Vaccine 37 (2019) 7269–7279

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Effect on cellular recruitment and the innate immune response by combining saponin, monophosphoryl lipid-A and Incomplete Freund's Adjuvant with *Leishmania* (*Viannia*) *braziliensis* antigens for a vaccine formulation

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ARTICLE INFO

Article history:
Received 17 October 2018
Received in revised form 17 September 2019
Accepted 20 September 2019
Available online 28 September 2019

Keywords:
Adjuvants
Saponin
Monophosphoryl lipid
Incomplete Freund Adjuvant
Cellular infiltration
Cytokines
Immune response

ABSTRACT

The poor immunogenicity displayed by some antigens has encouraged the development of strategies to improve the immune response and safety of vaccine candidates, resulting in an intense search for substances that potentiate vaccine response. Adjuvants have these properties helping vaccine candidates to induce a strong, durable, and fast immune response. In this study, we evaluated the specific immune response of adjuvants alone, Saponin (SAP), Incomplete Freund's Adjuvant (IFA) and Monophosphoryl lipid-A SE (MPL-SE®) and in combination with total antigen of *L. braziliensis* (LB): LBSAP, LBIFA and LBMPL. The specific immune response induced by these compositions demonstrated that they were powerfully immunogenic, increasing cellular infiltration in the skin. Draining lymph nodes cultures showed that LBIFA and LBMPL have higher ability to increase the capacity of APCs to present antigens, with increased frequency of $CD11c^{+}CD86^{+}$ cells. SAP, MPL, LBSAP, LBIFA and LBMPL could activate lymphocytes increasing expression of CD69 and CD25. LBSAP group was an excellent inducer of pro-inflammatory cytokines at 24 h. At 48 h, higher cytokines production was observed in IFA, LBIFA, MPL and LBMPL groups. Our data demonstrate that LBSAP and LBMPL are potential formulations to be tested in other experimental models. Also, the data obtained could expand the knowledge about immune response after sensitization and also contribute to the development of safe, immunogenic and effective vaccines.

1. Introduction

Adjuvants are co-administered with vaccines to enhance the immune response to the target antigen through the activation of innate immune system. As a result, this stimulation activates immune resident cells which produce cytokines and chemokines, resulting in a cell recruitment as well as APC recognition. After that, these APCs trigger the antigen presentation in lymph nodes increasing the adaptive immune responses [1]. Therefore, it seems that the key to a vaccine response closer to the ideal should be in use/combination of the ideal adjuvant or in search of new adjuvants. The choice of a correct adjuvant is extremely important in a vaccine design to avoid losing promising vaccine antigen

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https://doi.org/10.1016/j.vaccine.2019.09.067
0264-410X/

candidates [2]. In this sense, it is very important to know the effects of adjuvants before proposing associations with vaccine antigens. However, the current challenge faced in the search of adjuvants is to find the "perfect mix" with safe adjuvants that work in synergy, leading to an immune response in the desired direction and in the correct cells [3,4].

Following the progress in immunology, new approaches for adjuvant screening have been applied, for example, the study of adjuvant-induced inflammatory characteristics has become important for the interpretation of induction immunogenic capacity and the understanding of local side effects associated. Thus, the leukocyte recruitment is an important component of this process [5]. In this sense, cytokines such as IL-12, IFN- γ and TNF- α display an important role activating T effector cells to eliminate intracellular parasites [6].

In the field of parasitic diseases, it is important to emphasize that understanding similar patterns of protective response in murine VL, canine and human models is essential for the study of new vaccines. Work carried out by our group demonstrated the importance of the study of cell migration induced by the LBSap and Leishmune® vaccine constituents in hamster [7] and dog [8] models. The antigen used in the LBSAP vaccine consists of crude antigens of *L. (V.) braziliensis* (strain MHOM/BR/75/M2903). This antigen is composed mainly by proteins, glycoproteins, glycolipids, and lipids. Many studies of our group have shown the stimulation potential of the LB antigen in local and systemic immune response [9–11]. These antigens when associated with saponin adjuvant are capable of promoting cellular immune response and reducing infection rate [12] or stimulating the immune system modulation, triggering a cellular response involving $CD8^+$ T lymphocytes, which is very important for resistance to infection by *L. infantum* [13].

Considering the importance to investigate the immunological mechanisms triggered by adjuvants, this study proposed to develop an experimental strategy focused on bioprospecting of adjuvants. In the future, this strategy will enable the selection of adjuvants with safety features and acceptable immunogenicity to compose an anti-*Leishmania* vaccine. Moreover, we evaluated the specific immune response in Balb/c mouse model against the vaccine combination *L. (V.) braziliensis* antigens plus adjuvants in the skin, draining lymph nodes and blood at 24 and 48 h. Importantly, understanding the processes triggered by inflammatory cells in different compartments (skin, draining lymph nodes and blood) and the production of cytokines that mediate the inflammatory process induced by immunostimulatory substances will expand the knowledge of these vaccine additives.

2. Material and methods

2.1. Animals and sensitization protocol

Male outbred Swiss albino mice (8–10 weeks old) were obtained from the Centro de Ciência Animal (CCA/UFOP) and maintained in ventilated racks, water and food *ad libitum* throughout the study. The study protocol was approved by the Ethical Committee for the Use of Experimental Animals of the Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil, under the protocol number 008/2009.

Intradermal inoculum was performed in mice in the back with a single dose of adjuvants, LB antigen, and the association of adjuvants plus LB antigen, after 24- and 48-hours mice were sacrificed, and further analyses were done (Fig. 1). To evaluate the specific immune response to the vaccine composition, the animals were divided into eight experimental groups (n = 6 animals/group/time): Control group, inoculated with 50 μL of 0.9% sterile saline; LB group, inoculated with vaccine antigen (total antigen from promastigotes of *L. (V.) braziliensis*) in the concentration of 60 μg/dose; SAP group, inoculated with 100 μg/dose of Saponin (Sigma Chemical Co., St. Louis, MO); LBSAP group, inoculated with the association LB vaccine antigen and Saponin adjuvant; IFA group, inoculated with 50 μg/dose of Incomplete Freund's Adjuvant (Sigma Chemical Co.); LBIFA group, inoculated with the association LB vaccine antigen and Incomplete Freund's Adjuvant; MPL group, inoculated with 50 μg/dose of Monophosphoryl lipid-A stable solution (Corixa, Hamilton, MT); LBMPL group, inoculated with the association LB vaccine antigen and MPL-SE® adjuvant. The doses used for each adjuvant were inoculated in a total volume of 50 μL per animals. Mice were euthanized at specific time points after injection, skin samples were collected for histological

