Journal Pre-proof

Immunophenotyping of peripheral blood monocytes could help identify a baseline pro-inflammatory profile in women with recurrent reproductive failure

Alejandra Comins-Boo, Lorena Valdeolivas, Fernando Pérez-Pla, Ignacio Cristóbal, Nabil Subhi-Issa, Ángeles Domínguez-Soto, Lydia Pilar-Suárez, Pilar Gasca-Escorial, Marta Calvo-Urrutia, Miguel Fernández-Arquero, Miguel Ángel Herráiz, Ángel Corbí, Silvia Sánchez-Ramón

PII: S0165-0378(22)00264-9

DOI: https://doi.org/10.1016/j.jri.2022.103735

Reference: JRI103735

To appear in: *Journal of Reproductive Immunology*

Received date: 22 November 2021
Revised date: 23 July 2022
Accepted date: 19 August 2022

Please cite this article as: Alejandra Comins-Boo, Lorena Valdeolivas, Fernando Pérez-Pla, Ignacio Cristóbal, Nabil Subhi-Issa, Ángeles Domínguez-Soto, Lydia Pilar-Suárez, Pilar Gasca-Escorial, Marta Calvo-Urrutia, Miguel Fernández-Arquero, Miguel Ángel Herráiz, Ángel Corbí and Silvia Sánchez-Ramón, Immunophenotyping of peripheral blood monocytes could help identify a baseline pro-inflammatory profile in women with recurrent reproductive failure, *Journal of Reproductive Immunology,* (2021) doi:https://doi.org/10.1016/j.jri.2022.103735

Immunophenotyping of peripheral blood monocytes could help identify a baseline pro-inflammatory profile in women with recurrent reproductive failure.

Alejandra Comins-Boo[1,2], Lorena Valdeolivas[1,2], Fernando Pérez-Pla[3], Ignacio Cristóbal[4], Nabil Subhi-Issa[1,2], Ángeles Domínguez-Soto[5], Lydia Pilar-Suárez[4], Pilar Gasca-Escorial[4], Marta Calvo-Urrutia[4], Miguel Fernández-Arquero[1,2], Miguel Ángel Herráiz[4], Ángel Corbí[5] and Silvia Sánchez-Ramón[1,2]

[1]Department of Immunology, IML, and IdSSC, Hospital Clínico San Carlos. Madrid, Spain. [2]Department of Immunology, Ophthalmology, and ENT, School of Medicine, Complutense University School of Medicine. Madrid, Spain.
[3]Department of Applied Mathematics and Computational Science, University of Cantabria, Spain. [4] Department of Obstetrics and Gynecology, Hospital Clínico San Carlos. Madrid, Spain.
[5]Molecular Microbiology and Infection Biology Department, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain.

Correspondence:

Silvia Sánchez-Ramón, MD, PhD
Department of Clinical Immunology
Laboratory Medicine Institute
Hospital Clinico San Carlos and IdISSC
Calle Profesor Martín Lagos SN
28040-Madrid, Spain
Phone: +34 91 3303000 Ext 3342
Fax: +34 91 3303879
E-mail: ssramon@salud.madrid.org

Funding information

The project received a research grant from the Carlos III Institute of Health, Ministry of Economy and Competitiveness (Spain), awarded on the 2016 call under the Health Strategy Action 2016-2017, within the National Research Program oriented to Societal Challenges, within the Technical, Scientific and Innovation Research National Plan 2013-2016, with reference PI16/01428, and was co-supported by The European Regional Development Fund (ERDF)

Abstract

Recurrent pregnancy loss (RPL) and recurrent implantation failure (RIF) are two well-defined clinical entities, but the role of the monocytes in their pathophysiology needs to be clarified. This study aimed to evaluate the role of the three monocyte subsets (classical, intermediate, and non-classical) and relevant cytokines/chemokines in a cohort of RPL and RIF women to better characterize a baseline proinflammatory profile that could define inflammatory pathophysiology in these two different conditions. We evaluated 108 non-pregnant women: 53 RPL, 24 RIF, and 31 fertile healthy controls (HC). Multiparametric flow cytometry was used to quantify the frequency of surface chemokine receptors (CCR2, CCR5, and CX3CR1) on the monocyte subsets. Cytokines were assessed in plasma samples using a multiplex assay. The $CX3CR1^{+}$ and $CCR5^{+}$ intermediate monocytes were significantly higher in RPL and RIF compared to HC. A significant positive correlation was observed between $CX3CR1^{+}$ intermediate monocytes and IL-17A (P=.03, r=.43). The Boruta algorithm followed by a multivariate logistic regression model was used to select the most relevant variables that could help define RPL and RIF: in RPL were CX3CR1 non-classical monocytes, TGF-β1, and CCR5 intermediate monocytes; in RIF: CCR5 intermediate monocytes and TGF-β3. The combination of these variables could predict RPL and RIF with 90% and 82%, respectively. Our study suggests that a combination of specific blood monocyte subsets and cytokines could aid in identifying RPL and RIF women with a pro-inflammatory profile. These findings could provide a more integrated understanding of these pathologies. Further investigation and validation in independent cohorts are warranted.

Keywords: Recurrent Pregnancy Loss; Recurrent Implantation Failure; Monocytes; CCR5; CX3CR1

Introduction

Classically, recurrent reproductive failure (RRF) encompasses two different clinical conditions: recurrent pregnancy loss (RPL) and recurrent implantation failure (RIF) in women undergoing *in vitro* fertilization (IVF) techniques. The exact prevalence of RPL is quite challenging to estimate because it depends on the definitions and criteria used and the characteristics of the study population. Some groups have reported that RPL affects 2%–5% of couples (El Hachem et al., 2017). Limited studies have addressed RIF's incidence or prevalence (Bashiri et al., 2018) due to the variations in definitions. Some authors propose a prevalence of 10% (Somigliana et al., 2018).

It is well-established that these two entities have different etiologies and pathophysiology. Growing evidence highlights that immune dysregulation is associated with both RPL and RIF, although through diverse processes. Recently, it has been described how these conditions have a different immune

profile (Vomstein et al., 2020). Vomstein et al. showed lower peripheral T regulatory cells in RPL and higher uterine NK cells (uNK) in RIF patients. Other authors have found a differential expression of selected genes between RPL and RIF (Lédée et al., 2011). They observed that in RIF, cell-mediated immune response and nervous system development and function are highly dysregulated, while in RPL, the dysregulations mainly affect the humoral immune response, organ and tissue development, and muscular system development and function. Scarce data have been published comparing the role of monocytes in RPL and RIF, respectively.

RPL is a heterogeneous reproductive condition with multiple etiologies and contributing factors. However, in 50% of the patients, the case remains unexplained. (Mekinian et al., 2016; Rai and Regan, 2006). On the other hand, several risk factors for RIF that could be classified into maternal and embryonic factors have been described. (Bashiri et al., 2018; Simon and Laufer, 2012)

The disturbance of the maternal immune tolerance to the allogenic fetus could contribute to their etiopathogenesis (Mekinian et al., 2016). During normal pregnancy, there is an increase in innate immune cells at the feto-maternal interface, such as uterine natural killer (uNK) cells (~70%) and macrophages (Tang et al., 2015a) (20-30% of maternal immune cells). Monocytes are short-lived cells that emerge from a myelomonocytic precursor in the bone marrow, and they comprise about 5-10% of the total circulating blood leukocytes (M. M. Faas and de Vos, 2017). In the last few years, growing evidence shows that circulating monocytes infiltrate the decidua at the onset of pregnancy and differentiate into macrophages or dendritic cells (DCs). Decidual macrophages contribute to tolerance to fetal antigens. They are involved in essential processes for a successful pregnancy, like trophoblast invasion and tissue and vascular remodeling (Nagamatsu and Schust, 2010). In the last years, the M1/M2 macrophage polarization has been described based on their phenotypical and functional characteristics (Barros et al., 2013; Mills et al., 2000), and their development is regulated by several chemokines (Xuan et al., 2015). M1 macrophages are primarily involved in inflammatory responses with the secretion of TNF-α and IL-12. Meanwhile, M2 macrophages have immunosuppressive activity and participate in apoptosis or tissue remodeling (Mantovani et al., 2013).

Pregnancy has three distinct immunological phases characterized by distinct biological processes: the implantation requires a pro-inflammatory state, while the second phase is predominantly anti-inflammatory, and finally, a predominantly pro-inflammatory state governs parturition again (Förger and Villiger, 2020; Mor et al., 2011). Cytokines and cell surface receptors mediate communication between trophoblastic and decidual cells. Cytokines are elicited by trophoblastic and immune cells present within the decidua (natural killer cells, macrophages, and lymphocytes) (Saini et al., 2011). According

to that, there is a correlation between the M1/M2 macrophage polarization and the inflammatory phase during pregnancy. During the pre-implantation stage, activated M1 macrophages produce inflammatory cytokines such as IL-6, IL-1β, and TNF-α, promoting embryo implantation. Later and progressively, there is a switch towards the M2 subtype, promoting an anti-inflammatory state. Finally, M1 macrophages predominate over the M2 subset during the parturition period, predominantly inflammatory (Zhang et al., 2017).

Several cytokines and chemokines regulate the recruitment of monocytes to the decidua: monocyte chemoattractant protein-1 (MCP-1, CCL2), macrophage inflammatory protein 1-α (MIP-1α, CCL3), and regulated on activation normal T cell expressed and secreted (RANTES, CCL5) (Nagamatsu and Schust, 2010). C-C motif chemokine receptor-2 (CCR2) is a chemokine receptor expressed on monocytes (Björkander et al., 2013) and found on NK cells, immature DCs, and activated T cells. CCR2 is the receptor of MCP-1/CCL2, the main chemokine recruiting monocytes to tissues and produced by trophoblasts (Fest et al., 2007). One of the main functions of CCR2 is to mediate monocyte chemotaxis during inflammation (Yang et al., 2014). Monocytes also express the C-C motif chemokine receptor-5 (CCR5), the RANTES/CCL5 receptor (Björkander et al., 2013). CX3CR1 is the fractalkine receptor (CX3CL1), which exists in two forms: a membrane-anchored form that functions as an adhesion molecule and a soluble form that acts as a chemoattractant (Panek et al., 2015). CX3CR1 is expressed in monocytes, T cells, NK cells, and platelets (Postea et al., 2012) and mediates leukocyte migration and adhesion (Imai et al., 1997).

