Activation of IL-10+ B cells: A novel immunomodulatory mechanism for therapeutic bacterial suspensions

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Abstract
Objectives: Bacterial components are used to improve immune responses in patients with respiratory infections. Pharmacological formulations of bacterial components include a mixture of bacterial antigens, some of which are complete inactivated bacteria, that is, named bacterial suspensions; while others are fragments of bacteria, which are presented as bacterial lysates. Although bacterial lysates have been broadly used as immune-stimulators, the biological support for the therapeutic effectiveness of bacterial suspension has not yet been studied. Thus, the aim of our study was to investigate the immunological activity induced by bacterial suspension.

Methods: This work was an exploratory translational study. Peripheral blood mononuclear cells were obtained from healthy donors and cultured in time–dose dependent assays with a commercial bacterial suspension. Flow cytometry was used for phenotypic analysis and for determining soluble cytokines in culture supernatants.

Results: We observed that bacterial suspension activates B cells in a dose-dependent manner. Peripheral blood mononuclear cells were able to secrete IL-6 and IL-10 after 24 h of bacterial suspension stimulation. TLR2 expression was observed mainly on CD19+ CD38lo B cells after 72 h of culture; remarkably, most of the TLR2+ CD19+ cells were also IL-10+.

Conclusion: Our findings suggest that bacterial suspension induces the activation of B cell subsets as well as the secretion of IL-6 and IL-10. Expression of TLR2 on CD19+ cells could act as an activation loop of IL-10+ B regulatory cells. The clinical implications of these findings are discussed at the end of this article.

Keywords
B cells, Bregs, IL-6, IL-10, bacterial suspension, immunomodulation

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Introduction
Bacterial antigens or inactivated bacteria from different species are widely used to prevent recurrent respiratory infections in children and to improve immune responses in elderly patients.¹,² Commercially differing in their pharmaceutical formulations, the complete inactivated bacteria are referred to as bacterial suspensions (BSs), while fragments of bacteria are referred to as bacterial lysates (BLs). BS and BL vary in the number and type of bacteria they contain, and depending on whether they are lysates or suspensions, their biological effects are different. Some BL preparations can induce IL-1β, IL-6, and TNF-α in vitro³ while others activate in vivo T and NK cells⁴ and produce a protective Th1/Th17 memory immune response,⁵ induce nasal human beta-defensins,⁶ or increase the percentage of circulating B cells.⁷ Although BL are broadly used as immune-stimulators, the biological argument about the therapeutic effectiveness of BS has not been studied yet. Recently, some strains of bacteria were reportedly able to enhance the therapeutic effects of antigen-specific immunotherapy in asthma by the
induction of B regulatory cells (Bregs). Bregs are a subset of B cells that are functionally defined by IL-10 secretion. These cell subpopulations are involved in the control of inflammation, autoimmunity, and regulation of immune responses. This study aimed to investigate the immunomodulatory activities of a commercially available BS on human mononuclear cells.

Material and methods

Study design

This study was an exploratory translational study aimed to explore the immunological activity induced by BS on peripheral blood mononuclear cells (PBMCs) obtained from healthy donors.

Healthy donors

Convenience sampling was used in this study, and five healthy volunteers participated as healthy donors (three men and two women, age range 27–30 years old). Healthy donors were included after a complete medical evaluation. All included subjects had no familial or personal history of atopy, none of them presented with chronic diseases, and all were free of infectious diseases at the time of sampling. Laboratory explorations showed complete blood counts inside normal ranges for age and sex. All participants provided their informed consent for blood sampling after written information was provided. The Medical Investigation (CI-046-2016), Ethics (CEI-2016/10/03), and Biosecurity (CB-046-2016) Committees of the Institute of Ophthalmology “Conde de Valenciana Foundation” in Mexico City approved this study.

Reagents

RPMI-1640 culture medium, Concanavalin A (Con A), and salts were from Sigma Chemical Co. (St. Louis, MO, USA). Lymphoprep (Ficoll 1.077 density) was obtained from Nycomed Pharma (Nyegaard, Oslo, Norway). L-glutamine, 2-mercaptoethanol, and sodium pyruvate were purchased from Gibco BRL. (Rockville, MD, USA). Fetal calf serum was from HyClone Labs. (Logan, UT, USA). Bacterial suspension (Polivacc), a heat-killed whole bacteria suspension, was purchased from IPI ASAC Pharmaceutical Immunology (Alicante, Spain). Brefeldin A was obtained from BD Biosciences (San Jose, CA, USA).

