Maturation Phenotype of Peripheral Blood Monocyte/Macrophage After Stimulation with Lipopolysaccharides in Irritable Bowel Syndrome

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Background/Aims
Abnormal immune regulation and increased intestinal permeability augmenting the passage of bacterial molecules that can activate immune cells, such as monocytes/macrophages, have been reported in irritable bowel syndrome (IBS). The aim was to compare the maturation phenotype of monocytes/macrophages (CD14+) from IBS patients and controls in the presence or absence of Escherichia coli lipopolysaccharides (LPS), in vitro.

Methods
Mononuclear cells were isolated from peripheral blood of 20 Rome II-IBS patients and 19 controls and cultured with or without LPS for 72 hours. The maturation phenotype was examined by flow cytometry as follows: M1-Early (CD11c+CD206–), M2-Advanced (CD11c–CD206+CX3CR1+); expression of membrane markers was reported as mean fluorescence intensity (MFI). The Mann-Whitney test was used and significance was set at P < 0.05.

Results
In CD14+ cells, CD11c expression decreased with vs without LPS both in IBS (MFI: 8766.0 ± 730.2 vs 12 920.0 ± 949.2, P < 0.001) and controls (8233.0 ± 613.9 vs 13 750.0 ± 743.3, P < 0.001). M1-Early cells without LPS, showed lower CD11c expression in IBS than controls (MFI: 11 540.0 ± 537.5 vs 13 860.0 ± 893.7, P = 0.040), while both groups showed less CD11c in response to LPS (P < 0.01). Furthermore, the percentage of “Intermediate” (CD11c+CD206–CX3CR1+) cells without LPS, was higher in IBS than controls (IBS = 9.5 ± 1.5% vs C = 4.9 ± 1.4%, P < 0.001). Finally, fractalkine receptor (CX3CR1) expression on M2-Advanced cells was increased when treated with LPS in controls but not in IBS (P < 0.001).

Conclusions
The initial phase of monocyte/macrophage maturation appears to be more advanced in IBS compared to controls. However, the decreased CX3CR1 in patients with IBS, compared to controls, when stimulated with LPS suggests a state of immune activation in IBS. (J Neurogastroenterol Motil 2017;23:281-288)

Key Words
Fractalkine receptor; Irritable bowel syndrome; Lipopolysaccharides; Monocytes

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Introduction

Despite the high prevalence of functional gastrointestinal disorders, as is the case with irritable bowel syndrome (IBS), the underlying biologic mechanisms are not completely understood. Nevertheless, the recognition of post-infectious IBS (PI-IBS) and the risk for presenting IBS after antibiotic use, suggest an association of this disorder with the presence of an altered microbiota microbiome and the consequent generation or exacerbation of IBS symptomatology. This knowledge brought about a change of focus in the search for the factors involved in IBS, directing attention to the different microbial species living in the intestine and their relation to the immune cells recognition and response in terms of cytokines or chemokines. As a consequence, numerous efforts have been made to understand the participation of: (1) the types of immune response (innate and acquired), the state of inflammation, and immune regulation; (2) the behavior of the pathogenic and commensal bacterial communities, their response and symptomatology after the use of antibiotics and probiotics; and (3) the alterations in intestinal epithelium permeability in the genesis of IBS. The immunologic hypothesis inferred in relation to IBS is that of a transformation to a presumed state of low-grade inflammation and/or immune activation. For example, there is an imbalance in pro- and anti-inflammatory cytokines in IBS which is subtype and sex dependent. Even though none of the proposals is completely accepted, evidence has shown that at least one subgroup of patients can present with these alterations.

Thus, it is necessary to examine the response of the cell groups involved in immune regulation in IBS, which includes the monocytes/macrophages population. The maturation process of monocytes into macrophages that takes place in the intestine is carried out by a continuous flow of monocytes coming from the blood stream. This monocyte recruitment provides a constant intestinal replenishment of macrophage populations that gradually mature in a cascade-like manner until they reach the level of resident macrophages. Our hypothesis was that patients with IBS may show an alteration in the maturation cascade of monocytes into macrophages in response to bacterial components in charge of triggering this disorder. Therefore our aim was to analyze the maturation phenotype of monocytes/macrophages (CD14+) from the peripheral blood of patients with IBS and controls, by in vitro exposing CD14+ cells to Escherichia coli-derived lipopolysaccharides (LPS).