Three monocyte subsets have been described (Ziegler-Heitbrock et al., 2010) based on the expression of the lipopolysaccharide (LPS) receptor, CD14, and the Fcγ-III receptor, CD16: classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$), and non-classical monocytes ($CD14^{+}CD16^{++}$). Classical monocytes are the most common subset, representing 80-95% of total monocytes, and express a higher percentage of CCR2 and lower CX3CR1 (Weber et al., 2000; Yang et al., 2014). They are mainly phagocytic and preferentially express genes involved in angiogenesis and wound healing (Wong et al., 2011). The intermediate monocyte subset represents about 2-11% of total blood monocytes (Yang et al., 2014). They express high levels of CX3CR1 and the cytokine receptor CCR5 and display an inflammatory function with a high capacity to produce and release IL-1β and TNFα. The non-classical subset accounts for 2-8% of total monocytes and expresses high CX3CR1 and low CCR2 (Yang et al., 2014). They are recruited to non-inflamed tissues by CX3CR1 and are characterized by patrolling the resting vasculature, removing cell debris, and repairing the endothelium (Wolf et al., 2019). They are also characterized by secreting high amounts of

proinflammatory cytokines after TLR activation (Wong et al., 2011). It had been hypothesized that intermediate and non-classical monocytes arise from classical monocytes (Patel et al., 2017).

The main goal of this study was to evaluate the role of the three monocyte subsets and relevant cytokines/chemokines in a cohort of patients with RPL and RIF to better characterize a group of patients with a baseline proinflammatory profile that could define inflammatory pathophysiology (iRPL and iRIF).

Material and methods

Study population

Women referred to our Reproductive Immunology Unit of the San Carlos Clinical Hospital due to prior history of RRF were evaluated from 2017 to 2018. Those patients that fulfilled the criteria of RPL and RIF were considered for the study. A group of non-pregnant healthy control women (HC) between 20 and 45 years old was constituted as the control group with two or more children (proven fertility) and no spontaneous miscarriage. RPL was defined as the loss of two or more pregnancies, including non-visualized pregnancy losses (biochemical pregnancy losses with urine or serum positive pregnancy test but no ultrasound confirmation), according to the European Society for Human Reproduction and Embryology (ESHRE) (ESHRE, 2017) and in agreement with the last Practice Committee published by the American Society for Reproductive Medicine (ASRM) (Committee of the American Society for Reproductive Medicine, 2020). RIF was defined as the failure to achieve embryo implantation in at least three consecutive IVF attempts, in which one to two embryos of high-grade quality are transferred in each repeated IVF cycle, according to the ASRM (Simon and Laufer, 2012). According to this, RIF was defined as the failure to achieve a clinical pregnancy after transferring at least 4 good-quality embryos in at least 3 fresh or frozen cycles in women below 40 years of age (Coughlan, 2018).

As routine care, a full fertility screening was performed for all women and their partners. This screening included complete medical history and physical examination, per routine clinical practice, pelvic ultrasound scan to assess ovarian morphology and the uterine cavity, and hysterosalpingography if indicated. The genetic evaluation included the karyotype of parents and tests for inherited thrombophilia (factor V Leiden, prothrombin G20210A mutation), serum homocysteine, and deficiencies of protein C, protein S, and antithrombin III. In addition, the hormonal analysis was performed for thyroid-stimulating hormone (TSH), free thyroxin (fT4), estradiol (E2), progesterone (P4), and vitamin D. An immunological screening included antinuclear antibodies (by indirect immunofluorescence; HELIOS, Aesku), IgG and IgM anticardiolipin and anti-beta-2-glycoprotein

antibodies (Bio-Rad) and lupus anticoagulant, antithyroid antibodies (anti-peroxidase and anti-thyroglobulin antibodies), IgA antitransglutaminase-2 and IgG anti-deamidated gliadin peptide, and serum complement C3 and C4, was performed as per the routine analysis. The Ethics Committee of our hospital approved the study protocol, and all subjects provided signed informed consent.

Analysis of peripheral lymphocyte subpopulations

Multiparametric flow cytometry was used to quantify the frequency of peripheral blood monocytes and their subsets based on the expression of their surface markers. The data were obtained and analyzed using FACSCanto II with the BD FACSDiva software (BD Biosciences, San José, CA, USA) and Kaluza software (version 2.1, Beckman Coulter, Brea, CA, USA). Briefly, blood samples were extracted in EDTA vacuum tubes and processed within 2 hours of collection. Peripheral blood lymphocytes were stained with the following monoclonal antibodies according to the manufacturer's recommendations: CD14-APC-Cy7 (BD Pharmingen), CD16-FITC (Cytognos), HLA-DR-BV510 (BD Bioscience), CD69-PE-Cy7 (BD Bioscience), CCR2-APC (BD Bioscience), CCR5-BV421 (BD Horizon), and CX3CR1-PerCP Cy5.5 (Biolegend, San Diego, CA, USA).

Subpopulations of human monocytes were obtained according to their size, granulation, expression of HLA-DR, and expression of CD14 and CD16, obtaining three subpopulations: Classical monocytes ($CD14^{++}CD16^{-}$), the intermediate monocytes ($CD14^{++}CD16^{+}$), and the non-classical monocytes ($CD14^{low}CD16^{+}$), defined as the percentage of the total monocyte population (Figure 1). The surface markers CCR2, CCR5, CX3CR1, and CD69, were expressed as percentages on each monocyte subset.

Multiplex cytokine assay

Determination of cytokines was performed in plasma samples using a cytokine multiplex assay (Bio-Rad, Hercules, CA, USA). The assay was based on magnetic beads and conducted following the manufacturer's instructions. Briefly, the samples were centrifuged at 1,000 x g at 4°C for 15 min. Next, the plasma was collected and froze at –20°C until processing. Wells of a 96-well filter plate were loaded with 50 µl of 1:4 diluted plasma and 50 µl of the diluted beads mix and incubated on a shaker at 850 rpm for 30 minutes at room temperature. Wells were then washed three times with 100 µl wash buffer. Then, samples were incubated with 25 µl of biotinylated detection antibody at 850 rpm for 30 minutes at room temperature. After three washes, 50 µl of streptavidin-phycoerythrin was added to each well and incubated for 10 minutes at 850 rpm at room temperature in the dark. After three more washes, the beads were resuspended in 125 µl assay buffer for measurement with Luminex MAGPIX

(Luminex, Austin, TX, USA). The complete cytokine panel and the detection limits (LOD) are shown in table 1.

Statistics

Descriptive data are presented as median and interquartile range (IQR) when data are non-normally distributed and mean and standard deviation (SD) when they are normally distributed. The normality of the distribution of data values was assessed with the Kolmogorov–Smirnov and Shapiro-Wilk normality test. The differences among groups were analyzed by Kruskal-Wallis analysis of ranks for nonparametric data following Dunn's post hoc test, and for parametric data, one-way ANOVA and post hoc Tukey's multiple comparisons test were performed. Pearson's correlation was used to analyze the relation between plasma cytokines/chemokines and the frequency of the chemokine receptors in the monocyte subsets. We used SPSS software (Version 27.0, SPSS Inc, Chicago, IL, USA) to perform the statistical analysis. Graphics were made using Rstudio (ggplot2 and corrplot packages) and GraphPad Prism (version 9.3.1, GraphPad Software, San Diego, California, USA). The Boruta algorithm (Kursa and Rudnicki, 2010) in Rstudio was used to make the feature selection, followed by a multivariate logistic regression analysis to develop the predictive model. Receiver operating characteristic (ROC) curves were used to validate the selected variables for predicting the development of RPL or RIF on the optimum sensitivity and specificity. Statistical significance was determined as $P<.05$ ($^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$).

Results

Epidemiological and clinical characteristics of the study subjects

In this study, we evaluated 53 non-pregnant women who rigorously fulfilled the definition of RPL and 24 with RIF, consecutively studied at the Reproductive Immunology Unit at Hospital Clinico San Carlos, Madrid. In addition, a group of 31 non-pregnant fertile women with at least two pregnancies at term and without any adverse pregnancy event was included as a control group. The clinical and epidemiological features of all these three groups are shown in Table 2.

Phenotype characterization of the monocyte subsets in our cohort

The gating strategy for monocyte identification is shown in Figure 1A. According to previously published studies (Yang et al., 2014), our cohort of patients and controls confirmed that the percentage of CCR2 was higher on circulating classical monocytes than on the other subsets. In addition, the

percentage of CX3CR1 and CCR5 was higher on the intermediate subset. Finally, a higher percentage of CX3CR1 was found on the non-classical monocytes and lower CCR2 and CCR5 (Figure 1B).

The percentage of the CCR2$^+$, CX3CR1$^+$, and CCR5$^+$classical monocyte subset was higher in the reproductive failure groups

A higher frequency of peripheral monocytes (Figure 2A) was observed in RPL and RIF compared to HC (2.48 (1.53-4.65), only significant between HC and RIF (4.54 (3.48-5.22), P=.001). There were no significant differences in the frequency of classical monocytes between groups, although we observed a higher frequency of intermediate monocytes in RPL (median (IQR), 6.58 (5.01-8.32)) and RIF (6.36 (4.83-8.59)) than HC (5.42 (4.16-6.68)), but not statistically significant (P=.097). We also observed no significant differences in the percentage of non-classical monocytes between groups although there was a trend (P=.079) slightly higher in HC (Figure 2B). Interestingly, there was a significantly increased proportion of classical monocyte CCR2$^+$ in RPL (P<.001) and RIF (P<.001) compared to HC (Figure 2C). We also found that the classical monocytes expressing CX3CR1 were significantly higher in RPL (P<.001) and RIF (P<.001) compared to HC. The same pattern was observed in the classical monocytes expressing CCR5 (Figure 2C).

The frequency of the intermediate monocytes expressing CX3CR1 and CCR5 was increased in the reproductive failure groups

The percentage of intermediate monocytes expressing CX3CR1 (Figure 2D) was significantly higher in RPL (84.43 (76.76-92.42), P=.001) and RIF (85.02 (76.96-93.72), P=.001) compared to HC (74.48 (59.36-83.36)). In addition, the frequency of the intermediate monocytes CCR5$^+$ was significantly increased in both groups (P<.001) compared to HC (Figure 2D).

The fractalkine receptor (CX3CR1) on the non-classical monocyte subset was higher in the reproductive failure groups

We found that the percentage of the non-classical monocyte subset expressing CX3CR1 was significantly higher in RPL (P=.039) and RIF (P.002) compared to HC (Figure 2E).