PBMCs

Whole heparinized peripheral blood was diluted 1:2 (vol/vol) in phosphate buffered saline (PBS), pH 7.2. PBMCs were separated on a Ficoll density gradient by centrifugation at 300 g for 30 min at room temperature. After centrifugation, the cells at the interface were collected, washed twice, and counted using a handheld automated cell counter (Millipore Co., Billerica, MA, USA), and viability was assessed by eosin dye exclusion.

Cell cultures

PBMCs were cultured in 24-well flat-bottomed cell culture plates (Costar, Cambridge, MA, USA) at 5 x 10^6 cells/well in RPMI-1640 medium supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum. Commercial BS were added at different concentrations, starting from a dilution stock of 2.6 x 10^9 of inactivated bacteria/mL according to the manufacturer. The BS concentrations used were 19.5 x 10^6, 13 x 10^6, 7.8 x 10^6, 3.9 x 10^6, and 1.5 x 10^6 bacteria/mL. Protein concentration was determined using a Bio Rad Protein Assay Kit (Bio Rad Laboratories, Philadelphia, PA). The initial stock concentration was at 4.84 µg/mL; then, we calculated protein concentrations per microliter, and the final concentrations were 36.3, 24.2, 14.5, 7.2, and 3.6 ng/mL.

The BS used in this study contained a total of 14 different inactivated bacteria (BS-14): Streptococcus pneumoniae, Klebsiella pneumoniae, Brahmanna catarrhalis, Staphylococcus aureus, Haemophilus influenza, Streptococcus alpha and beta, Enterococcus faecalis (Syn. Streptococcus faecalis), Staphylococcus epidermidis, Bordetella pertussis, Proteus sp., Pseudomonas sp., Escherichia coli, and Corynebacterium pseudodiphtheria. Con A mitogen (2 µg/mL) was used as a positive control for PBMCs stimulation. Brefeldin A (1 µg/10^6 cells) was added 5 h before culture ended. Cells were harvested every 24 h and were processed to determine CD19, CD38, CD69, TLR2, or IL-10 expression by flow cytometry. Supernatants were collected and stored at −70°C to determine soluble cytokine levels.

Immunofluorescence staining of cell-surface markers

Three or four-color staining was performed on harvested cells by direct immunofluorescence using PE CY5.5- or FITC-labeled anti-CD3 and anti-CD19 monoclonal antibodies (mAbs, e-Biosciences, BioLegend, San Diego, CA, USA) and either APC and/or PE-labeled mAbs against CD38 or CD69, and TLR2 (BD Biosciences, San Jose, CA, USA) for 30 min at 4°C. After incubation, the cells were washed twice with PBS, supplemented with 0.2% Bovine serum albumin and 0.2% sodium Azide (PBA), and immediately analyzed by flow cytometry.

Immunofluorescence staining of intracellular markers

After extracellular staining was performed, the cells were fixed and permeabilized with BD Cytofix/Cytoperm™ solution according to manufacturer’s instructions. Then, the cells...
were incubated with PE-labeled anti-human IL-10 antibody and immediately acquired by flow cytometry.

**Flow cytometric analysis**

All cells were analyzed for the expression of phenotypic markers on a FACSCalibur cytometer (Becton Dickinson, San Jose, CA) using CellQuest software version 10.0 (BD Biosciences), and 10,000 events were counted. To analyze the staining of cell-surface markers, lymphocytes were first gated by their physical properties (forward and side scatter). To determine B cells, a second gate was drawn based on the immunofluorescence characteristics of the gated cells to determine if the cells were CD3⁺ or CD3⁻ cells. The CD3⁻ cells were selected, and a CD19 dot plot was created to analyze CD38, CD69, TLR2, or IL-10 staining on CD19⁺ cells. Data are presented as dot-plots or histograms. Control stains were performed using isotype-matched mAb of unrelated specificity. Background staining was <1% and was subtracted from experimental values.