Materials and Methods

Study Population

Patients between 18 to 60 years of age that have previously been evaluated at our laboratory, and consulted at the colorectal clinic of the Hospital General de México, Dr. Eduardo Liceaga, within the time frame of May to October 2011, were studied. This hospital is a tertiary care center and is considered the most prominent within the public health system in Mexico. Independently of the reason for consultation, all patients seen at the colorectal clinic routinely undergo rigid rectosigmoidoscopy. Those patients that showed no endoscopic evidence of any organic disease were eligible to participate in our study. Also, their medical history had to be negative for inflammatory bowel disease (IBD), celiac disease, microscopic colitis, or gastrointestinal cancer. Likewise, those patients with current signs or symptoms of gastrointestinal or respiratory infectious diseases, those using proton pump inhibitors, antispasmodics, prokinetics, antibiotics, antidepressants, or anti-inflammatory or immunomodulatory agents were excluded from the study. The patients that met the above criteria and voluntarily accepted to participate in the study signed informed consent for their inclusion.

Rome II Modular Questionnaire

All the subjects fulfilling the above criteria answered the Rome II Modular Questionnaire validated in Mexico to further categorize them as IBS or controls, regardless of the reason for their medical consultation. IBS was considered when there was abdominal pain or discomfort for at least 12 weeks in the last 12 months that was associated with improvement after defecation and/or associated with a change in defecation frequency and/or associated with a change in stool consistency. The patients with IBS were then classified as diarrhea-predominant IBS (IBS-D) or constipation-predominant IBS (IBS-C), whereas the individuals that did not fit the IBS-D or IBS-C characteristics were classified as alternating/mixed IBS (IBS-A/M). In addition, IBS diagnosis was confirmed by a gastroenterologist with experience in IBS (M.S.). Furthermore, none of the patients had a history of gastroenteritis as a triggering factor of IBS (in other words, PI-IBS). The controls were subjects that did not meet criteria for IBS or any other functional gastrointestinal disorder according to the Rome II Modular Questionnaire, and those that had no history of any chronic gastrointestinal disorders as previously described (Study Population), or any other chronic pain syndrome.
Isolation of the Mononuclear Cells

Peripheral venous blood samples were collected from each of the study subjects and were packed in sterile EDTA vacutainers (BD Biosciences, San Jose, CA, USA). The mononuclear cells (MNCs) were immediately isolated for centrifugation at the concentration gradient. Fifteen milliliters of blood were diluted in a filtered phosphate-buffered saline solution (PBS) (volume 1:1) and a layer was carefully added to 15 mL of Histopaque-1077 (Sigma, St Louis-MO, USA) in a 50 mL sterile centrifugation tube and centrifuged at 400 g for 20 minutes at room temperature. The MNCs were washed, once with lysis buffer and twice with PBS, to finally evaluate their viability through the trypan blue exclusion test. The MNCs were resuspended at a volume of $2 \times 10^6$ cells/mL in complete medium (RPMI 1640; Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Gibco), 100 IU/mL penicillin, and 10 mg/mL streptomycin (Gibco).

Cell Cultures

The MNCs were cultured in 24-well plaques (Costar, Corning Inc, Wilkes Barre, PA, USA) with no stimulus or with 50 mg/mL of LPS from E. coli (Sigma-Aldrich, St. Louis, MO, USA). The reason for using that particular ligand was the association that has been reported between toll-like receptor 4 stimulation and IBS. The cell cultures were incubated for 72 hours at 35°C, 5% CO2, and a humidified atmosphere. Finally, the resulting cells were preserved in dimethyl sulfoxide 10% at –80°C until measuring time.