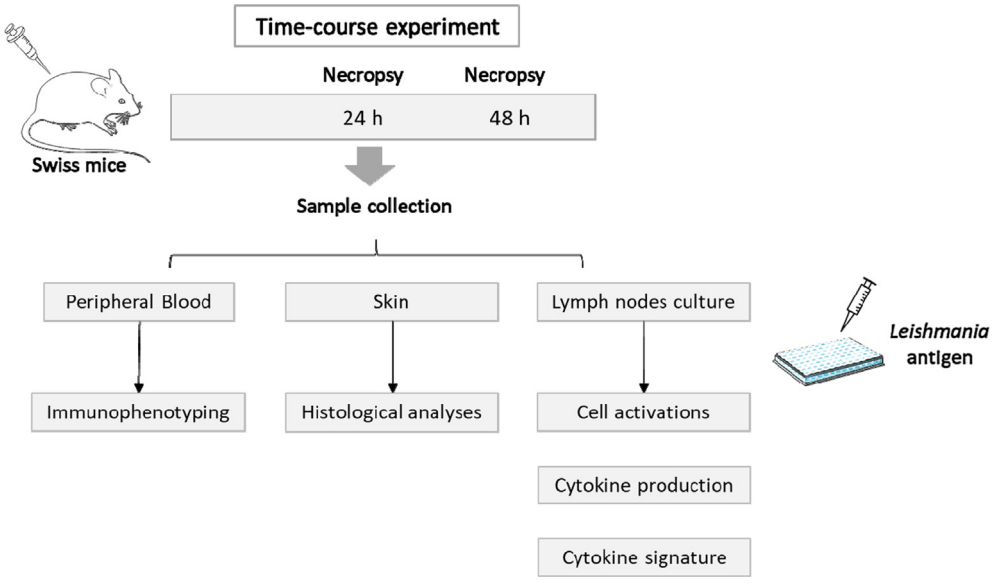

Fig. 1. Study design representing the sensitization protocol in swiss mice. Animals received a single dose of adjuvants, LB antigen or the association of adjuvant plus LB antigen. After 24 and 48 h the mice were sacrificed, and further analyses were taken. Peripheral blood was used for immunophenotyping, skin samples was used for histological analyses and lymph nodes were taken for cell culture after stimulation with *Leishmania* antigen to evaluate cell activations, cytokines production in the supernatant and cytokine signature.

analyses, the blood was collected for peripheral blood cells immunophenotyping and draining lymph nodes were collected for evaluation of cell activation and cytokine assessment. The experiment was performed in duplicate for each time point.

It is important to note that, squalene emulsion (SE) has the role to keep the stability of the MPL adjuvant as showed in different works [14,15] for this reason we do not immunize a group of animals with this component alone.

2.2. Production of vaccine antigen

The vaccine antigen used in this study was produced from culture of the *L. (V.) braziliensis* strain (MHOM/BR/75/M2903) cultivated in culture medium blood agar, Nicolle-Novy-Neal (NNN) associated with the Liver Infusion Tryptose (LIT) according to Giunchetti et al 2007 [9]. The disruption of promastigotes was continued for ultrasound (Sonifier ® Cell Disruptor - Brason Sonic Power Co. USA) using five cycles of 1 min at 40 Watt at intervals of 15 s. The vaccine antigen was aliquoted and frozen at −80 °C freezer (Forma Scientific, USA). Protein concentration was measured by Lowry's method (1951). The concentration of total antigen suspension was used for each dose of 60 μg/dose in sterile saline.

2.3. Histological examination

Histological analyses were performed according to [16]. Briefly, cellular infiltration and differential counts of inflammatory cells were assessed in skin biopsies from the inoculation sites at 24 and 48 h after injections. Image analyses of the slides were performed using the Leica Qwin V3 (Leica Microsystems Ltd.), counting cell nuclei.

2.4. Blood puncture and immunophenotyping of peripheral blood

Whole peripheral blood was collected in EDTA from the animals (6 animals/group/time) 24 and 48 h after sensitization. The immunophenotypic profile of the populations and subpopulations of mouse peripheral blood cells was performed according to Vieira et al 2012 [17]. Briefly, the respective antibodies, Anti-CD3 (PE Hamster anti-mouse CD3, clone 145-2C11, BioLegend), anti-CD4 (PerCP-CyTM 5.5 Rat anti-mouse CD4, clone RM4-5, PharmingenTM BD), anti-CD8 (FITC Rat anti-Mouse CD8a, clone 5:10, Catalg), anti-CD14 (FITC rat anti-mouse CD14, clone Sa2-8, eBioscience), anti-CD19 (FITC rat anti-mouse CD19, clone 6D5, Catalg) and anti-CD49b (FITC Rat Anti-Mouse CD49b, clone DX5, BD PharmingenTM) were put into polystyrene tubes, and the blood was added. After vortex homogenization, the samples were incubated for 30 min at room temperature in the dark. After erythrocytes lysis, the preparations were centrifuged at 600 × g/7 min at room temperature. The supernatant was discarded, and the cells washed with 3 ml of PBS (pH 7.2–7.4), centrifuged at 600 × g/7 min at room temperature. The leukocytes were fixed with 200 μL in FACS FIX solution and stored at 4 °C prior to flow cytometry acquisition and analysis. Phenotypic and morphometric parameters of cells present in each tube were determined by flow cytometry (FACScalibur ® - Becton Dickinson). The software CELLQuest® (Franklin Lakes, NJ, USA) was used for data acquisition and analysis of results from 15,000 events/sample.

2.4.1. Phenotypic analysis of lymph node cells after in vitro stimulation with antigen of L. (L.) infantum

Draining lymph nodes (axillary and lateral) were removed and macerated in RPMI 1640 medium to obtain cells, which were centrifuged at 600 *g* at 10 °C for 7 min and resuspended in 1 ml volume for counting in a Neubauer chamber (BOECO, Germany) with Turk liquid. The cells of the draining lymph nodes were adjusted to a suspension containing 5×10^5 cells/well. Then 250 μL of this suspension were distributed in duplicate in microculture plates with 48 wells flat bottom. This cell suspension was cultured with 250 μL of RPMI medium (supplemented with fetal bovine serum, penicillin, β-mercaptoethanol, and L-glutamine) and diluted with 25 μL of stimuli (10μg/mL of mitogen concanavalin-A or 25 μg/mL of sonicate antigen *L. (L.) infantum*).

Cells were incubated for 72 h in CO_2 incubator (5%) at 37 °C. After incubation, the plates were centrifuged at 600*g* at 10 °C for 10 min and the supernatant was collected in microtubes and stored in a freezer at −80 °C for subsequent quantitation of cytokines. The cell suspension for immunophenotyping was removed from the microplate with the aid of Pasteur pipette to polystyrene tubes (Falcon ® 2054, Becton Dickinson, San Diego, USA) with 1.5 ml of RPMI 1640 for subsequent centrifugation at 600*g* at 10° C for 10 min. The supernatant was rejected, and cells were resuspended to a volume of 500 μL.

Monoclonal antibodies conjugated to fluorochromes anti-cells used in this study were: Anti-CD3 (PE Hamster anti-Mouse CD3, clone 145-2C11, Biolegend), anti-CD-4 (PercP-Cy ™ 5.5 Rat anti-Mouse CD4, clone RM4-5, BD Pharmingen™), anti-CD8 (FITC Rat anti-Mouse CD8a, clone 5H10, Catalg), anti-CD14 (FITC Rat anti-Mouse CD14, clone Sa2-8, eBioscience), anti-CD19 (FITC Rat anti-Mouse CD19, clone 6D5, Catalg), anti-CD11c (APC Hamster Anti-Mouse CD11c, clone HL, BD Pharmingen™), anti-F4/80 (FITC Anti-Mouse F4/80 Antigen, clone BM8, eBioscience). The markers related to cell activation were: anti-CD86 (PE Rat anti-Mouse CD86, clone GL1, BD Pharmingen™), anti-CD69 (Hamster anti-Mouse CD69, clone H1.2F3, BD Pharmingen™) e anti-CD25 (PE Rat anti-Mouse CD25, clone 3C7, BD Pharmingen™).