Monocyte subsets and cytokines correlations

The crucial role of cytokines and chemokines during different phases of pregnancy is well known. The present study evaluated the plasma levels of different cytokines/chemokines and the differential production in a subgroup of RPL, RIF, and HC subjects at baseline (Table 3). Furthermore, we studied the correlations between these cytokines/chemokines' mediators and the different blood monocyte

subsets.

We found lower levels of IL-1ra in RPL and RIF compared to HC, although they did not reach statistically significant differences (P=.057). Other anti-inflammatory cytokines such as IL-4 and IL-13 were also decreased in RPL and RIF, with no statistically significant effect. By contrast, we observed significantly higher levels of PDGF-BB, TGF- β1, and TGF-β3 in RPL and RIF, compared to HC. We also found that the levels of IL-18 were significantly increased in HC compared to RPL (P=.028) and significant differences in the levels of IL-8 between RPL and RIF (P=.044).

Besides, we observed differential associations between monocyte subsets and the studied plasma cytokines in the three groups (Figure 3). Firstly, we found common significant positive correlations in all three groups: eotaxin levels significantly correlated with IL-4 or IL17A that correlated with PDGF-BB and TNF- α.

In the HC group (Figure 3A), we found significant positive correlations between the $CX3CR1^+$ classical monocytes and BasicFGF (P=.002, r=0.78), IL-9 (P=.008, r=0.67), IL-17A (P=.04, r=0.66), MIP-1β (P=.003, r=0.73), PDGF-BB (P=<.001, r=0.89), RANTES (P=.01, r=0.64), TNF-α (P=.02, r=0.62), TGF-β1 (P=.04, r=0.55) and TGF-β2 (P=.02, r=0.60). This pattern of correlation also occurred in BasicFGF, which positively correlated with IFN-α (P=.03, r=0.60), IL-9, IL-17A, MIP-1β, PDGF-BB, RANTES, TNF-α, and in this case with TGF-β3. Of note, this correlation pattern did not appear in RIF and only partially in RPL (with IFN-α, IL-17A, PDGF-BB, and TNF-α). No correlations were observed between $CX3CR1^+$, $CCR2^+$, and $CCR5^+$ intermediate monocytes and the studied cytokines/chemokines. Interestingly, highly significant correlations were found between PDGF-BB and TGF-β1 (P<.001, r=0.83), TGF-β2 (P=.004, r=0.69), and TGF-β3 (P=.002, r=0.73) in HC and RIF, being only weak with TGF-β3 in RPL (P=.05, r=0.37).

Otherwise, some correlations were present only in the RPL group (Figure 3B). In this group, we found a significantly positive correlation between $CX3CR1^+$ intermediate monocytes and plasma IL-17A (P=.03, r=0.43). Moreover, the $CCR5^+$ intermediate monocytes correlated negatively with TGF-β1 (P=.01, r= -0.47) and TGF-β3 (P=.001, r= -0.58) and the $CCR2^+$ classical monocytes exerted a negative significant correlation with IL-8 (P=.01, r= -0.87) and TRAIL (P=.04, r= -0.40). As expected, the percentage of CCR2 in all monocyte subsets (classical, intermediate, and non-classical) were positively correlated with the CCR2 ligand, MCP-1 (P=.01, r=0.46; P=.04, r=0.39, and P=.02, r=0.44, respectively). There was also a significant positive correlation between IP10 and TRAIL (P=<.001, r=0.67) and a significative negative correlation between IFN-γ and IL-9 also present in RIF, although

not significant. In the HC group, there was a positive correlation between IFN-γ and IL-9, which was also not significant.

By contrast, in the RIF group (Figure 3C), $CCR2^+$ intermediate monocytes presented a significant positive correlation with the proinflammatory cytokines: IFN-α (P=.009, r=0.64), IP-10 (P=.01, r=0.62), and TNF-α (P=.004, r=0.69). Moreover, non-classical $CCR2^+$ monocytes correlated with IL-8 (P=.01, r=0.96) and IL-1β (P=.04, r=0.96) and intermediate $CCR5^+$ monocytes also positively correlated with IL-7 (P=.04, r=0.54). Otherwise, a significant negative correlation was observed between classical $CX3CR1^+$ monocytes and IL-1β (P=.003, r= -0.99).

Feature selection and prediction model

The Boruta algorithm selected the potentially relevant predictors among all studied variables. The variables considered unimportant by the Boruta algorithm were no longer taken for further consideration in the study. The algorithm selected four important variables for the RPL group (Figure 4A): TGF-β1, CD69 classical monocytes, CCR5 intermediate monocytes, CX3CR1 classical monocytes, and four tentative: TGF-β3, CX3CR1 non-classical monocytes, IL-18, and MIP-1β. The variables selected by the algorithm for the RIF group (Figure 4B) were one important, CCR5 intermediate monocytes, and two tentatives: TGF-β3 and MIP-1β.

Then, we run a multivariate logistic regression analysis and ROC curves (Supplementary figure 1) to evaluate the performance of these two proposed models, one for RPL and the other for RIF. The multivariate logistic regression analysis showed that TGF-β1, CCR5 intermediate monocytes, and CX3CR1 non-classical monocytes remained independent predictors of RPL and TGF-β3 and CCR5 intermediate monocytes for RIF (Table 4). The predictive model elicited a global accuracy of 90% y 82% for RPL and RIF, respectively, with higher sensitivity and specificity than every variable alone (Table 4).

Discussion

To the best of our knowledge, we have identified a subgroup of RPL and RIF patients with a baseline active inflammatory profile by analyzing peripheral blood monocytes and plasma cytokines levels for the first time. To evaluate which of the variables included in the study are the most relevant to characterize the RPL and the RIF groups, we used the Boruta algorithm. Eight variables in RPL (TGF-β1, CD69 classical monocytes, CCR5 intermediate monocytes, CX3CR1 classical monocytes, TGF-β3, CX3CR1 non-classical monocytes, IL-18 and MIP-1β) and three in RIF (CCR5 intermediate

monocytes, TGF-β3 and MIP-1β) were identified as most relevant. Then, multivariate logistic regression analysis was applied to evaluate the performance of the two proposed models. Finally, the variables included in the multivariate model were: TGF-β1, intermediate CCR5, and non-classical CX3CR1 monocytes for RPL and TGF-β3 and intermediate CCR5 monocytes for RIF. The combination of these variables could predict RPL and RIF with 90.0% and 82.1% accuracy, respectively. Our model provided a more accurate identification of clinically meaningful outcomes for individual biomarkers, which might allow early identification and the choice of immunomodulatory strategies in selected patients on a personalized approach.

Given the complexity of immune adaptation to pregnancy, the functional relevance of a baseline pro-inflammatory profile of blood monocytes is challenging to interpret. RPL and RIF exhibit totally different pathologies. We can hypothesize in terms of the "degree" of inflammation. For example, in RPL, implantation occurs, but the fetus may be lost later due to the failure to mount the essential immune tolerance. On the other hand, in RIF, implantation fails, which is inherently an inflammatory process. However, too much inflammation can be deleterious, for instance, in the setting of autoimmune diseases. In fact, anti-inflammatory treatments are frequently used in active inflammation patients with RIF and autoimmune disease. In this context, an excessive baseline proinflammatory status can convey the same outcome in two different situations and by different mechanisms, such as RPL and RIF. We found that the proportion of total monocytes was significantly higher in RIF compared to HC. Our results show that the intermediate monocyte subset, called proinflammatory, was increased in RPL and RIF compared to HC, although this increment was not significant. Other authors have described (Tang et al., 2015b) the correlation between intermediate monocytes with the severity of preeclampsia, pointing to this population as a relevant player in the pathogenesis of this pregnancy complication. Elevated peripheral blood intermediate monocytes have also been involved in several inflammatory and autoimmune diseases, like rheumatoid arthritis (Kawanaka et al., 2002; Rossol et al., 2012), acute Kawasaki disease (Katayama et al., 2000), sarcoidosis (Okamoto et al., 2003), and type 1 diabetes (Groen et al., 2015).

In humans, the placenta is hemochorial, implying an intimate contact between the trophoblast (embryo/fetal) tissue and the maternal systemic immune system (Marijke M. Faas and de Vos, 2017). In addition, most maternal immune cells at the placental bed are innate immune cells, mainly uNK cells and macrophages (Trundley and Moffett, 2004). Different authors defend the trophoblast's and the placenta's ability, together with soluble actors, to mature monocytes towards deciduous macrophages (Aldo et al., 2014). Therefore, monocytes are recruited into the uterus to generate macrophages with

essential immunological functions (Nagamatsu and Schust, 2010).

It has been reported that the adherence of monocytes to the syncytiotrophoblast is mediated by fractalkine (CX3CL1) (Siwetz et al., 2015), among other factors. These interactions may contribute to the monocyte activation at the maternal-fetal interface. The differential expression of chemokine receptors (CX3CR1, CCR2, CCR5) in monocytes seems to be related to different migratory cell patterns from the blood to the tissues in response to inflammation. In this study, the percentages of CCR5 and CX3CR1 intermediate monocytes were significantly higher in RIF and RPL groups than in HC, suggesting enhanced migratory capabilities of this subset and their potential role in inflammatory pregnancy morbidity. CCR5 binds the chemokine CCL5/RANTES, which activates signaling pathways that can induce migration and monocyte recruitment to inflamed tissues (Shi and Pamer, 2011; Tacke et al., 2007). According to that, levels of RANTES were higher in RPL and RIF than in HC, although without statistical significance. In human decidual stromal cells CCR5 is highly expressed, and CCL5 is secreted by decidual tissue and acts as a chemoattractant for invading trophoblasts (Du et al., 2014). A significantly higher expression of CCR5 in the intermediate monocyte subset in pre-eclamptic pregnant women than normal pregnancy (Al-ofi et al., 2012) has been reported. CCL5/RANTES promotes a correct pro-implantation microenvironment that influences trophoblast cell survival and modulates the balance of maternal regulatory and effector T lymphocytes in favor of maternal tolerance. However, an increase in CCL5 and CCR5 has been described in the transcriptome of villitis of unknown etiology placentas, a destructive inflammatory lesion of the villous placenta (Grasso et al., 2014; Kim et al., 2009). Therefore, RANTES seems to be a key player in successful implantation, but it must be present at an optimal concentration (Ramhorst et al., 2006). Some authors previously showed that pregnant and non-pregnant women with systemic lupus erythematosus (SLE) had a higher expression of CCR5 on inflammatory monocytes ($CD16^{+}$) compared with healthy women (Björkander et al., 2013). This finding could indicate that women with SLE have a more activated phenotype underlying an ongoing inflammatory process. Other studies pointed out the role of intermediate monocytes in several inflammatory diseases such as obesity (Devêvre et al., 2015), osteoarthritis (Raghu et al., 2017), or type 1 diabetes mellitus (Ren et al., 2017).