**Determination of soluble cytokines**

After stimulation with BS-14, supernatants were collected, and the cytokines, IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A were measured with cytometric bead arrays, according to the manufacturer’s instructions (Human Th1/Th2/TH17 Kit, BD Biosciences, Franklin Lakes, NJ, USA). Cytokine concentrations were analyzed by flow cytometry in a FACSVerse Cytometer with FCAP Array software, version 3.0 (BD Biosciences).

**Statistical analysis**

T-tests or Mann–Whitney U tests were used to detect significant differences. The analysis was performed with Prism8 v. 8.2.0 (GraphPad Software, San Diego, CA). Differences were considered statistically significant when the test yielded p values less than 0.05.

**Results**

**BS preferentially activates B cells in a dose-dependent manner**

We began by determining the percentage of CD3⁺ and CD19⁺ cells that were activated after BS-14 stimulation in time–dose in vitro assays. Under these culture conditions, we determined the percentage of cells expressing CD69 (Figure 1). As shown in Table 1, 4.4-fold higher CD69 expression was observed in CD19⁺ cells than in CD3⁺ cells at 24 h with the lowest BS-14 concentration (p = 0.0004). In addition, the percentage of CD19⁺ CD69⁺ cells increased in a dose-dependent manner at all evaluated times, but most significantly at 24 h (Table 1).

**BS induced the activation of CD19⁺ CD38Lo B cells and CD19⁺ CD38Med B cells**

To determine changes in B cell subsets after BS-14 stimulation, we assessed CD38 expression at different times. We did not observe significant differences in the frequency of CD19⁺ CD38⁺ cells at any time or at any tested concentration of BS-14. However, when we analyzed the frequency of CD69 on CD19⁺ CD38⁺ B cell subsets, we observed a significantly higher percentage of CD19⁺ CD38Lo⁺ CD69⁺ B cells and CD19⁺ CD38Med⁺ CD69⁺ B cells at 24h with the majority of the BS-14 concentrations tested (Table 2), and at 48 and 72 h, only with the highest concentration evaluated. No significant differences were observed in the CD19⁺ CD38Hi⁺ CD69⁺ cell frequencies (Figure 2).

**IL-10 and IL-6 are cytokines induced after BS-14 stimulation**

To identify the cytokine profile induced by BS-14 stimulation, we determined the secreted cytokines IL-2, IL-4, IL-6,
Table 1. Frequency of CD3+ CD69+ cells and CD19+ CD69+ cells after BS-14 stimulation.

| [ ] ng/mL | 24 h | 48 h | 72 h | p  | 24 h | 48 h | 72 h | p |
|-----------|------|------|------|----|------|------|------|----|
| RPMI      | 1.6 ± 0.8 | 6.7 ± 2.2 | 6.3 ± 0.8 | –  | 5.5 ± 3.8 | 4.4 ± 1 | 5.3 ± 3 | – |
| 3.6       | 4.8 ± 1.5 | 9.7 ± 3.4 | 8.5 ± 2.8 | –  | 21.4 ± 2.1 | 7.7 ± 4 | 11.8 ± 4.9 | *0.006 |
| 7.2       | 6.1 ± 2.4 | 10.3 ± 3 | 9.3 ± 4.8 | –  | 23.9 ± 7.7 | 10 ± 3.4 | 19 ± 10.2 | *0.04 |
| 14.5      | 6.6 ± 0.8 | 11.2 ± 2 | 11.6 ± 5.3 | –  | 31.3 ± 6.7 | 13.1 ± 5.9 | 21.4 ± 10.4 | †0.02 |
| 24.2      | 8.3 ± 1.5 | 12.8 ± 6.7 | 12.4 ± 5.3 | –  | 39.1 ± 3.5 | 22 ± 11 | 27.6 ± 9.3 | **0.03 |
| 36.3      | 7.0 ± 0.8 | 9.4 ± 1.5 | 13.9 ± 4.3 | –  | 47.8 ± 6.7 | 21.5 ± 3 | 30.3 ± 7.9 | 0.003 |
| Con A     | 14.5 ± 7.3 | 32.7 ± 11.3 | 41.8 ± 6.3 | *0.04 | 35.4 ± 9.8 | 40.5 ± 10.5 | 59.6 ± 14.7 | *0.03 |

Roswell Park Memorial Institute (RPMI) 1640 Medium. Mean ± SD. The p columns indicate the degree of significance between the compared cell populations. Symbols indicate comparisons between the time of cultured cells.