Flow Cytometry

The MNCs preserved at –80°C were thawed using the modified Germann et al. technique. In short, the cells were rapidly thawed in a water bath (37°C), diluted in PBS (volume 1:10), centrifuged at 400 g for 5 minutes, and resuspended in 50 μL of PBS. Briefly, as the monocytes/macrophages (CD14+ cells were used for this study) mature, they differentially express a series of membrane receptors, measured in terms of mean fluorescence intensity (MFI), as is the case with CD11c, CD206, and the fraktaline receptor (CX3CR1). These receptors let us examine the degree of development within the maturation cascade. In the most immature state, the cells are negative for the 3 markers. CD11c is more rapidly expressed (CD11c+CD206−, conventionally known as M1, which under the cascade proposal is more an early state) and as the cell matures, the receptor again declines. CD206 and CX3CR1 are slowly expressed as the cells continue to develop (CD11c−CD206+, conventionally known as M2), which under the proposal of cascade maturation corresponds to a more advanced state, the same as CX3CR1, whose expression is related to mature phenotypes, mainly immune regulators. The proposed maturation cascade is depicted in Figure 1.

Statistical Analysis

The continuous variables were expressed as mean ± SD. The data were analyzed using the non-parametric Mann-Whitney test. Statistical significance was set at a value of $P < 0.05$. The SPSS version 17.0 (SPSS, Chicago, IL, USA) statistical program was employed. The study protocol was approved by the Ethics and Research Committee of the Hospital General de México, Dr. Eduardo Liceaga. In addition, a post hoc analysis for Mann-Whitney test (G*Power 3.1.9.2; Institut für Experimentelle Psychologie, Christian-Albrechts-Universität Kiel, Kiel, Germany) was conducted to evaluate the statistical power of the obtained results between the IBS and controls. The power was higher than 0.94 for the statistically significant differences.

Figure 1. Schematic representation of the proposed maturation cascade of monocytes/macrophages. In normal conditions where no immune activation is seen, monocytes-macrophages exhibit a M1-Early state of maturation characterized by increased CD11c expression (CD11c+CD206). As immune activation raises, monocytes-macrophages progressively lose CD11c expression and show moderate expression of both CD206 and CX3CR1, thus constituting an intermediate maturation phenotype (CD11c−CD206−CX3CR1) (lower part in gray). In IBS patients, monocytes-macrophages display a more activated basal state based on a lower expression of CD11c in M1 cells in cultures without LPS stimulation. However an altered immune regulatory state is observed in IBS suggested by a lower expression of CX3CR1 (upper part in red). A, M1-early maturation phenotype; B, intermediate maturation phenotype; and C, M2-advanced maturation phenotype.
**Results**

The Table describes the general characteristics of the study groups. Age and sex distribution were similar between the IBS patients and the controls. Almost half (45%) of the patients with IBS were classified as IBS-A/M, followed by IBS-C (40%), and IBS-D (15%).

**Monocyte/Macrophage Phenotype**

Based on the previously described maturation cascade, in CD14+ cells cultured with LPS compared with those in cultures with no LPS stimulation, we found a lower expression of CD11c. These findings were observed in the IBS patients (8766.0 ± 730.2 vs 12 920.0 ± 949.2, P < 0.001), as well as for the controls (8233.0 ± 613.9 vs 13 750.0 ± 743.1, P < 0.001), with no statistically significant differences between the groups (IBS vs Controls) (Fig. 2). Based on CD11c and CD206 expression, the subgroups were mainly composed of CD11c+CD206+ (intermediate state), followed by CD11c+CD206− (advanced state), and a smaller proportion of CD11c−CD206+ (early state).

In M1 cells (CD11c+CD206+) cultured without LPS stimulation, the CD11c receptor mean fluorescence intensity (MFI) was lower in patients with IBS than in controls (11 540.0 ± 537.5 vs 13 860.0 ± 893.7, P = 0.038) (Fig. 3). In addition, upon analyzing the cultures stimulated with LPS, CD11c expression decreased both in IBS and controls with no significant differences between them (Fig. 3). Moreover, without LPS stimulation the percentage of intermediate cells (CD11c+CD206+CX3CR1+−) was higher in IBS than controls (9.5 ± 1.5% vs 4.9 ± 1.4%; P = 0.022). Such a clear difference was not observed when studying monocytes stimulated with LPS (Fig. 4). Finally, in relation to fractalkine receptor (CX3CR1) expression on M2-Advanced cells, when the difference between with and without LPS stimulation was calculated, IBS showed a slightly decrease in CX3CR1 while there was an increase in controls an increase (P < 0.001) (Fig. 5).