Classical monocytes comprise 90% of the circulating monocytes, and their main functions are phagocytosis and inflammatory effectors. They preferentially express the chemokine receptor CCR2 (Tang et al., 2015a). CCR2 and its ligand have been described as crucial for recruiting inflammatory monocytes in several diseases like cardiovascular disease (Verweij et al., 2018). In our cohort, RPL and RIF patients showed a significantly increased percentage of CCR2 classical monocytes compared

to HC. This finding could indicate a proinflammatory profile underlying these diseases. Decidual stromal cells express CCL2 (also known as MCP-1, CCR2 ligand) and its receptor, CCR2 (Du et al., 2014). CCL2 is upregulated by pregnancy hormones like progesterone and β-hCG, playing an essential role in the maternal-fetal interface (He et al., 2007). Furthermore, it has been described that CCL2 controls Th2 polarization by inducing IL-4 production and suppressing IFN-γ secretion by decidual stromal cells (Gu et al., 2000; He et al., 2012).

Non-classical monocytes characterized by low expression of CD14 and high expression of CD16 display the highest CX3CR1 (fractalkine receptor) expression, which is an important receptor for adhesion and migration. In addition, they have a patrolling function and efficiently invade inflamed tissue via CX3CR1 that interacts with vascular endothelium allowing rapid tissue invasion in case of damage (Auffray et al., 2007). CX3CR1 patrolling monocytes respond to fractalkine (CX3CR1 ligand) that can function as an apoptotic recognition receptor and mediate the removal of damaged endothelial cells under inflammatory conditions (Thomas et al., 2015). In our cohort, the percentage of CX3CR1 non-classical monocytes was significantly higher in RPL and RIF compared with HC. Recent works have found that non-classical monocytes are significantly increased in pre-eclamptic groups than in healthy controls (Alahakoon et al., 2018; Jabalie et al., 2019).

We found a significantly higher level of TGF-β1 in the plasma of RPL and RIF patients compared to HC. It has been described by other authors how in non-pregnant women with severe recurrent miscarriage, the levels of TGF-β1 were elevated compared to controls (Ogasawara et al., 2000), in line with our findings. One potential explanation could be that the levels of TGF-β1 are higher during normal pregnancy, but excessive production could lead to spontaneous miscarriage. We also found that the plasma levels of TGF-β3 were higher in RPL and RIF than in HC.

There was a significant positive correlation between TGF-β1, TGF-β3, and PDGF-BB in HC and RIF, only between PDGF-BB and TGF-β3 in RPL. It has been described that the levels of TGF-β1 and PDGF-BB increase in peripheral blood of systemic lupus erythematosus patients (Yuan et al., 2017). We found that PDGF-BB and TGF-β3 were significantly increased in RPL and RIF compared with HC. This finding was in agreement with a recent work that described higher levels of TGF-β in RPL patients in plasma samples (Zhu et al., 2019). TGF-β is a growth inhibitor for several cell types, including endothelial cells and lymphocytes, and it is also a potent pro-fibrotic factor (Najem et al., 2020). TGF-β secreted by decidual stromal cells may regulate trophoblast invasion in normal pregnancy, but excessive production of this molecule by aberrantly activated macrophages may prematurely restrict the invasive capacity of this molecule in trophoblast cells (Renaud and Graham, 2008). TGF-β3 inhibits

trophoblast differentiation toward an invasive phenotype in first-trimester human placental explants. It has been described that pre-eclamptic placentae overexpress TGF-β3 (Caniggia et al., 1999). Besides, it has been proposed that the ability of TGF-β1 to educate T regulatory (Treg) in the feto-maternal interface could be reduced in RPL patients, and dysregulation of TGF-β1 may be related to the development of RPL (Yang et al., 2021). Some significant positive correlations appeared in the three groups, indicating maybe a basic immunological status. One was eotaxin with IL-4, and the other was PDGF-BB with the pro-inflammatory cytokines IL-17A and TNF-α. The presence of eotaxin at the maternal-fetal interface and its role during placentation has been described (Chau et al., 2013). An intriguing finding is the presence of some correlations between functional cell subsets and certain analytes within the HC group that are not present in the RPL and RIF groups. Of note is the positive correlation between classical monocytes $CX3CR1^{+}$ and several cytokines and growth factors, like MIP1β, PDGF-BB, RANTES, or TGF-β1 and TGF-β3. These correlations might be related to the cytokine milieu favoring the recruitment of these monocytes, which were dysregulated in the pathologic groups. Another potential explanation might be a defect in the monocyte's differentiation in the studied groups of women.

PDGF-BB is a growth factor that elicits chemotaxis and chemokinesis of endometrial stromal cells at the implantation site (Schwenke et al., 2013). Additionally, it has been described how PDGF-BB acts with TGFβ to potentiate TNF-α-induce release of pro-inflammatory cytokines (van der Kroef et al., 2020). This finding could explain the positive correlation between PDGF-BB and TNF-α in our cohort.

In this study, the anti-inflammatory cytokines IL-1ra, IL-4, and IL-13 were higher in HC than in RPL and RIF. These results agree with previous studies (Ahmadi et al., 2017; Saito et al., 2010). Our data, however, were not in line with other authors regarding TNF-α and IFN-γ (Al Jameil et al., 2018; Daher et al., 2004; Kumar et al., 2014), which are lightly raised in the HC group compared to RPL and RIF.

The present study shows some limitations. One of the weaknesses is the relatively small size of the cohort. Another limitation was that we performed the analysis in peripheral blood and not in endometrial samples, which lost local underlying inflammatory processes without systemic changes. However, biomarkers in peripheral blood are easier to perform and reproduce, which is optimal in the clinical setting. Finally, a validation group will be essential to cross-validate these findings. Another limitation is that cytokines were identified in plasma samples as we sought blood biomarkers, and therefore, we could not discriminate which cells were secreting these cytokines. Cytokines often operate in a local tissue milieu at very low concentrations and have short half-lives. In further studies, we will choose a more precise method to measure functional data, like isolation of monocytes and in-

vitro activation of peripheral blood monocytes stimulated by various ligands and measuring the production of cytokines. We have included parous women as HC. It is worthy of note that after pregnancy, there is a development of long-term immunological memory, as has been published previously (Huang et al., 2021) through epigenetic modifications that occurred in some specific immune cell subpopulations, like NK or T cells. This memory may help produce a specific immunity capable of alloimmunization and tolerogenic phenotype that can benefit subsequent pregnancies.

In conclusion, this study showed a combination of biomarkers, including blood monocyte subsets and cytokines, that may reflect a pro-inflammatory profile in RPL and RIF women. We built a predictive model with a global accuracy of 90% y 82% for RPL and RIF, respectively, as a potential diagnostic tool to use in the clinic. These findings could provide a better understanding of these pathologies and establish a correct therapeutic intervention.

Acknowledgments

We are indebted to all the patients that participated in this study.

References

Ahmadi, M., Abdolmohammadi-vahid, S., Ghaebi, M., Aghebati-Maleki, L., Afkham, A., Danaii, S., Abdollahi-Fard, S., Heidari, L., Jadidi-Niaragh, F., Younesi, V., Nouri, M., Yousefi, M., 2017. Effect of Intravenous immunoglobulin on Th1 and Th2 lymphocytes and improvement of pregnancy outcome in recurrent pregnancy loss (RPL). Biomed. Pharmacother. 92, 1095–1102. https://doi.org/10.1016/j.biopha.2017.06.001

Al-ofi, E., Coffelt, S.B., Anumba, D.O., 2012. Monocyte subpopulations from pre-eclamptic patients are abnormally skewed and exhibit exaggerated responses to toll-like receptor ligands. PLoS One 7. https://doi.org/10.1371/journal.pone.0042217

Al Jameil, N., Tabassum, H., AlMayouf, H., Alshenefy, A., Almohizea, M.M., Ali, M.N., 2018. Identification of serum cytokines as markers in women with recurrent pregnancy loss or miscarriage using MILLIPLEX analysis. Biomed. Res. 29, 3512–3517. https://doi.org/10.4066/biomedicalresearch.29-18-1030

Alahakoon, T.I., Medbury, H., Williams, H., Fewings, N., Wang, X.M., Lee, V.W., 2018. Distribution of monocyte subsets and polarization in preeclampsia and intrauterine fetal growth restriction. J. Obstet. Gynaecol. Res. 44, 2135–2148. https://doi.org/10.1111/jog.13770

Aldo, P.B., Racicot, K., Craviero, V., Guller, S., Romero, R., Mor, G., 2014. Trophoblast induces monocyte differentiation into CD14+/CD16+ macrophages. Am. J. Reprod. Immunol. 72, 270–284. https://doi.org/10.1111/aji.12288

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., Geissmann, F., 2007. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science (80-.). 317, 666–670. https://doi.org/10.1126/science.1142883

Barros, M.H.M., Hauck, F., Dreyer, J.H., Kempkes, B., Niedobitek, G., 2013. Macrophage polarisation: An immunohistochemical approach for identifying M1 and M2 macrophages. PLoS One 8. https://doi.org/10.1371/journal.pone.0080908

Bashiri, A., Halper, K.I., Orvieto, R., 2018. Recurrent Implantation Failure-update overview on etiology, diagnosis, treatment and future directions. Reprod. Biol. Endocrinol. 16. https://doi.org/10.1186/s12958-018-0414-2

Björkander, S., Heidari-Hamedani, G., Bremme, K., Gunnarsson, I., Holmlund, U., 2013. Peripheral monocyte expression of the chemokine receptors CCR2, CCR5 and CXCR3 is altered at parturition in healthy Women and in women with systemic lupus erythematosus. Scand. J. Immunol. 77, 200–212. https://doi.org/10.1111/sji.12021

Caniggia, I., Grisaru-Gravnosky, S., Kuliszewsky, M., Post, M., Lye, S.J., 1999. Inhibition of TGF-β3 restores the invasive capability of extravillous trophoblasts in preeclamptic pregnancies. J. Clin. Invest. 103, 1641–1650. https://doi.org/10.1172/JCI6380

Chau, S.E., Murthi, P., Wong, M.H., Whitley, G.S., Brennecke, S.P., Keogh, R.J., 2013. Control of extravillous trophoblast function by the eotaxins CCL11, CCL24 and CCL26. Hum. Reprod. 28, 1497–1507.