Table 2. Frequency of CD69 on CD19+ CD38lo/Med B cells and on CD19+ CD38med B cells after BS-14 stimulation.

| Time | [ ] ng/mL | RPMI | 3.6 | 7.2 | 14.5 | 24.2 | 36.3 | Con A | p |
|------|-----------|------|-----|-----|------|------|------|-------|----|
| CD19+ CD38lo/CD69+ | 24 h | 9.9 ± 5 | 15.2 ± 5 | 21.6 ± 6 | 28.2 ± 4 | 36.7 ± 4 | 44 ± 9 | 35.8 ± 9 | *0.04 |
| CD19+ CD38med/CD69+ | 48 h | 6.4 ± 4 | 13.2 ± 10 | 10.3 ± 7 | 16 ± 5 | 14.2 ± 2 | 22 ± 9 | 39.9 ± 2 | *<0.005 |
| CD19+ CD38med/CD69+ | 72 h | 5.4 ± 3 | 22.1 ± 14 | 37.5 ± 20 | 47.1 ± 21 | 51 ± 21 | 53.7 ± 11 | 58.7 ± 12 | *<0.001 |
| CD19+ CD38med/CD69+ | 24 h | 7.4 ± 2 | 17.8 ± 6 | 24.8 ± 7 | 32.6 ± 6 | 39.6 ± 2 | 48.4 ± 9 | 36 ± 11 | *<0.001 |
| CD19+ CD38med/CD69+ | 48 h | 1.6 ± 1 | 5.2 ± 3 | 8.2 ± 3 | 12 ± 7 | 16.4 ± 4 | 21 ± 5 | 38.5 ± 16 | *0.01 |
| CD19+ CD38med/CD69+ | 72 h | 4.8 ± 3 | 13.7 ± 13 | 17 ± 13 | 16.5 ± 14 | 23.8 ± 12 | 27.1 ± 11 | 66.3 ± 11 | *0.001 |

Roswell Park Memorial Institute (RPMI) 1640 Medium. Mean ± SD. The p columns indicate the degree of significance between the compared cell populations. Symbols indicate comparisons between the concentration of BS-14 in cultured cells.

IL-10, TNF-α, IFN-γ, and IL-17A by cytometric bead arrays. A significantly increased concentration of IL-6 was observed at 24 and 48 h after BS-14 stimulation. Interestingly, secretion of IL-10 was significantly increased at 24 h (results are depicted in Table 3). IL-2, IL-4, and IL-17A were not detected at any time following any of the BS-14 treatments. No significant changes in cytokine secretion were observed at 72 h.

TLR2 is mainly expressed on CD19+ CD38lo/Med B cells after 72 h of BS-14 stimulation

We evaluated the frequency of TLR2 expression on B cell subpopulations at 24, 48, and 72 h after BS-14 stimuli. We observed that TLR2 expression increased by 11.9-fold with 24.2 ng/mL and significantly increased by 12.4-fold with 36.3 ng/mL on CD19+ CD38lo B cells at 72 h. In addition, it increased by 5.2-fold on CD19+ CD38med B cells at 72 h with only 36.3 ng/mL (Figure 3 and Table 4). Interestingly, when we analyzed for IL-10 expression in TLR2− and TLR2+ B cells, we observed that the TLR2+ B cells were also IL-10+, and after stimulation, the mean fluorescence intensity (MFI) was increased even though the percentage did not significantly change (Figure 4).