**Discussion**

Even though the model of monocyte/macrophage cultures from peripheral blood that we used in the current study does not

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**Table. Demographic Characteristics of the Study Groups**

| IBS | Controls | P-value |
|-----|----------|---------|
| n = 20 | n = 19 | |
| Sex (W/M) | 15/5 | 13/6 | 0.580 |
| Age (mean ± SD, yr) | 41.9 ± 12.3 | 40.7 ± 9.8 | 0.730 |
| IBS subtype (C/D/A) | 8/3/9 | – | – |

IBS, irritable bowel syndrome; W, women; M, men; C, constipation; D, diarrhea; A, alternating/mixed.
exact replicate the conditions of a response given by macrophages living in the lamina propria of the bowel, our study is based on the fact that the lamina propria macrophages are composed of monocytes recruited from the peripheral blood that enter the intestine mainly through the TGF-β and IL-8 produced by intestinal epithelial cells. Thus, analyzing a model of MNCs stimulated with LPS is a logical focus for speculating the possible response of these cells to bacterial stimuli and their comparison between IBS patients and controls.

Likewise, it is important to point out that in the 2 studies using a murine model, Bain et al and Tamoutounour et al proposed that the conditions of the intestine lead to the development of the macrophage in a cascade-like fashion. This is a linear concept in which complete macrophage development up to a tolerant state is possible in a normal lamina propria, whereas an inflammatory state mainly promotes the maturation of proinflammatory response macrophages. This condition suggests that when the monocytes/macrophages are stimulated, as was carried out in our study, it could be possible to observe whether the process of cell development varies between IBS patients and controls, within the logic of a stimulated environment and maturation cascade. Based on the above, the maturation process registered in the present study can be summarized as follows: (1) the presence of LPS induces monocyte/macrophage maturation compared with the cultures with no stimulation, as suggested by less CD11c expression in CD14+ cells in the presence of LPS in both IBS and controls; (2) a more activated basal state is suggested in the monocytes/macrophages of patients with IBS with respect to healthy controls, based on the lower expression of CD11c in M1 cells in cultures without LPS stimulation in IBS vs controls; and (3) a faster development to an immune regulatory state is observed in the controls compared with the IBS patients, as suggested by the presence of an increased expression of the fractalkine receptor (CX3CR1) in response to LPS only in controls but not in the IBS (Fig. 1).

Therefore, in line with the proposed cascade fashion development, it could be speculated that the presence of the different membrane markers found in the current study, suggests that there is a difference between patients with IBS and the controls in the monocyte/macrophage maturation process. In this sense, the CD11c, CD206, and CX3CR1 markers are not expressed in peripheral monocytes, but rather in a state of residence in the bowel, which in our study is very likely associated with the cell-culture conditions. In the bowel, the macrophages express the CD11c marker, although its presence is associated with differentiated macrophages. In general, it is not found in the cells with the M2-like phenotype, whose main function is the regulation of inflammation mediated by T cells. That is to say, as the macrophage matures, there is an increase expression of CD11c, and as the cell continues to develop, the CD11c expression declines until it completely disappears.

![Figure 4](image1.png)

**Figure 4.** Percentage of CD11c+ CD206+ CX3CR1+ monocytes, in cultures with and without lipopolysaccharide (LPS) stimulation from irritable bowel syndrome (IBS) patients and controls. In cultures that are not stimulated with LPS from patients with IBS, there is a greater frequency (%) of monocytes/macrophages that express the different membrane receptors, compared with the controls. No statistically significant difference was found between the groups in cultures with LPS.