https://doi.org/10.1093/humrep/det060

Committee of the American Society for Reproductive Medicine, P., 2020. Definitions of infertility and recurrent pregnancy loss: a committee opinion. Fertil. Steril. 113, 533–535. https://doi.org/10.1016/j.fertnstert.2019.11.025

Coughlan, C., 2018. What to do when good-quality embryos repeatedly fail to implant. Best Pract. Res. Clin. Obstet. Gynaecol. https://doi.org/10.1016/j.bpobgyn.2018.07.004

Daher, S., Denardi, K.D.A.G., Blotta, M.H.S.L., Mamoni, R.L., Reck, A.P.M., Camano, L., Mattar, R., 2004. Cytokines in recurrent pregnancy loss. J. Reprod. Immunol. https://doi.org/10.1016/j.jri.2003.10.004

Devêvre, E.F., Renovato-Martins, M., Clément, K., Sautès-Fridman, C., Cremer, I., Poitou, C., 2015. Profiling of the Three Circulating Monocyte Subpopulations in Human Obesity. J. Immunol. 194, 3917–3923. https://doi.org/10.4049/jimmunol.1402655

Du, M.R., Wang, S.C., Li, D.J., 2014. The integrative roles of chemokines at the maternal-fetal interface in early pregnancy. Cell. Mol. Immunol. https://doi.org/10.1038/cmi.2014.68

El Hachem, H., Crepaux, V., May-Panloup, P., Descamps, P., Legendre, G., Bouet, P.E., 2017. Recurrent pregnancy loss: Current perspectives. Int. J. Womens. Health. https://doi.org/10.2147/IJWH.S100817

ESHRE, 2017. Recurrent pregnancy loss. Guideline of the European Society of Human Reproduction and Embryology. Eur. Soc. Hum. Reprod. Embryol. 20, 0–153.

Faas, M. M., de Vos, P., 2017. Maternal monocytes in pregnancy and preeclampsia in humans and in rats. J. Reprod. Immunol. https://doi.org/10.1016/j.jri.2016.06.009

Faas, Marijke M., de Vos, P., 2017. Uterine NK cells and macrophages in pregnancy. Placenta 56, 44–52. https://doi.org/10.1016/j.placenta.2017.03.001

Fest, S., Aldo, P.B., Abrahams, V.M., Visintin, I., Alvero, A., Chen, R., Chavez, S.L., Romero, R., Mor, G., 2007. Trophoblast-macrophage interactions: A regulatory network for the protection of pregnancy. Am. J. Reprod. Immunol. 57, 55–66. https://doi.org/10.1111/j.1600-0897.2006.00446.x

Förger, F., Villiger, P.M., 2020. Immunological adaptations in pregnancy that modulate rheumatoid arthritis disease activity. Nat. Rev. Rheumatol. 16, 113–122. https://doi.org/10.1038/s41584-019-0351-2

Grasso, E., Paparini, D., Hauk, V., Salamone, G., Leiros, C.P., Ramhorst, R., 2014. Differential migration and activation profile of monocytes after trophoblast interaction. PLoS One 9, e97147. https://doi.org/10.1371/journal.pone.0097147

Groen, B., Van Der Wijk, A.E., Van Den Berg, P.P., Lefrandt, J.D., Van Den Berg, G., Sollie, K.M., De Vos, P., Links, T.P., Faas, M.M., 2015. Immunological Adaptations to Pregnancy in Women with Type 1 Diabetes. Sci. Rep. 5. https://doi.org/10.1038/srep13618

Gu, L., Tseng, S., Horner, R.M., Tam, C., Loda, M., Rollins, B.J., 2000. Control of T(H) 2 polarization by the chemokine monocyte chemoattractant protein-1. Nature 404, 407–411. https://doi.org/10.1038/35006097

He, Y.Y., Du, M.R., Guo, P.F., He, X.J., Zhou, W.H., Zhu, X.Y., Li, D.J., 2007. Regulation of C-C motif chemokine ligand 2 and its receptor in human decidual stromal cells by pregnancy-associated hormones in early gestation. Hum. Reprod. 22, 2733–2742. https://doi.org/10.1093/humrep/dem208

He, Y.Y., He, X.J., Guo, P.F., Du, M.R., Shao, J., Li, M.Q., Li, D.J., 2012. The decidual stromal cells-secreted CCL2 induces and maintains decidual leukocytes into Th2 bias in human early pregnancy. Clin. Immunol. 145, 161–173. https://doi.org/10.1016/j.clim.2012.07.017

Huang, X., Wang, L., Zhao, S., Liu, H., Chen, S., Wu, L., Liu, L., Ding, J., Yang, H., Maxwell, A., Yin, Z., Mor, G., Liao, A., 2021. Pregnancy Induces an Immunological Memory Characterized by Maternal Immune Alterations Through Specific Genes Methylation. Front. Immunol. 12, 2156. https://doi.org/10.3389/fimmu.2021.686676

Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiyama, H., Schall, T.J., Yoshie, O., 1997. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. Cell. https://doi.org/10.1016/S0092-8674(00)80438-9

Jabalie, G., Ahmadi, M., Koushaeian, L., Eghbal-Fard, S., Mehdizadeh, A., Kamrani, A., Abdollahi-Fard, S., Farzadi, L., Hojjat- Farsangi, M., Nouri, M., Yousefi, M., 2019. Metabolic syndrome mediates proinflammatory responses of inflammatory cells in preeclampsia. Am. J. Reprod. Immunol. 81. https://doi.org/10.1111/aji.13086

Katayama, K., Matsubara, T., Fujiwara, M., Koga, M., Furukawa, S., 2000. CD14+CD16+ monocyte subpopulation in Kawasaki disease. Clin. Exp. Immunol. https://doi.org/10.1046/j.1365-2249.2000.01321.x

Kawanaka, N., Yamamura, M., Aita, T., Morita, Y., Okamoto, A., Kawashima, M., Iwahashi, M., Ueno, A., Ohmoto, Y., Makino, H., 2002. CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. Arthritis Rheum. https://doi.org/10.1002/art.10545

Kim, M.J., Romero, R., Kim, C.J., Tarca, A.L., Chhauy, S., LaJeunesse, C., Lee, D.-C., Draghici, S., Gotsch, F., Kusanovic, J.P., Hassan, S.S., Kim, J.-S., 2009. Villitis of Unknown Etiology Is Associated with a Distinct Pattern of Chemokine Up-Regulation in the Feto-Maternal and Placental Compartments: Implications for Conjoint Maternal Allograft Rejection and Maternal Anti-Fetal Graft-versus-Host Disease. J. Immunol. 182, 3919–3927. https://doi.org/10.4049/jimmunol.0803834

Kumar, A., Begum, N., Prasad, S., Aggarwal, S., Sharma, S., 2014. Oral dydrogesterone treatment during early pregnancy to prevent recurrent pregnancy loss and its role in modulation of cytokine production: A double-blind, randomized, parallel, placebo-controlled trial. Fertil. Steril. 102, 1357-1363.e3. https://doi.org/10.1016/j.fertnstert.2014.07.1251

Kursa, M.B., Rudnicki, W.R., 2010. Feature selection with the boruta package. J. Stat. Softw. 36, 1–13. https://doi.org/10.18637/jss.v036.i11

Lédée, N., Munaut, C., Aubert, J., Sérazin, V., Rahmati, M., Chaouat, G., Sandra, O., Foidart, J.M., 2011. Specific and extensive endometrial deregulation is present before conception in IVF/ICSI repeated implantation

failures (IF) or recurrent miscarriages. J. Pathol. 225, 554–564. https://doi.org/10.1002/path.2948

Mantovani, A., Biswas, S.K., Galdiero, M.R., Sica, A., Locati, M., 2013. Macrophage plasticity and polarization in tissue repair and remodelling. J. Pathol. https://doi.org/10.1002/path.4133

Mekinian, A., Cohen, J., Alijotas-Reig, J., Carbillon, L., Nicaise-Roland, P., Kayem, G., Daraï, E., Fain, O., Bornes, M., 2016. Unexplained Recurrent Miscarriage and Recurrent Implantation Failure: Is There a Place for Immunomodulation? Am. J. Reprod. Immunol. https://doi.org/10.1111/aji.12493

Mills, C.D., Kincaid, K., Alt, J.M., Heilman, M.J., Hill, A.M., 2000. M-1/M-2 Macrophages and the Th1/Th2 Paradigm. J. Immunol. https://doi.org/10.4049/jimmunol.164.12.6166

Mor, G., Cardenas, I., Abrahams, V., Guller, S., 2011. Inflammation and pregnancy: The role of the immune system at the implantation site. Ann. N. Y. Acad. Sci. 1221, 80–87. https://doi.org/10.1111/j.1749-6632.2010.05938.x

Nagamatsu, T., Schust, D.J., 2010. The Immunomodulatory Roles of Macrophages at the Maternal-Fetal Interface. Reprod. Sci. https://doi.org/10.1177/1933719109349962

Najem, M.Y., Couturaud, F., Lemarié, C.A., 2020. Cytokine and chemokine regulation of venous thromboembolism. J. Thromb. Haemost. https://doi.org/10.1111/jth.14759

Okamoto, H., Mizuno, K., Horio, T., 2003. Circulating CD14+ CD16+ monocytes are expanded in sarcoidosis patients. J. Dermatol. 30, 503–509. https://doi.org/10.1111/j.1346-8138.2003.tb00424.x

Panek, C.A., Ramos, M.V., Mejias, M.P., Abrey-Recalde, M.J., Fernandez-Brando, R.J., Gori, M.S., Salamone, G.V., Palermo, M.S., 2015. Differential expression of the fractalkine chemokine receptor (CX 3 CR1) in human monocytes during differentiation. Cell. Mol. Immunol. 12, 669–680. https://doi.org/10.1038/cmi.2014.116

Patel, A.A., Zhang, Y., Fullerton, J.N., Boelen, L., Rongvaux, A., Maini, A.A., Bigley, V., Flavell, R.A., Gilroy, D.W., Asquith, B., Macallan, D., Yona, S., 2017. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. J. Exp. Med. 214, 1913–1923. https://doi.org/10.1084/jem.20170355

Postea, O., Vasina, E.M., Cauwenberghs, S., Projahn, D., Liehn, E.A., Lievens, D., Theelen, W., Kramp, B.K., Butoi, E.D., Soehnlein, O., Heemskerk, J.W.M., Ludwig, A., Weber, C., Koenen, R.R., 2012. Contribution of platelet CX3CR1 to platelet-monocyte complex formation and vascular recruitment during hyperlipidemia. Arterioscler. Thromb. Vasc. Biol. 32, 1186–1193. https://doi.org/10.1161/ATVBAHA.111.243485