Discussion

BLs have long been used to prevent recurrent respiratory tract infections in children and to improve immune responses in elderly patients.1,2 Large numbers of BLs are commercially available, but the amount of bacteria and the type of bacterial mixture in BL induces a broad range of effects on the immune system.3–7 Here, we studied a commercial formulation of 14 inactivated bacteria (BS-14) commonly used as immune-stimulant. The mechanism by which BS-14 impacts the immune response had not been previously evaluated, and this is the first work that explored it.
First, we analyzed the frequency of CD69 on CD3+ and CD19+ cells after BS-14 stimulation in PBMCs. It is well known that CD69 is an activation antigen that is induced early after activation.10,11 Our results showed that B cells were mainly activated by BS-14, and most of these activated B cells had CD38Lo or CD38Med expression. CD38 is an ecto-enzyme that is expressed in early B cells and in mature B cells.12 The staining of CD38 allows for distinction between precursor cells (CD38+), transitional cells (CD38++), and plasmablast cells (CD38+++),13 suggesting that activated B cells by BS-14 were mainly precursor and transitional B cells. Similar results were reported by Lanzilli et al.,4 who evaluated a formulation of BL that were orally administered in tablets and, after 100 days of treatment, they reported changes in the frequency of circulating T, NK, and B cells. Interestingly, the observed B cells were phenotypically early precursors (CD19+ CD27– IgM+/–) and early memory B cells (CD19+ CD27+ IgM+).

Figure 2. Increased CD69 frequency on CD19+CD38+ cells after BS-14 stimulation. CD19+ cells were gated according to CD38 expression and separated into three cell subsets: CD38lo, CD38med, and CD38hi. Histograms from CD19+CD38+ cells subpopulation are shown. The x-axis denotes the frequency of CD69 on the gated cells. PBMCs were stimulated with BS-14 at 36.3 ng/mL and evaluated after 48 h of stimulation. Con A mitogen was used as a positive stimulation control. Representative FACS plots and histograms from five different healthy individuals are shown and were performed in triplicate.
In addition, in our work after BS-14 stimulation, the cytokines detected in the supernatant were mainly IL-6 and IL-10. IL-6 is a pro-inflammatory cytokine that enhances Ig secretion by activated B cells and causes differentiation into plasma cells\textsuperscript{14} while IL-10 is an anti-inflammatory cytokine with a central role in regulating immune responses to pathogens.\textsuperscript{15} Interestingly, IL-6 and IL-10 can be induced after TLR2 ligation\textsuperscript{16–18} and both cytokines are involved in the activation of a subset of memory B cells.\textsuperscript{19,20} In this context, whether the activation of transitional B cells after BS stimulation corresponds to a switched memory phenotype (CD19\textsuperscript{+} CD27\textsuperscript{+} IgD– IgM–)\textsuperscript{13} is not known, and further investigation is needed.

On the contrary, in this work, we observed that TLR2\textsuperscript{+} B cells were the most important source of IL-10. TLR2 is an innate receptor that recognizes specific components conserved among microorganisms.\textsuperscript{21} After BS-14 stimulation, TLR2 was mainly expressed on precursor B cells at 72 h. Some of the bacteria contained in BS-14 can induce TLR2, such as \textit{Klebsiella pneumonia} and \textit{Pseudomonas} sp.\textsuperscript{16,22–24} Remarkably, it has been reported that some TLR2 ligands induce IL-10, thereby leading to activation of regulatory B cells and the attenuation of T effector functions, which contribute to immune regulation.\textsuperscript{25} It is possible that BS-14 stimulation functionally expands a subgroup of Bregs characterized by TLR2 expression and IL-10 production.

### Table 3. Cytokine concentration in the supernatants of cells stimulated with BS-14.

|          | RPMI 3.6 | 7.2  | 14.5 | 24.2 | 36.3 | Con A | p      |
|----------|----------|------|------|------|------|-------|--------|
| IL-6     | 24 h     | 6.7 ± 3.9*§ | 26,899 ± 5788* | 29,894 ± 14,989* | 33,158 ± 10,293 | 36,126 ± 12,139 | 33,588 ± 7538* | 4.2 ± 1.9* | *0.02 | 0.01 |
|          | 48 h     | 3.9 ± 1.7*§ | 26,601 ± 9756 | 39,280 ± 11,490* | 37,475 ± 10,309* | 28,984 ± 27,201 | 35,331 ± 8704* | 6166 ± 5999 | *0.03 | 0.02 |
| IL-10    | 24 h     | ND   | 1178 ± 479.3 | 925.5 ± 523.1* | 1049 ± 509.2* | 784.8 ± 363* | 638.1 ± 276.2* | 16.6 ± 12.2* | *0.03 |
|          | 48 h     | ND   | 939.2 ± 486.4 | 971.7 ± 607.3 | 702.2 ± 591.4 | 654.6 ± 189.7 | 578.7 ± 297 | 133.4 ± 71.3 | – |
| TNF-a    | 24 h     | ND   | 248.4 ± 202.2 | 399.2 ± 247.7 | 553.2 ± 338.2 | 386.8 ± 192.6 | 491 ± 292.2 | 40.9 ± 37.2 | – |
|          | 48 h     | ND   | 58.90 ± 43    | 68.48 ± 47    | 33.7 ± 9.4    | 193.9 ± 163.8 | 97.35 ± 36 | 341.2 ± 183.1 | – |
| IFN-g    | 24 h     | ND   | ND            | ND            | ND            | 16.4 ± 4.8    | 21.1 ± 6.8 | 30.2 ± 26.6 | – |
|          | 48 h     | ND   | 5.5 ± 1.3     | 21 ± 17.6     | 68.7 ± 56.7   | 44.2 ± 23.3   | 216.7 ± 153.6 | – |

