed cells were then stimulated with ATP (3 mM, 30 min at 37°C) and supernatants were collected for

cytokine determination. Cells were fixed with 2% paraformaldehyde and stained for the detection of intracellular ASC specks by the time-of-flight inflammasome evaluation technique as previously described.[39] The rabbit polyclonal anti-ASC (N-15)-R antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was used as primary antibody and the donkey antirabbit Alexa 488 (Thermo Fisher Scientific, Waltham, MA, USA) as secondary antibody. Peritoneal macrophages were detected with positive staining for APC-vio770 mouse antihuman CD33 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). Stained cells were acquired on a BD FACSCanto cytometer.

Cytokine release analysis

Human IL-1β was measured in cell supernatants from LPS + ATP-stimulated cells using an ELISA kit (Thermo Fisher Scientific). Human IL-6 and tumor necrosis factor-α were measured from LPS-stimulated cells using the Diaplex cytometric multiplexed fluorescent bead-based immunoassay (Diaclone, Besançon, France) and analyzed in a BD FACSCanto flow cytometer.

Statistical analysis

Categorical variables are reported as frequencies or percentages. Continuous variables are reported as median and interquartile range or mean ± s.e.m., and are graphically represented as histograms or box diagrams, where the top and bottom of the box are the 25th and 75th percentile (the lower and upper quartiles, respectively), the band inside the box is the 50th percentile (the median), the end of the whiskers represent the lowest datum still within 1.5 interquartile range of the lower quartile and the highest datum still within 1.5 interquartile range of the upper quartile. The observations considered as outliers are not represented. Statistical differences were analyzed using the Mann–Whitney *U*-test or Wilcoxon signed-rank test. All reported *P*-values are two-sided, and *P*-values lower than 0.05 were considered to indicate statistical significance. All calculations were performed using the SPSS 19.0 (IBM, New York, NY, USA) and GraphPad Prism 6 software (GraphPad, Chicago, IL, USA). Reporting of the study conforms to STROBE and EQUATOR guidelines.[40]

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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