Raghu, H., Lepus, C.M., Wang, Q., Wong, H.H., Lingampalli, N., Oliviero, F., Punzi, L., Giori, N.J., Goodman, S.B., Chu, C.R., Sokolove, J.B., Robinson, W.H., 2017. CCL2/CCR2, but not CCL5/CCR5, mediates monocyte recruitment, inflammation and cartilage destruction in osteoarthritis. Ann. Rheum. Dis. https://doi.org/10.1136/annrheumdis-2016-210426

Rai, R., Regan, L., 2006. Recurrent miscarriage. Lancet 368, 601–611. https://doi.org/10.1016/S0140-6736(06)69204-0

Ramhorst, R., Patel, R., Corigliano, A., Etchepareborda, J.J., Fainboim, L., Schust, D., 2006. Induction of maternal tolerance to fetal alloantigens by RANTES production. Am. J. Reprod. Immunol. 56, 302–311. https://doi.org/10.1111/J.1600-0897.2006.00430.X

Ren, X., Mou, W., Su, C., Chen, X., Zhang, H., Cao, B., Li, X., Wu, D., Ni, X., Gui, J., Gong, C., 2017. Increase in peripheral blood intermediate monocytes is associated with the development of recent-onset type 1 diabetes mellitus in children. Int. J. Biol. Sci. https://doi.org/10.7150/ijbs.15659

Renaud, S.J., Graham, C.H., 2008. The role of macrophages in utero-placental interactions during normal and pathological pregnancy. Immunol. Invest. https://doi.org/10.1080/08820130802191375

Rossol, M., Kraus, S., Pierer, M., Baerwald, C., Wagner, U., 2012. The CD14 brightCD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. Arthritis Rheum. https://doi.org/10.1002/art.33418

Saini, V., Arora, S., Yadav, A., Bhattacharjee, J., 2011. Cytokines in recurrent pregnancy loss. Clin. Chim. Acta 412, 702–708. https://doi.org/10.1016/j.cca.2011.01.002

Saito, S., Nakashima, A., Shima, T., Ito, M., 2010. Th1/Th2/Th17 and Regulatory T-Cell Paradigm in Pregnancy. Am. J. Reprod. Immunol. https://doi.org/10.1111/j.1600-0897.2010.00852.x

Schwenke, M., Knöfler, M., Velicky, P., Weimar, C.H.E., Kruse, M., Samalecos, A., Wolf, A., Macklon, N.S., Bamberger, A.M., Gellersen, B., 2013. Control of Human Endometrial Stromal Cell Motility by PDGF-BB, HB-EGF and Trophoblast-Secreted Factors. PLoS One 8. https://doi.org/10.1371/journal.pone.0054336

Shi, C., Pamer, E.G., 2011. Monocyte recruitment during infection and inflammation. Nat. Rev. Immunol. https://doi.org/10.1038/nri3070

Simon, A., Laufer, N., 2012. Repeated implantation failure: Clinical approach. Fertil. Steril. https://doi.org/10.1016/j.fertnstert.2012.03.010

Siwetz, M., Sundl, M., Kolb, D., Hiden, U., Herse, F., Huppertz, B., Gauster, M., 2015. Placental fractalkine mediates adhesion of THP-1 monocytes to villous trophoblast. Histochem. Cell Biol. 143, 565–574. https://doi.org/10.1007/s00418-014-1304-0

Somigliana, E., Vigano, P., Busnelli, A., Paffoni, A., Vegetti, W., Vercellini, P., 2018. Repeated implantation failure at the crossroad between statistics, clinics and over-diagnosis. Reprod. Biomed. Online 36, 32–38. https://doi.org/10.1016/j.rbmo.2017.09.012

Tacke, F., Alvarez, D., Kaplan, T.J., Jakubzick, C., Spanbroek, R., Llodra, J., Garin, A., Liu, J., Mack, M., Van Rooijen, N., Lira, S.A., Habenicht, A.J., Randolph, G.J., 2007. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. J. Clin. Invest. 117, 185–194. https://doi.org/10.1172/JCI28549

Tang, M.X., Hu, X.H., Liu, Z.Z., Kwak-Kim, J., Liao, A.H., 2015a. What are the roles of macrophages and

monocytes in human pregnancy? J. Reprod. Immunol. https://doi.org/10.1016/j.jri.2015.08.001

Tang, M.X., Zhang, Y.H., Hu, L., Kwak-Kim, J., Liao, A.H., 2015b. CD14++CD16+HLA-DR+ Monocytes in Peripheral Blood are Quantitatively Correlated with the Severity of Pre-eclampsia. Am. J. Reprod. Immunol. 74, 116–122. https://doi.org/10.1111/aji.12389

Thomas, G., Tacke, R., Hedrick, C.C., Hanna, R.N., 2015. Nonclassical Patrolling Monocyte Function in the Vasculature. Arterioscler. Thromb. Vasc. Biol. 35, 1306–1316. https://doi.org/10.1161/ATVBAHA.114.304650

Trundley, A., Moffett, A., 2004. Human uterine leukocytes and pregnancy. Tissue Antigens. https://doi.org/10.1111/j.1399-0039.2004.00170.x

van der Kroef, M., Carvalheiro, T., Rossato, M., de Wit, F., Cossu, M., Chouri, E., Wichers, C.G.K., Bekker, C.P.J., Beretta, L., Vazirpanah, N., Trombetta, E., Radstake, T.R.D.J., Angiolilli, C., 2020. CXCL4 triggers monocytes and macrophages to produce PDGF-BB, culminating in fibroblast activation: Implications for systemic sclerosis. J. Autoimmun. 111, 102444. https://doi.org/10.1016/j.jaut.2020.102444

Verweij, S.L., Duivenvoorden, R., Stiekema, L.C.A., Nurmohamed, N.S., Van Der Valk, F.M., Versloot, M., Verberne, H.J., Stroes, E.S.G., Nahrendorf, M., Bekkering, S., Bernelot Moens, S.J., 2018. CCR2 expression on circulating monocytes is associated with arterial wall inflammation assessed by 18F-FDG PET/CT in patients at risk for cardiovascular disease. Cardiovasc. Res. 114, 468–475. https://doi.org/10.1093/cvr/cvx224

Vomstein, K., Voss, P., Molnar, K., Ainsworth, A., Daniel, V., Strowitzki, T., Toth, B., Kuon, R.J., 2020. Two of a kind? Immunological and clinical risk factors differ between recurrent implantation failure and recurrent miscarriage. J. Reprod. Immunol. https://doi.org/10.1016/j.jri.2020.103166

Weber, C., Belge, K.U., Von Hundelshausen, P., Draude, G., Steppich, B., Mack, M., Frankenberger, M., Weber, K.S.C., Ziegler-Heitbrock, H.W.L., 2000. Differential chemokine receptor expression and function in human monocyte subpopulations. J. Leukoc. Biol. 67, 699–704. https://doi.org/10.1002/jlb.67.5.699

Wolf, A.A., Yáñez, A., Barman, P.K., Goodridge, H.S., 2019. The ontogeny of monocyte subsets. Front. Immunol. https://doi.org/10.3389/fimmu.2019.01642

Wong, K.L., Tai, J.J.Y., Wong, W.C., Han, H., Sem, X., Yeap, W.H., Kourilsky, P., Wong, S.C., 2011. Gene expression profiling reveals the defining features of the classical, intermediate,and nonclassical human monocyte subsets. Blood 118. https://doi.org/10.1182/blood-2010-12-326355

Xuan, W., Qu, Q., Zheng, B., Xiong, S., Fan, G.-H., 2015. The chemotaxis of M1 and M2 macrophages is regulated by different chemokines. J. Leukoc. Biol. 97, 61–69. https://doi.org/10.1189/jlb.1a0314-170r

Yang, D., Dai, F., Yuan, M., Zheng, Y., Liu, S., Deng, Z., Tan, W., Chen, L., Zhang, Q., Zhao, X., Cheng, Y., 2021. Role of Transforming Growth Factor-β1 in Regulating Fetal-Maternal Immune Tolerance in Normal and Pathological Pregnancy. Front. Immunol. https://doi.org/10.3389/fimmu.2021.689181

Yang, J., Zhang, L., Yu, C., Yang, X.F., Wang, H., 2014. Monocyte and macrophage differentiation: Circulation

inflammatory monocyte as biomarker for inflammatory diseases. Biomark. Res. 2, 1. https://doi.org/10.1186/2050-7771-2-1

Yuan, Y., Yang, M., Wang, K., Sun, J., Song, L., Diao, X., Jiang, Z., Cheng, G., Wang, X., 2017. Excessive activation of the TLR9/TGF-β1/PDGF-B pathway in the peripheral blood of patients with systemic lupus erythematosus. Arthritis Res. Ther. 19. https://doi.org/10.1186/s13075-017-1238-8

Zhang, Y.H., He, M., Wang, Y., Liao, A.H., 2017. Modulators of the balance between M1 and M2 macrophages during pregnancy. Front. Immunol. https://doi.org/10.3389/fimmu.2017.00120

Zhu, L., Aly, M., Kuon, R.J., Toth, B., Wang, H., Karakizlis, H., Weimer, R., Morath, C., Ibrahim, E., Ekpoom, N., Opelz, G., Daniel, V., 2019. Patients with idiopathic recurrent miscarriage have abnormally high TGFß+ blood NK, NKT and T cells in the presence of abnormally low TGFß plasma levels. BMC Immunol. 20. https://doi.org/10.1186/s12865-019-0290-3

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D.N., Leenen, P.J.M., Liu, Y.J., MacPherson, G., Randolph, G.J., Scherberich, J., Schmitz, J., Shortman, K., Sozzani, S., Strobl, H., Zembala, M., Austyn, J.M., Lutz, M.B., 2010. Nomenclature of monocytes and dendritic cells in blood. Blood. https://doi.org/10.1182/blood-2010-02-258558

Tables

Table 1. Cytokines and chemokines and their limits of detection (LOD).