The assay was carried out incubating the 50 μL of cell suspension and 5 μL of each antibody for 30 min at room temperature and protected from light. After this time, 1 ml of PBS-W was added and centrifuged at 600*g* for 10 min at 10 °C. The cells were resuspended in 200 μL of fixative solution. The reading was performed in flow cytometer and 20,000 events per sample were acquired. The program CellQuest ® (Franklin Lakes, NJ, USA) was used to acquire the data and Flow Jo software (BD, Bioscience) for analysis. Unspecific binding was monitored by isotype controls. Autofluorescence was monitored using a negative control were the cell suspension was incubated without fluorochrome-labeled mAbs. Flow cytometry compensation was carried out by previous instrument settings using calibration bead (CaliBrite® - Becton Dickinson, San Jose, CA, USA). The experiments were performed in duplicate (6 animals/group/time).

2.5. Cytometric bead array

Cytokine levels were measured using Cytometric Bead Array (BD Biosciences) in culture supernatant of lymph node cells after *in vitro* stimulation with antigen of *L. (L.) infantum* according to the manufacturer's recommendations. The cytokines assessed were interleukin IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α. Standard curves for each cytokine in picograms per milliliter (pg/mL) were plotted. The concentrations of each test sample were calculated using the FCAP software array (BD Biosciences).

2.6. Cytokine signature analysis

Based on the cytokine results obtained after culture supernatant of lymph node cells, we performed a cytokine signatures of mice sensitized with distinct stimuli. Firstly, we assessed the percentages of low and high cytokine producers, as previously suggested by Silva, Martins [18], using the global median as the cut off values to segregate the producers. The percentage of both animals

sensitized and control group showing high frequency was calculated for each parameter. The ascendant curve of high frequency subjects on control group was then used as the reference curve to identify changes in the overall immune patterns from the other groups. Each axis represents the frequency (%) of mice showing high cytokine production of a specific stimuli.

2.7. Statistical analysis

One-way ANOVA followed by Tukey's test were performed to analyze the differences between the specific groups. *P* values were calculated using PRISM software (GraphPad, San Diego, CA). Comparative analyzes were performed between CS and LB groups in relation to adjuvants (SAP, MPL and IFA) or in relation to the vaccine composition (LBSAP, LBIFA and LBMPL). Comparisons were made between the adjuvant group and the association with total antigen of *L. (V.) braziliensis* corresponding (SAP/LBSAP, IFA/LBIFA and MPL/LBMPL).

3. Results

Herein, mice were sensitized with SAP, IFA and MPL adjuvants alone and in association with *L. braziliensis* antigen (LBSAP, LBIFA and LBMPL) as vaccine to investigate the immune response, immunohistopathological and inflammatory profile in blood, skin and lymph nodes at 24 and 48 h. Regarding an *ex vivo* analysis, at 24 h, in peripheral blood there was an increase in frequency of $CD4^+$ T-lymphocytes in the following groups: IFA, MPL, LBSAP, LBIFA and LBMPL when compared to control group. Concerning $CD8^+$ T lymphocytes there was an increase in the percentage in LBMPL group compared to the control, LB and LBIFA groups. There was an increase of T $CD8^+$ in LBSAP group when compared to SAP group (Supplementary Fig. S1). At 48 h, we observed an increase in frequency of NK cells ($CD49^+$) in LBIFA and LBMPL compared with LBSAP group (Supplementary Fig. S1).

3.1. Adjuvants used alone or in association with L. braziliensis antigens as vaccines displayed an increased number of cells migrated to the skin of sensitized mice

The skin histological analysis through quantification of the cellular infiltration demonstrated a significant increase in the cells number of the animals sensitized with SAP, IFA and MPL adjuvants when compared with control and LB groups in both time points (24 and 48 h). This event was also observed when mice were immunized with the *L. braziliensis* antigen combined with LBSAP, LBIFA and LBMPL adjuvants. The comparative analysis among adjuvants showed that MPL induced higher cellular infiltration compared with SAP (24hs) and with IFA (48 hs) as shown in Fig. 2(A and B). Likewise, we observed that the association of *L. braziliensis* antigen was more efficient when the MPL was employed (LBMPL) as adjuvant to improve higher cell recruitment when compared with others (LBSAP and LBIFA) in both time points (Fig. 2A–C).

3.2. The IFA and MPL adjuvants when combined with L. braziliensis antigen (LBIFA and LBMPL) as a vaccine stimulated an increase of dendritic cells ($CD11c^+$) and their activation ($CD11c^+CD86^+$) in draining lymph nodes culture

The lymph nodes are an important organ in the early stage of the immune response by presenting antigens. To investigate whether adjuvants and/or adjuvant plus antigen could induce dendritic cell (DC) and macrophage activation we analyzed, by flow cytometry, the phenotypic characteristics of these activated cells from draining lymph nodes culture. The percentage of activated cells in the lymph node culture was evaluated by analysis of $CD11c^+CD86^+$ and $F4/80^+CD86^+$ phenotypes (Fig. 3A). The data are presented as Cell Index, which is the value ratio of stimulated culture/control culture analyzes (Fig. 3B). Our results highlighted that there is an increase of the dendritic cells ($CD11c^+$) in LBIFA, MPL and LBMPL groups compared to LB group in the 24 h time point. When the activation of these cells was evaluated, there were increased $CD11c^+CD86^+$ indexes in LBIFA and LBMPL regarding LBSAP group. However, no differences were observed when the $CD86^+$ activation marker in macrophages cells was evaluated. On the other hand, we observed an increase of macrophages ($F4/80^+$) in saponin group in 24hs when compared with the LB, IFA and MPL groups. However, in 48hs the SAP group exhibited decreased $F4/80^+$ macrophages in comparison with the LB and MPL groups (Fig. 3B).

3.3. Mice immunized with L. braziliensis plus saponin as adjuvant (LBSAP), highlighted an increase of $CD8^+$ T-lymphocyte, higher activation marker ($CD69^+$) in $CD4^+$ T-lymphocyte and B-lymphocytes ($CD19^+$) from draining lymph nodes in the in vitro context

In order to investigate the activation of T and B-lymphocytes in the lymph node of mice sensitized with adjuvants and/or vaccines we performed the evaluation of CD25 and CD69 phenotype using flow cytometry at the 24 and 48 h time points (Fig. 4A and B). Our results showed that, in 24 h an increase of T-$CD4^+CD25^+$ phenotype was observed in mice immunized with LB when compared to SAP, IFA and LBIFA groups. When we compared the adjuvants, we observed in MPL group an increase of T-$CD4^+CD25^+$ in relation to SAP and IFA groups. This increase was also observed in mice immunized with LBSAP and LBMPL when compared with LBIFA ones (Fig. 4A). The saponin adjuvant was the only one that, when combined with the *L. braziliensis* antigen (LBSAP), was able to promote an increase of $CD4^+CD25^+$ as compared with the mice sensitized with the saponin alone. The LBSAP group also displayed an increased expression of $CD19^+CD25^+$ B-lymphocytes in comparison with control and LBIFA groups (Fig. 4A).