![Figure 5](image2.png)

**Figure 5.** Difference in the fractalkine receptor CX3CR1 expression, with vs without lipopolysaccharide (LPS) stimulation, in irritable bowel syndrome (IBS) patients and controls. The relation between fractalkine receptor expression in cultures with LPS compared to those without LPS (LPS - without LPS), suggests that it is increased in the controls (thus the mean fluorescence intensity: mean fluorescence intensity (MFI) has a positive value), whereas the expression is not increased (MFI is negative or near zero) the cells of patients with IBS.
wise, CD206 and CX3CR1 marker expression is increased as the cells mature in the bowel and they are equally associated with the M2-like phenotype, which can involve metastable state functions with the dynamics of the microbiota, as well as a constant remodeling of the intestinal tissue. 

In accordance with all of the above, it is to be expected that the CD206⁺CX3CR1⁻ subpopulation (in relation to the level of cellular activation and percentage) is the one that presents with a greater state of development, whereas CD206⁺CX3CR1⁺ is the less advanced. Therefore, the general observation of the reduced expression of CD11c in the cells cultured with LPS in our study suggests a maturation process in the presence of a bacterial stimulus, as is to be expected in the cascade proposal of monocyte development. Likewise, the significantly higher MFI of CD11c in M1 control cells cultured without LPS stimulation suggests that, under basal conditions, the cells of the controls are less developed than those from patients with IBS.

On the other hand, despite the fact that in the M2 subpopulation the level of CX3CR1 receptor expression is slightly higher in the cells stimulated with LPS from patients with IBS compared with the controls, upon subtracting the CX3CR1 receptor expression of the CD11c⁺CD206⁺ cells stimulated with LPS from the non-stimulated cells, a reduction in CX3CR1 expression in IBS is suggested, which is contrary to what observed in the controls. This is concordant with the previous hypothesis that there are different initial states of maturation between IBS and controls and that they become more alike after LPS stimulation. There is most likely a faster response in the controls toward a tolerance state in the presence of the TLR ligand, LPS, despite being “behind” in the state with no stimulation. In other words, even though the data suggest that in the absence of bacterial stimulus the patients with IBS can present a superior activation state, in the presence of LPS it becomes more advanced. Therefore, the general observation of the reduced expression of CD11c in the cells cultured with LPS in our study suggests a maturation process in the presence of a bacterial stimulus, as is to be expected in the cascade proposal of monocyte development. Likewise, the significantly higher MFI of CD11c in M1 control cells cultured without LPS stimulation suggests that, under basal conditions, the cells of the controls are less developed than those from patients with IBS.

In conclusion, monocyte/macrophage, maturation appears to be more advanced in patients with IBS compared to controls in cultures without stimulation. However, the contact with PAMPs, such as E. coli-derived LPS, generated a greater maturation process in the monocytes/macrophages in the controls, but not in IBS. This last result suggests an alteration in immune regulation in patients with IBS.

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Conflicts of interest: Max Schmulson has received grant supports from Alfa Wassermann, Nestle Ltd, and Nycomed/Takeda Mexico. He has served on the Advisory Board of Alfa Wassermann and has been a consultant for Almirall, Commonwealth Laboratories Inc, Commonwealth Diagnostics International Inc,
Janssen, Nestle Ltd, Novartis, Procter and Gamble, Senosiain, and Takeda Mexico. He has also been a speaker for Alfa Wassermann, Janssen, Mayoli-Spindler, and Takeda Mexico. Joselín Hernández-Ruiz has received grant support from Alfa Wassermann. Yolanda López-Vidal has been a consultant for Alfa Wassermann. Oscar Rodríguez-Fandiño, Luis Chárua-Guindic, and Galileo Escobedo have nothing to declare.

Author contributions: Oscar Rodríguez-Fandiño: patient selection and enrollment, sample collection, and processing, analyzing, and interpreting the data and drafting the manuscript; Joselín Hernández-Ruiz: planning and study conceptualization, flow cytometry analyses, data analyses and interpretation; Yolanda López-Vidal: analyzing and interpreting the data; Luis Chárua: patient selection and enrollment, sample collection; Galileo Escobedo: data analysis and interpretation; and Max J Schmulson: study planning, conceptualization and funding, patient selection, analyzing and interpreting the data, and drafting the manuscript. All authors have approved the final draft.

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