Cytokine/Chemokine	***LOD (pg/mL)***
Eotaxin	2.5
Basic FGF (FGF-2)	1.9
IFN-α	4.3
IFN-γ	6.4
IL-1β	0.6
IL-1ra	5.5
IL-4	0.7
IL-7	1.1
IL-8 (CXCL8)	1.0
IL-9	2.5
IL-13	0.7
IL-17	3.3
IL-18	0.2
IP-10 (CXCL10)	6.1
MCP-1 (CCL2)	1.1
MIP-1β (CCL4)	2.4
PDGF-BB	2.9
RANTES (CCL5)	1.8
TNF-α	6.0
TRAIL	2.1
TGF-β1	3.9
TGF-β2	1.9
TGF-β3	0.5

IL: interleukin; IL-1ra: IL-1 receptor antagonist; CXCL: C-X-C motif ligand; FGF: fibroblast growth factor; IFN: interferon; IP: interferon- γ-inducible protein; MCP: monocyte chemotactic protein; CCL: C-C chemokine motif ligand; MIP: macrophage inflammatory protein; PDGF-BB: platelet-derived growth factor-BB; RANTES: Regulated on activation, normal T cell expressed and secreted; TNF: tumor necrosis factor; TRAIL: TNF-related apoptosis-inducing ligand); TGF: transforming growth factor.

Table 2. Age and obstetrical histories of women with RPL, RIF, and healthy controls. Values are expressed as mean ± SD.

	RPL (n=53)	***RIF (n=24)***	***HC (n=31)***	***P***
Age (years)	36.7 ± 3.8	35.8 ± 4.5	37.9 ± 6.4	NS
No. of miscarriages	3.0 ± 1.3	0.3 ± 0.5	0	<.001
No. of IVF cycles	1.2 ± 2.0	3.0 ± 1.4	0	.005
No. of embryos	2.2 ± 1.4	9.0 ± 4.2	0	<.001

IVF: in vitro fertilization. NS: no significant.

Cytokine/Chemokine	***HC (n=14)***	***RPL (n=29)***	***RIF (n=15)***
Eotaxin	93.35 ± 43.89	70.17 ± 24.81	85.43 ± 39.33
Basic FGF (FGF-2)	124.13 ± 44.58	150.23 ± 42.16	143.03 ± 48.20
IFN-α	113.28 ± 20.62	111.82 ± 29.75	110.31 ± 26.49
IFN-γ	6.24 ± 3.82	5.29 ± 2.66	9.37 ± 15.69
IL-1β	1.97 ± 0.67	3.18 ± 3.79	2.14 ± 1.23
IL-1ra	597.75 ± 159.14	511.23 ± 166.24	511.41 ± 160.23
IL-4	5.96 ± 2.00	5.03 ± 1.17	5.35 ± 1.43
IL-7	74.63 ± 32.80	90.15 ± 27.59	74.34 ± 33.87
IL-8 (CXCL8)	19.77 ± 5.96	16.11 ± 2.36 #	24.59 ± 7.67
IL-9	364.34 ± 78.61	392.63 ± 43.16	390.29 ± 62.07
IL-13	5.41 ± 1.68	5.30 ± 2.29	4.84 ± 1.25
IL-17A	22.03 ± 9.03	18.90 ± 7.45	19.53 ± 9.64
IL-18	209.20 ± 95.57	151.65 ± 49.18 *	166.19 ± 65.50
IP-10 (CXCL10)	1204.42 ± 604.39	1074.64 ± 649.65	1060.47 ± 469.39
MCP-1 (CCL2)	56.48 ± 22.98	37.54 ± 17.39	54.10 ± 40.24
MIP-1β (CCL4)	265 (232.80-303.00)	297.70 (280.29-314.55)	295.77 (271.79-339.93)
PDGF-BB	1,659 (900.50-2,163.36)	2,926.04 (2,445.79-3,656.11) **	3,498 (1,295.76-5,308.11) *
RANTES (CCL5)	35,065.23 ± 12477.16	36,395.16 ± 11,869.85	39,185.62 ± 9,247.23
TNF-α	138.13 ± 37.66	135.15 ± 25.93	133.17 ± 28.64

TRAIL	181.33 ± 64.65	162.49 ± 51.03	149.18 ± 27.09
TGF- β1	15,566.70 (10,462.43-25,023.30)	29,968.50 (24,151.61-36,201.01) ***	25,816.66 (17,308.03-36,420.22) *
TGF- β2	1,191.34 ± 168.90	1,213.84 ± 281.91	1,179.53 ± 388.18
TGF- β3	140.28 (81.97-196.48)	244.12 (208.47-260.59) **	248.83 (142.81-276.95) **

Table 3. Levels of cytokines (pg/mL) in healthy controls (HC), recurrent pregnancy loss (RPL), and recurrent implantation failure (RIF). Data are displayed as mean ± standard deviation (SD) or median (interquartile range, IQR). P values are represented: * P<.05, ** P<.01, and ***P<.001. The symbol # means a statistically significant difference between RPL and RIF.

IL: interleukin; IL-1ra: IL-1 receptor antagonist; CXCL: C-X-C motif ligand; FGF: fibroblast growth factor; IFN: interferon; IP: interferon- γ-inducible protein; MCP: monocyte chemotactic protein; CCL: C-C chemokine motif ligand; MIP: macrophage inflammatory protein; PDGF-BB: platelet-derived growth factor-BB; TNF: tumor necrosis factor; TRAIL: TNF-related apoptosis-inducing ligand); TGF: transforming growth factor.

Table 4. Backward stepwise logistic regression and ROC analysis for RPL and RIF.

	Multivariate analysis				ROC analysis				
	Variable	**P**	**OR**	**CI**	**AUC**	**P**	**CI**	**Sens**	**Spec**
RPL Model	TGF-β1	0.011	1.000	1.000-1.000	0.934	<0.001	0.839-1.000	88.9	92.9
	CCR5 intermediate	0.038	1.065	1.004-1.131					
	CX3CR1 non-classical	0.035	1.137	1.009-1.282					
RIF Model	CCR5 intermediate	0.057	1.060	0.998-1.127	0.833	0.002	0.667-0.999	80.0	85.7
	TGF-β3	0.011	1.017	1.004-1.030					

RPL: recurrent pregnancy loss; RIF: recurrent implantation failure; OR: odds ratio; CI, confidence interval; AUC: area under the curve; Sens: sensitivity; Spec: specificity.

Figure legends

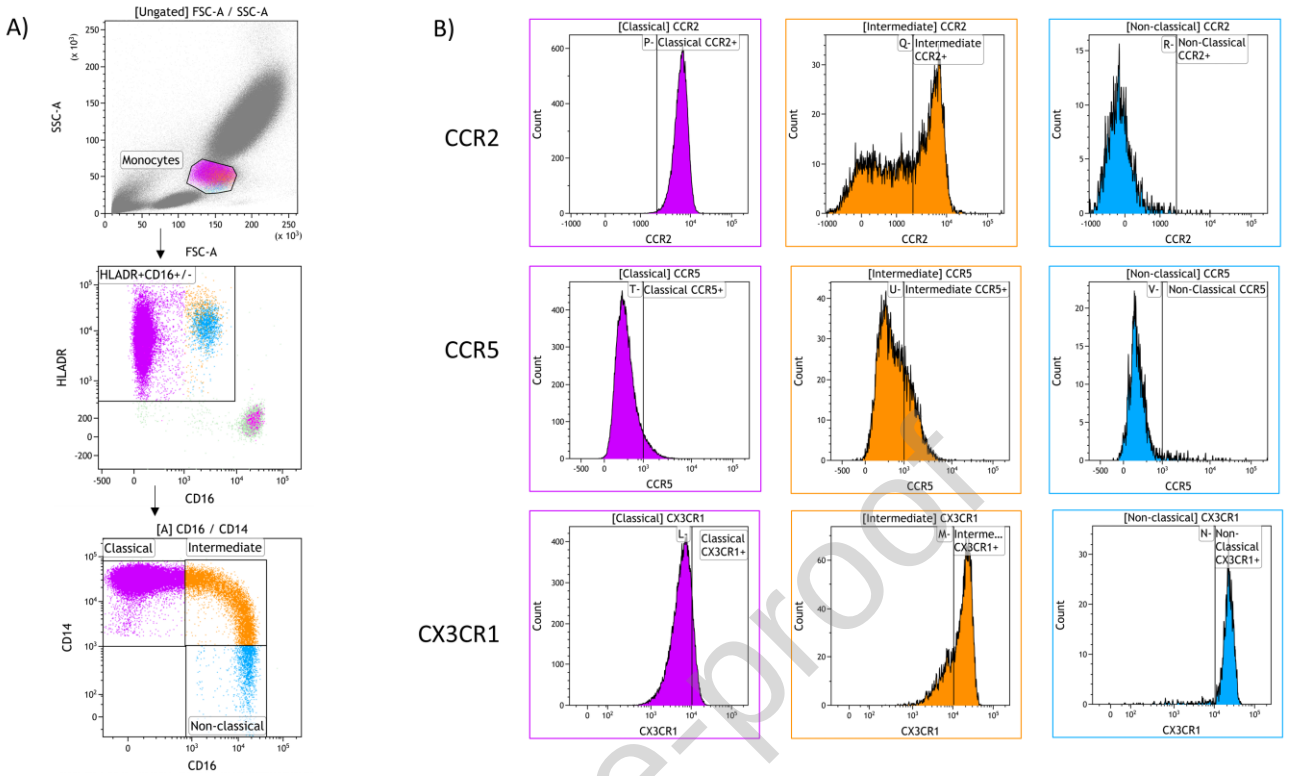

Figure 1. Gating strategy to identify the three monocyte subsets by flow cytometry. (A) First, forward scatter (FCS), and side scatter (SSC) dot plot was used to identify monocytes. Then, an HLA-DR/CD16 plot was used to exclude the HLA-DR-/CD16+ population. Subsequently, the remaining cells were shown on a CD14/CD16 plot and defined the three monocyte subsets (classical, purple; intermediate, orange; and non-classical, blue). (B) Histograms show the frequency of chemokine receptor-2 (CCR2), fractalkine receptor (CX3CR1), and chemokine receptor-5 (CCR5) in the three monocyte subsets.