In 48 h, an increase of $CD8^+$ T-lymphocytes was observed in the SAP group when compared to the control and MPL groups. Furthermore, in LBSAP group there was an increase of $CD8^+$ T-lymphocytes when compared to control, LB, LBIFA and LBMPL groups. The recent activation biomarkers of the immune response analysis showed an increase of the $CD4^+CD69^+$ phenotype expression in LBSAP group when compared to control and LB groups. We also observed increases in this phenotype profile in the animals immunized with LBIFA and LBMPL in comparison to the animals immunized with *L. braziliensis* antigen alone. Likewise, the LBSAP vaccine was also able to increase the expression of the $CD69^+$ phenotype in B-cells ($CD19^+$) when compared to SAP, LBIFA and LBMPL groups. This increased phenotype in $CD19^+CD69^+$ B-Cells was also observed in mice sensitized with MPL over the SAP group. As shown in Fig. 4B, the analysis of intermediate activation markers $CD25^+$ showed that they increased in both $CD4^+$ and $CD8^+$ T-lymphocytes in SAP and MPL groups in relation to the IFA. Moreover, there was an increase in the levels of $CD8^+CD25^+$ phenotype in SAP group as compared to the control and LBSAP animals (Fig. 4B).

3.4. Saponin when combined with the L. Braziliensis antigen (LBSAP) was able to promote a pro-inflammatory immune response profile, considering the increased levels of (IL-2, IFN-γ, IL-17) in the early time point (24hs)

The quantification of cytokines production by lymph node cell culture was performed using the quantification bead array assay (Fig. 5). As observed, in 24 h, animals sensitized with LBSAP pro-

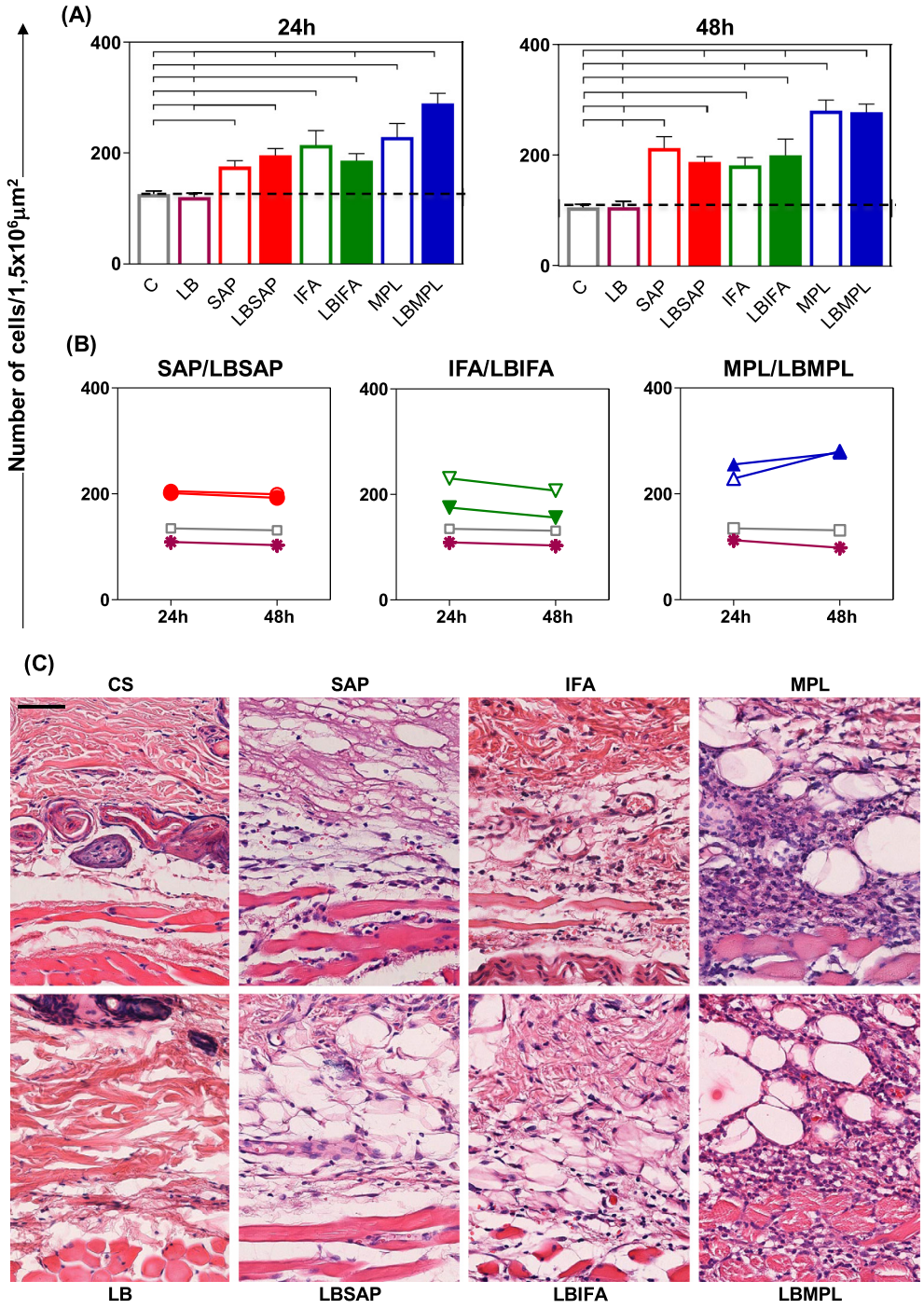

Fig. 2. **(A)** Quantification of the cell infiltrate in the skin of mice sensitized with different vaccine components: total antigen of *L. (V.) braziliensis* (LB, empty purple bar), saponin (SAP; empty red bar), SAP + LB (LBSAP; filled red bar), Incomplete Freund's Adjuvant (IFA; empty green bar), LB + IFA (LBIFA; filled green bar) and monophosphoryl Lipid A (MPL; empty blue bar) and LB + MPL (LBMPL; filled blue bar) at 24 and 48 h after sensitization. The control group (C; empty gray bar) was inoculated with saline. The dashed line represents the average number of cell nuclei quantified in histological sections of skin from control animals. Data presented are the mean ± SD from groups of six animals/evaluation time. Significant differences ($p < 0.05$) between the groups are represented by connector lines. **(B)** The graphics line represents the kinetics of infiltration at 24 and 48 h in the groups: CS (empty gray square), LB (purple asterisk), SAP (empty red circle), LBSAP (filled red circle), IFA (inverted empty green triangle), LBIFA (inverted filled green triangle), MPL (empty blue triangle) and LBMPL (filled blue triangle). **(C)** Representative photomicrographs of the cellular infiltrate at 48 h, Bar = 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

duced higher levels of IL-2 in relation to those of the other groups; IFN-γ only in relation to control group; IL-17 and IL-10 when compared to control and LB groups. The IFA, LBIFA, MPL and LBMPL groups only promoted a significant increase in the IL-17 cytokine when compared to the control and LB groups at this time point (Fig. 5).