Roswell Park Memorial Institute (RPMI) 1640 Medium.
ND: not detected, or below the limits of detection. The results are in pg/mL. Kit detection limits were as follows: IL-2, 2.6 pg/mL; IL-4, 4.9 pg/mL; IL-6, 2.4 pg/mL; IL-10, 4.5 pg/mL; IL-17A, 18.9 pg/mL; IFN, 3.7 pg/mL; and TNF-a, 3.8 pg/mL. Symbols indicate comparisons between the concentration of BS-14 in cultured cells.

**Figure 3.** Increased TLR2 frequency on CD19\textsuperscript{+} CD38\textsuperscript{+} cells after 72 h of BS-14 stimulation. Percentage of TLR2\textsuperscript{+} cells in (a) CD19\textsuperscript{+} CD38\textsuperscript{lo} B cells, and in (b) CD19\textsuperscript{+} CD38\textsuperscript{med} B cells. Significant differences are indicated (*). n = 5 different healthy individuals were evaluated, and all assays were performed in triplicate.
It is undeniable that other TLRs could be participating in the activation of IL-10+ B cells, as both gram-positive and gram-negative bacteria were present in the BS. In this context, it has been reported that peptidoglycan, and lipoteichoic acid from S. aureus, induces large quantities of IL-10 and proliferation of B1 cells. Moreover, prolonged stimulation by Lipopolysaccharide (LPS) induces clonal expansion of Bregs, and TLR4 ligation on Bregs could suppress CD4+ T cell proliferation. Interestingly, LPS stimulation promotes maturation of B10 pro-cells from the human blood into IL-10+ B competent cells, which parallels mouse regulatory B10 cells.

Thus, the clinical implications of our findings are relevant, as it is well known that the activation and expansion of Bregs are fundamental to control immune responses, and the mechanisms of suppression are IL-10 dependent. Moreover, it has been suggested that dysfunction or low frequencies of circulating Bregs are related to allergy and autoimmune diseases. Hence, BS-14 could be easily applied as an adjuvant therapy in patients with chronic inflammatory diseases to therapeutically downregulate immune response through Breg induction, and the evaluation of circulating Bregs could be proposed as efficacy biomarker.

**Limitations**

This research was performed with a small healthy donor sample size, as it was necessary to first explore the ability of

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**Table 4.** Frequency of TLR2 on CD19+CD38lo B cells and on CD19+CD38med B cells after BS-14 stimulation.