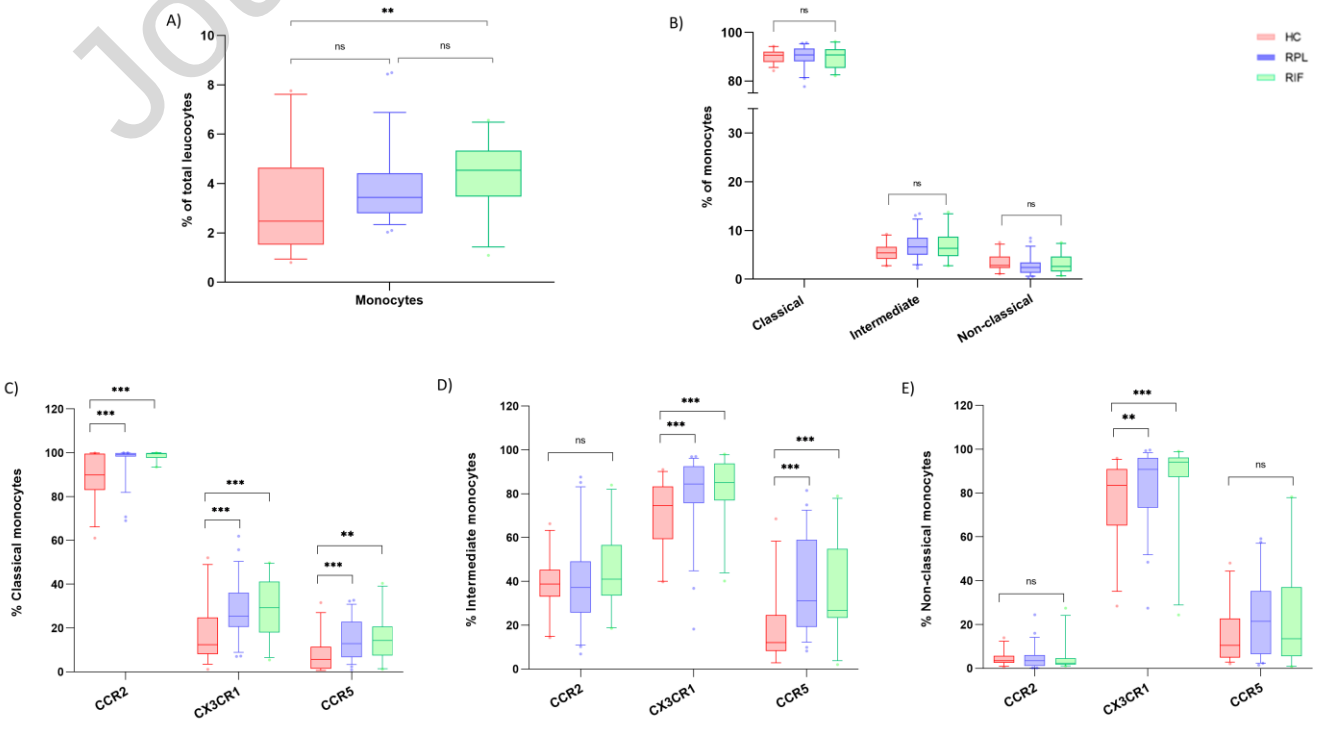

Figure 2. Box-plots depicting the percentage of the total monocytes (A) and the three monocytes subsets (B). The percentage of CCR2, CX3CR1, and CCR5 in the classical monocyte subset (C), the percentage of CCR2, CX3CR1, and CCR5 in the intermediate monocyte subset (D), and the percentage of CCR2, CX3CR1, and CCR5 in the non-classical monocyte subset (E) in the three study groups (HC in light pink, RPL in purple, and RIF in green). The whiskers represent the percentiles 5 and 95. P values are represented as follows: * P<.05, ** P<.01, ***P<.001.

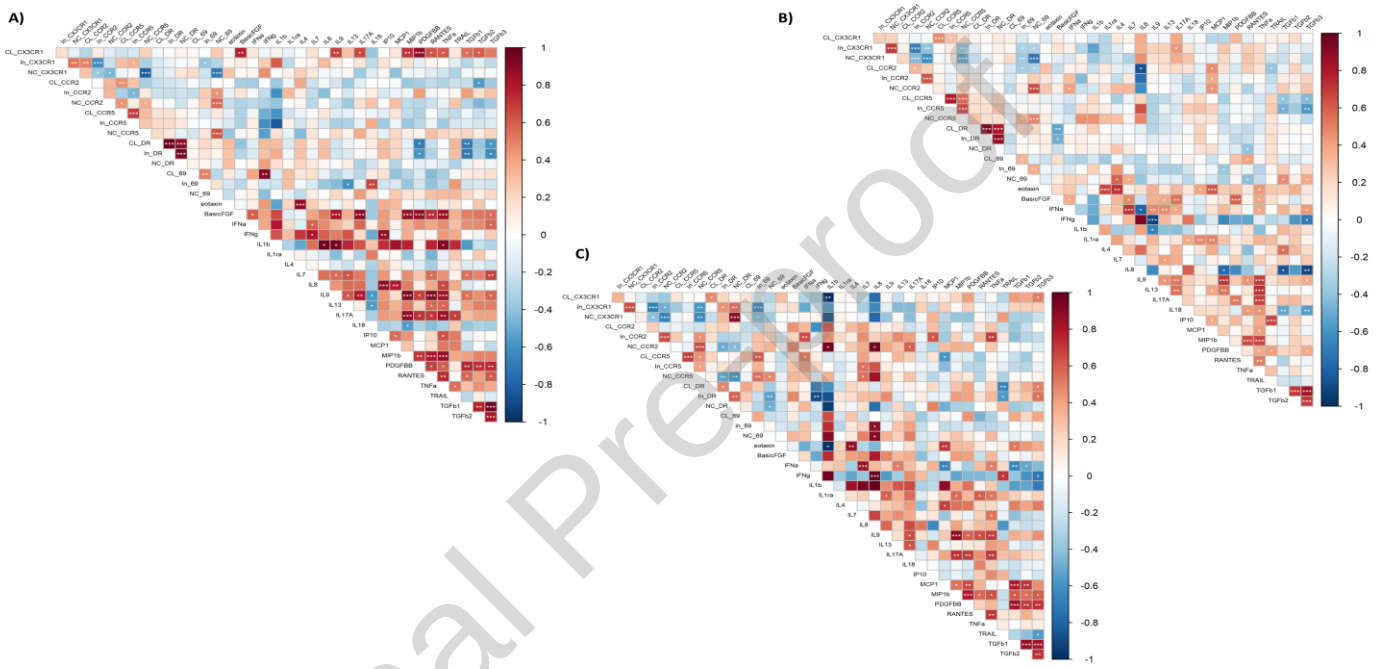

Figure 3. Comparison of the correlation map between monocyte subsets and cytokines/chemokines on healthy controls (A), recurrent pregnancy loss (RPL) (B), and recurrent implantation failure and (C). The circle color shows Pearson's correlation coefficient (red indicates a positive correlation and blue a negative correlation). Asterisks represent the statistically significant correlations (*p<.05, **p<.01, ***p<.001).

Abbreviations: CL: classical; In: intermediate; NC: non-classical; IL: interleukin; IL-1ra: IL-1 receptor antagonist; CXCL: C-X-C motif ligand; FGF: fibroblast growth factor; IFN: interferon; IP: interferon- γ-inducible protein; MCP: monocyte chemotactic protein; CCL: C-C chemokine motif ligand; MIP: macrophage inflammatory protein; PDGF-BB: platelet-derived growth factor-BB; TNF: tumor necrosis factor; TRAIL: TNF-related apoptosis-inducing ligand); TGF: transforming growth factor.

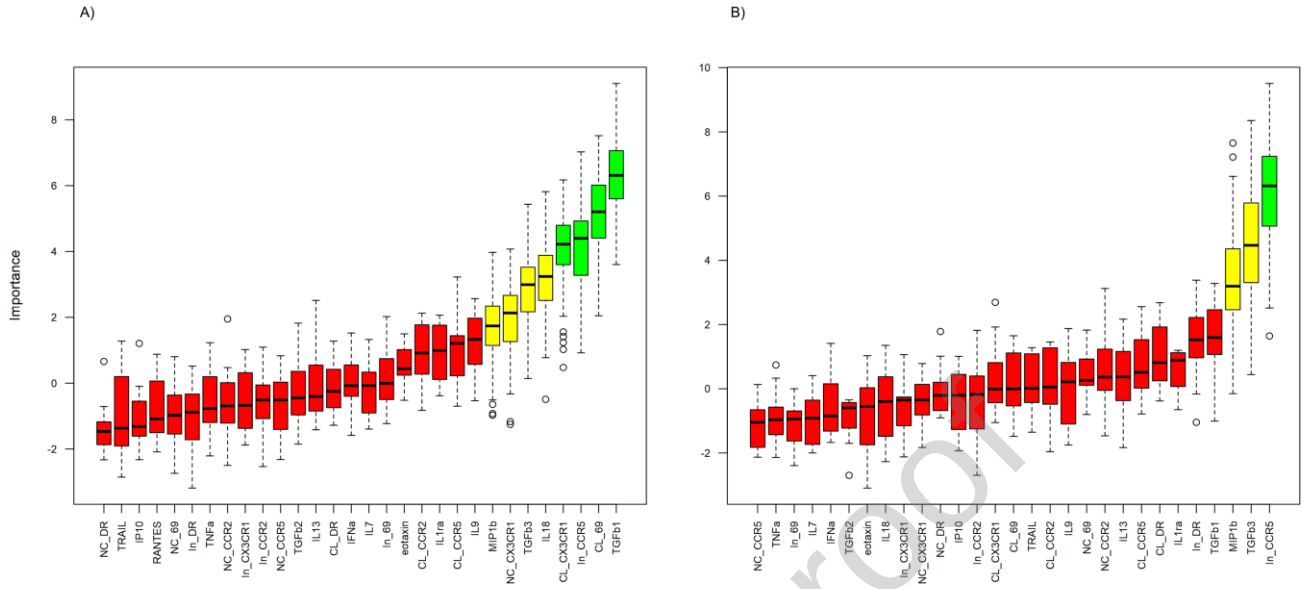

Figure 4. The Boruta algorithm plot shows the selected variables and, therefore, more relevant for RPL (A) and RIF (B) groups. The green box plots confirm important attributes, and the red box plots are considered unimportant. The yellow box plots are tentative, which means the algorithm was not able to conclude this importance.

Supplementary figure 1. ROC curve analysis of the univariate model of the variables selected by the multivariate logistic regression model in the RPL group (A) and the RIF group (B). The ROC curves of the variable combination obtained in the multivariate logistic regression analysis in the RPL (C) and RIF (D) groups were depicted.

Highlights

- Monocytes/macrophages play a critical role in pregnancy success.
- $CX3CR1^{+}$ and $CCR5^{+}$ intermediate monocytes were significantly higher in RPL than in healthy mothers.
- High intermediate monocytes may reflect a baseline pro-inflammatory state in a subgroup of patients.
- We present a Boruta algorithm as a valuable tool to identify women with inflammatory RPL.