In 48hrs time point, a reduction in IL-6 cytokine levels was observed in SAP, LBSAP, IFA and LBIFA groups when compared to control and LB groups. A general increased production of IL-2 cytokine was observed in all groups (SAP, LBSAP, IFA, LBIFA, MPL and LBMPL) in 48 h when compared to control and LB groups. At this time, a higher level in TNF-α production was observed in the

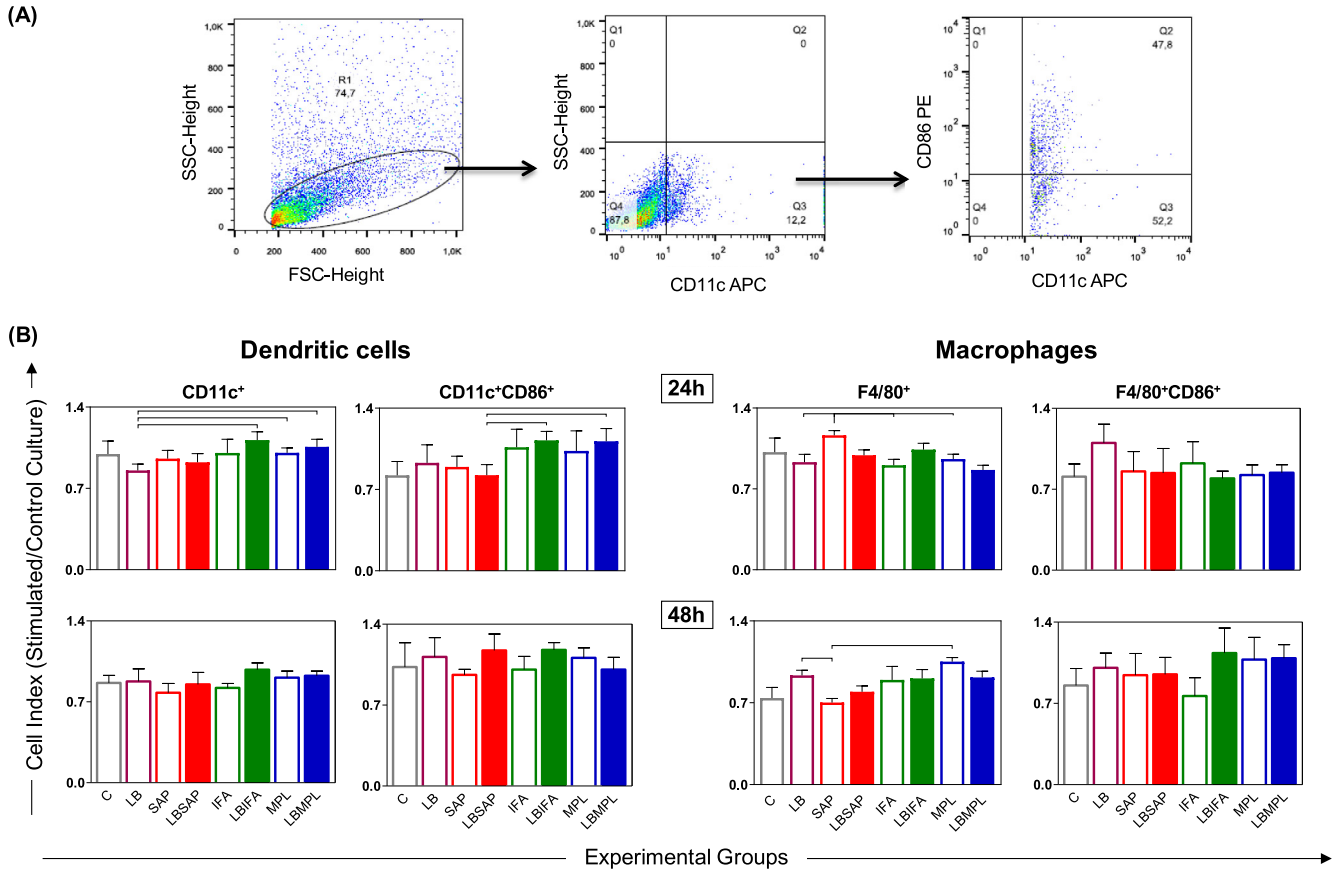

Fig. 3. Profile of the activation marker CD86 expressed in dendritic cells ($CD11c^+$) and macrophages ($F4/80^+$) in the draining lymph nodes culture of the groups sensitized with: total antigen of *L. (V.) braziliensis* (LB, empty purple bar), saponin (SAP; empty red bar), SAP + LB (LBSAP; filled red bar), Incomplete Freund's Adjuvant (IFA; empty green bar), LB + IFA (LBIFA; filled green bar) and monophosphoryl Lipid A (MPL; empty blue bar) and LB + MPL (LBMPL; filled blue bar) at 24 and 48 h after sensitization. The control group (C; gray bar) was inoculated with saline. **(A)** Illustrative figure of flow cytometry analysis of the dendritic cells and macrophage from draining lymph nodes culture. **(B)** Data presented are the mean ± SD from groups of six animals/evaluation time. Significant differences ($p < 0.05$) between the groups are represented by connector lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MPL group when compared with other adjuvants (SAP and IFA); and, in LBMPL vaccinated animals when compared to LBSAP and LBIFA vaccines. Furthermore, regarding IFN- γ production, we observed increased levels in 48 h in the LB group when compared with the SAP, LBSAP and IFA groups; in MPL group compared with SAP and IFA groups; and in LBIFA and LBMPL groups compared with LBSAP group. About IL-17 in 48 h, an increase of this cytokine was detected in the SAP, IFA, LBIFA, MPL and LBMPL groups compared to control and LB groups. The IL-10 analysis in 48 h showed lower levels of this cytokine in SAP, LBSAP and IFA groups in relation to control and LB groups. This reduction was also observed in SAP and IFA groups as compared to the control group; Interestingly, there was a reduction in the IL-10 levels in LBSAP group when compared with LBIFA group. Outstandingly, the MPL adjuvant used alone or combined with *L. braziliensis* antigen was able to increase the production of IL-10 in 48 h when compared to the other groups (Fig. 5).

3.5. The cytokine signatures revealed a distinct profile of immune response according to the type of sensitization employed in the animals

The cytokine signatures demonstrated that sensitization of animals at 24 h with LBSAP induced a plethora of cytokines with higher frequency of IL-2, IFN- γ, TNF, IL-6, IL-10 and IL-17 high producers as compared to the control group (Fig. 5A). Moreover, SAP only also induced a relevant increase of cytokine IL-2, IFN- γ, TNF, IL-6, IL-10 as compared to the control group. Interestingly, the sensitization of animals at 48 h pointed out the ability of LB group to induce a more frequency of TNF, IL-6, IL-10 and IFN- γ high producers as compared to the control group. When the sensitization was performed with LB combined with IFA or MPL at 24 h, the data demonstrated that the presence of LB altered the profile presented by the groups. While in the LBIFA there was increased frequency of IL-2 and IL-6 high producers, only an increase in the frequency of IL-2 high producer was observed in the LBMPL group as compared to the control group (Fig. 6B). Moreover, the sensitization of animals at 48 h showed in the LBIFA group an increased frequency of IL-10 and IFN- γ and in the LBMPL group an increased frequency of IL-10 as compared to the control group (Fig. 6B).

Finally, we summarized in a flowchart the results obtained in the present work with the focus in draining lymph node and peripheral blood from mice sensitized with SAP, IFA and MPL isolated or combined with *L. braziliensis* antigen and connecting with the other results of the cell recruitment and cytokines in the skin [16] as shown in Fig. 7. Taken together all those findings, we can better understand the role of the adjuvant in the immune response in the early and late time points after sensitization considering the distinct compartments targeted.