| Time | RPMI 3.6 | 7.2 | 14.5 | 24.2 | 36.3 | Con A | p     |
|------|----------|-----|------|------|------|-------|-------|
| CD19+ | 24 h     | 1.6 ± 1.6 | 3.2 ± 1.8 | 5.3 ± 2.8 | 5 ± 2.4 | 9.2 ± 3.3 | 3 ± 1 | 1.5 ± 1.1 | – |
| CD38lo | 48 h     | 5.8 ± 3.8 | 1.4 ± 0.4 | 7.7 ± 5.1 | 6.2 ± 3 | 6.9 ± 2.7 | 5.5 ± 1.5 | 10.4 ± 6.7 | – |
| TLR2+ | 72 h     | 2.9 ± 1.5* | 10.6 ± 4 | 17.8 ± 11 | 24.6 ± 11.9 | 35.4 ± 10.8* | 36.9 ± 8* | 30.6 ± 10.8 *0.02 |
| CD19+ | 24 h     | 1.7 ± 1.5 | 1.6 ± 1.6 | 1.9 ± 0.9 | 1.8 ± 1.6 | 4.9 ± 1 | 2.2 ± 0.9 | 0 ± 0 | – |
| CD38med | 48 h     | 2.3 ± 1.5 | 0.2 ± 0.1 | 1.1 ± 1 | 1.2 ± 1.1 | 2.7 ± 2.3 | 1.1 ± 0.8 | 1.7 ± 1.2 | – |
| TLR2+ | 72 h     | 1.4 ± 1.1* | 2 ± 0.9 | 3.2 ± 2.7 | 4.5 ± 2.7 | 6.4 ± 3.3 | 7.2 ± 1.3* | 28.7 ± 11.9 *0.01 |

Roswell Park Memorial Institute (RPMI) 1640 Medium.
Mean ± SD. Symbols indicate comparisons between the concentration of BS-14 in cultured cells.

**Figure 4.** TLR2+ cells are the main source of IL-10. After 72 h of stimulation with BS-14 at 36.3 ng/mL, TLR2 expression was assessed on CD19+ cells by flow cytometry. Intracellular IL-10 was evaluated in TLR2− and TLR2+ B cells. The histograms show CD19+ TLR2− IL-10+ (upper right) and CD19+ TLR2+ IL-10+ (low right), demonstrating that TLR2+ B cells are the primary source of IL-10. Representative FACS plots and histograms from five different healthy individuals, which were performed in triplicate.
BS-14 to induce functional changes in PBMCs. This unknown function of BS-14 in PBMCs was the reason that we performed our first exploration using Con A as a positive control. After we analyzed the results, we observed that BS-14 mainly activated B cells, rather than T cells. This finding was interesting because it is known that BL activates mainly T and NK cells, without activation of B cells.\textsuperscript{4,5,38} In this work, we explored the impact of BS-14 on PBMCs comparing with Con A, a polyclonal mitogen used to study activation of T cells.\textsuperscript{39} Therefore, we know that it is essential to perform new assays using a pokeweed mitogen (PKW) as a positive control, as PKW is a polyclonal mitogen used to evaluate B cell function since activation observed on B cells by Con A is a consequence to cellular cooperation.\textsuperscript{39} By performing these new assays on PBMCs or on isolated B cells, we will be able to know the real impact of BS on B cell function. Nevertheless, the use of Con A as a positive control on cultured cells did not change our findings related to the effect of BS-14 on B cells. It is undeniable that pharmacological formulation of bacteria in BL versus BS may activate the immune response differentially. Our study gives the first insights that bacterial formulations commonly used in clinical practice have different action mechanisms, and this can be explained if we understand the mechanism of activation of immune cells depending on the antigen. BLs are fragments of bacteria antigens and are quickly captured by antigen-presenting cells, then stimulating T cells; while the TLRs or even BCR could be participating in recognition of BS, activating mainly B cells. The last limitation in our study was that the manufacturer did not provide the relative proportions of the different strains contained in BS-14, and as all experiments were performed in an ex vivo model, more studies are needed to evaluate both the role of each bacterial strain on immune cells and the activation or expansion of IL-10 producing B cells in BS-14 treated patients.

**Conclusion**

Our findings demonstrate that BS-14 induces activation of precursor and transitional B cells, and secretion of IL-6 and IL-10, favoring differentiation of B memory cells. In addition, the expression of TLR2 on B cells could be involved in an activation loop of IL-10+ B cells (Bregs) (Figure 5). Our results provide evidence that at the cellular level, BS-14 could be used therapeutically to diminish pathogenic immune responses. To address this question, clinical studies are needed to elucidate the real impact of BS-14 and other bacterial formulations on the modulation of protective versus pathogenic immune responses.

**Authors’ Note**

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**Author Contributions**

A.S. designed and performed experiments; J.E.N. performed the experiments; H.V.-S. analyzed the data and wrote the paper; and M.C.J.-M. designed experiments, analyzed data, and wrote the paper.

**Declaration of conflicting interests**

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