4. Discussion

Nowadays adjuvants have been widely employed in vaccine compositions, although this large number of studies with adju-

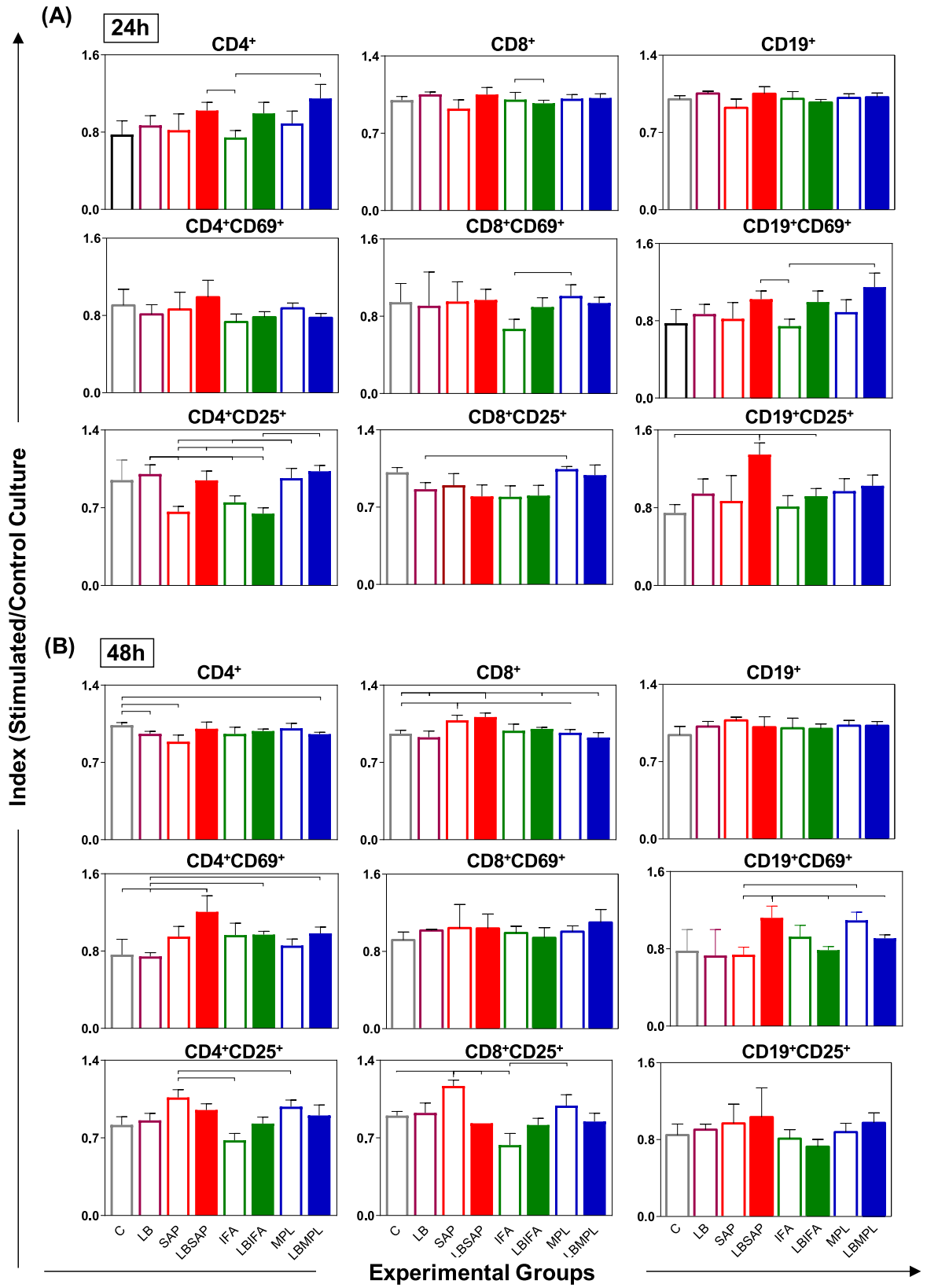

Fig. 4. Profile of the activation markers CD69 and CD25 expressed in T lymphocyte subsets ($CD4^+$ and $CD8^+$) and B lymphocytes ($CD19^+$) in draining lymph nodes culture of the groups sensitized with: total antigen of L. (V.) braziliensis (LB, empty purple bar), saponin (SAP; empty red bar), SAP + LB (LBSAP; filled red bar), Incomplete Freund's Adjuvant (IFA; empty green bar), LB + IFA (LBIFA; filled green bar) and monophosphoryl Lipid A (MPL; empty blue bar) and LB + MPL (LBMPL; filled blue bar) at 24 **(A)** and 48 **(B)** hours after sensitization. The control group (C; gray bar) was inoculated with saline. Data presented are the mean ± SD from groups of six animals/evaluation time. Significant differences ($p < 0.05$) between the groups are represented by connector lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

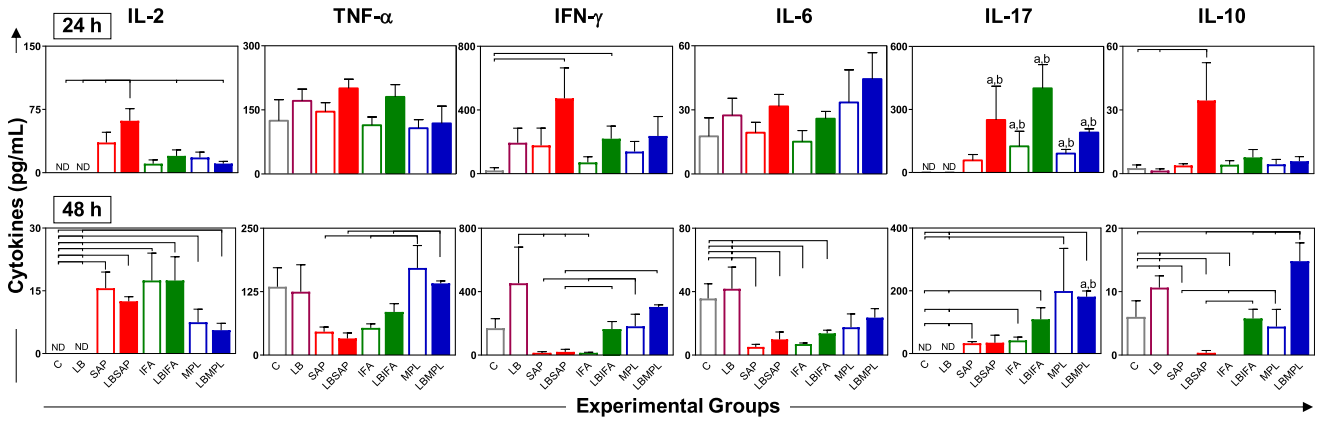

Fig. 5. Cytokine profile produced by draining lymph nodes cells culture in animals sensitized with: total antigen of L. (V.) braziliensis (LB, empty purple bar), saponin (SAP; empty red bar), SAP + LB (LBSAP; filled red bar), Incomplete Freund's Adjuvant (IFA; empty green bar), LB + IFA (LBIFA; filled green bar), monophosphoryl Lipid A (MPL; empty blue bar) and LB + MPL (LBMPL; filled blue bar) at 24 and 48 h after sensitization. The control group (C; gray bar) was inoculated with saline. Data presented are the mean ± SD from groups of six animals/evaluation time. Significant differences ($p < 0.05$) between the groups are represented by connector lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vants shows a lack of information about the precise mechanism by which the adjuvants can improve the stability, safety and immunogenicity of vaccines. So, understanding how vaccine adjuvants interact with components of the innate and acquired immune response will help in the selection and design of new vaccines [19,20]. Herein we provided relevant data about elements of innate and acquired immune response regarding the cellular microenvironment, specific immune response and inflammatory mediators induced by adjuvants alone (SAP, IFA and MPL) and in combination with total antigen of *L. (V.) braziliensis* (LBSAP, LBIFA and LBMPL).

To evaluate cellular recruitment, the quantification of cellular infiltrate was performed after sensitization of the mouse skin with different adjuvant or vaccine composition. The results after 24 and 48 h showed that MPL and LBMPL groups stood out showing most cell recruitment than the other groups. Similar result was found in previous study comparing the mechanism of various adjuvants in skin of mice demonstrating the ability of MPL to enhance cellular recruitment [16]. In addition, it could be observed that there is no difference between the adjuvant groups alone and vaccine combinations. This points out that LB alone cannot induce a sustained cellular recruitment and the adjuvant is considered an important part of the vaccine for leishmaniasis [8]. Thus, based on this finding it can be assumed that adjuvants have a higher capacity to induce cellular infiltrate without causing macroscopic adverse effects. These findings strongly corroborate with other studies and reinforce that adjuvants can trigger the innate immune response in the site of sensitization as shown by [21]. According to these results, it is important to highlight the occurrence of a notable difference between the administration of the antigen alone, resulting in low immunogenicity and the antigen administration associated with the adjuvant that promoted a cellular migration inducing inflammation [22]. This cellular recruitment mechanism promoted by the adjuvant is fundamental to generate inflammation in the site of inoculum, providing a high traffic of cells and prolonging the antigen bond, consequently the enhancement of antigen-APC interaction [23].

Other mechanisms that determine the development of an immune response requires the antigen translocation from inoculum site to the draining lymph nodes, where the antigen will be presented to T and B lymphocytes [24,25]. Thus, the analysis of antigen presenting cells (dendritic cells and macrophages) and expression of the costimulatory molecule (CD86) in the lymph nodes becomes relevant and contributes to understanding these phenomena. It is known that the signs of damage (inflammation, apoptotic and necrotic cells) induced by adjuvants recruit dendritic cells and macrophages to the site of administration to maintain tissue homeostasis [26] and possibly results in migration of these cells to the draining lymph nodes. Regarding dendritic cells, it was observed in 24 h a significant increase in indexes of these cells in IFA, LBIFA, MPL and LBMPL groups. However, the activation of these cells was increased only in LBIFA and LBMPL groups. Interestingly, this shows the importance of the antigen in vaccine formulation for activation of antigen-specific immune response, where even though the adjuvant could recruit dendritic cells, the activation only occurred in the presence of the antigen. This phenomenon is similar to what has been observed in other study where immunogenic epitopes, associated with adjuvant, promotes an activation of dendritic cells with *ex vivo* T lymphocytes proliferation [27]. Shen & Yang (2012) demonstrated that exposure of cells pre-treated with emulsions adjuvants induces maturation of dendritic cells with increased expression of costimulatory molecules (CD40, CD80 and CD86) [28]. In this way, dendritic cells activation may be characterized by recognition and processing vaccine antigen and differentiation into effector cells that are responsible for the immune mechanisms of activation and memory [29].

Concerning macrophages, our results demonstrated increased rates of these cells in 24 h in the SAP group, while at 48 h, this increase was observed in LB and MPL groups. This corroborates with other study showing that saponins have an early effect in macrophage recruitment to lymph nodes and macrophages are essential for maturation of dendritic cells [30]. However, there was no significant increase in CD86 expression on macrophages. Our results suggest that the vaccine antigen and the adjuvant alone were not able to induce an increase in CD86 expression. Our results agree with the study that observed an increase in CD40 expression in monocytes, but not in CD86 expression in formulations containing MPL in 24 h after sensitization [31].

To expand the understanding of the specific immune response, it was carried out the analysis of T and B lymphocytes profile and the activation markers CD69 and CD25. The rapid and transient induction of CD69/CD25 expression in T cells suggests an increase in the activation as well as differentiation [32]. The activation of an immune response is the result of different interactions involving many cell types. Mediated cell response by T lymphocytes is dependent on the interaction between these cells and APCs, promoting the proliferation and differentiation into effectors cells. The sustained link between T cells and APCs is critical to full activation, including entry into cell cycle and expression of cytokine

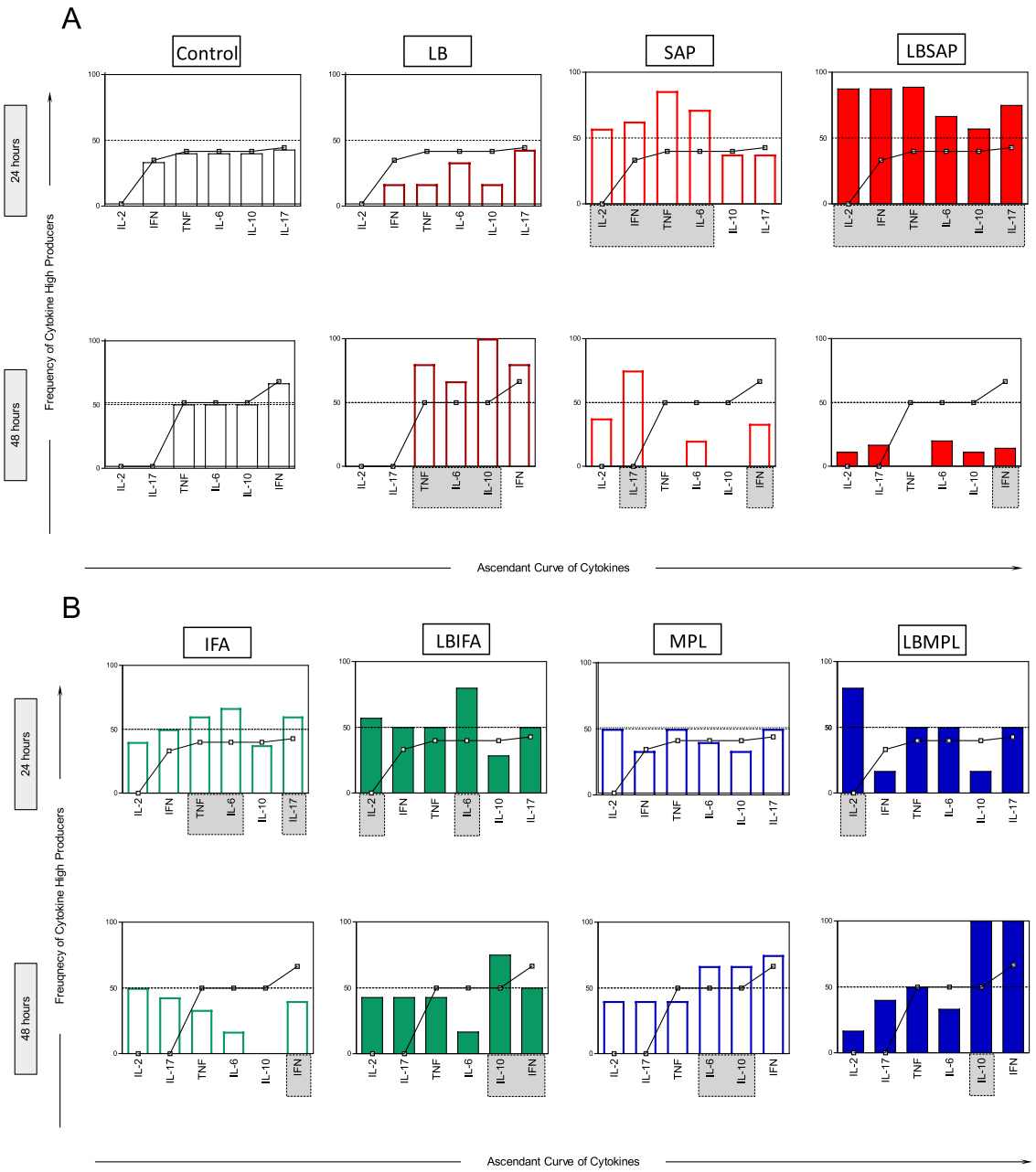

Fig. 6. Inflammatory and regulatory cytokine pattern of animals sensitized with: total antigen of L. (V.) braziliensis (LB, empty purple bar), saponin (SAP; empty red bar), SAP + LB (LBSAP; filled red bar), Incomplete Freund's Adjuvant (IFA; empty green bar), LB + IFA (LBIFA; filled green bar), monophosphoryl Lipid A (MPL; empty blue bar) and LB + MPL (LBMPL; filled blue bar) at 24 and 48 h after sensitization. The control group (C; empty blank bar) was inoculated with saline. The overall pattern of inflammatory and regulatory cytokines is presented as the ascendant percentages of mice with high cytokine levels for each experimental arm. Comparative analysis of the overall cytokine patterns found in the control group (lines with black rectangles) were further compared by overlapping the ascendant cytokine curves. Dotted lines highlight the 50th percentile was used as reference for comparative analysis. *Relevant differences were considered when the frequency for a given cytokine emerged outside the 50th percentile as compared to the reference cytokine pattern or signature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genes, although a transient recognition can also stimulate the expression of CD69 and CD25 [33]. Overall, our dendritic cell results agree with the increase of $CD4^+CD25^+$ in LB group in comparison with SAP, IFA at 24 h, and once again we showed that the presence of LB is crucial for the activation of T lymphocytes. Although the adjuvant alone could induce an activation of T lymphocytes, the vaccine formulations were capable to enhance this activation compared with LB group alone. So, the association between antigen and adjuvant is the most effective way to trigger a high expansion and activation of T cells in drain lymph nodes [34].

The immune response to a protein antigen may be determined by the environment in which it is found [35]. In this sense, the formation of a microenvironment with different cytokines can effectively direct the immune response. These data showed that at 24 h, the LBSAP group induced higher production of important cytokines to proliferate and activate lymphocytes such as IL-2, IFN- γ and the immunomodulatory cytokine IL-10. These results corroborate with the early $CD4^+$ and $CD8^+$ cells activation in this group. Regarding the data at 48 h, SAP and LBSAP groups demonstrated only IL-2 increased levels while LBMPL group demonstrated significant differences of IL-2, IFN- γ, TNF- α and IL-10

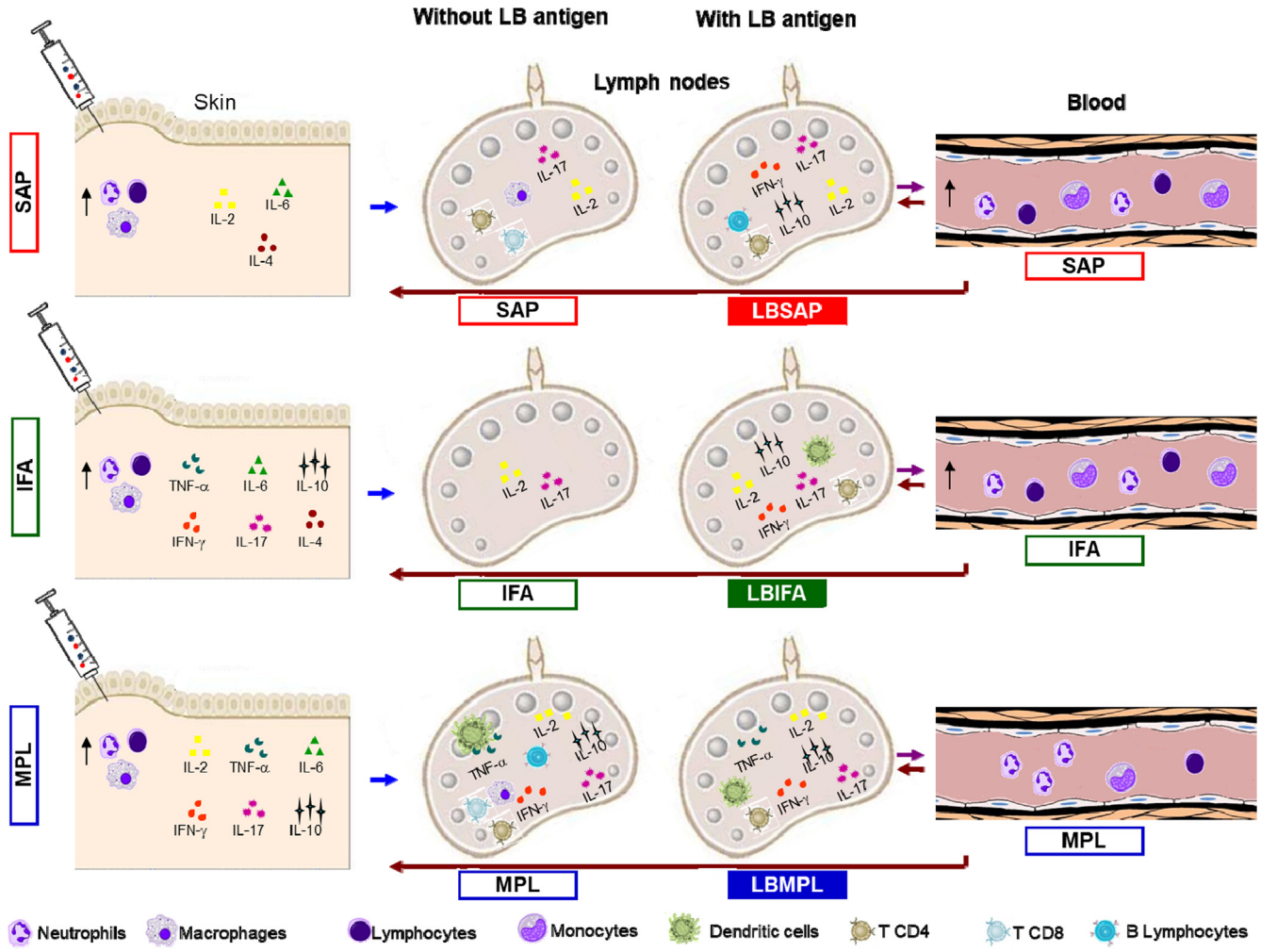

Fig. 7. Flowchart summarizing the main results observed in the present study and other results previously published in the skin, lymph nodes and blood of the animals sensitized with total antigen of L. (V.) braziliensis (LB), saponin (SAP), SAP + LB (LBSAP), Incomplete Freund's Adjuvant (IFA), LB + IFA (LBIFA) and monophosphoryl Lipid A (MPL) and LB + MPL (LBMPL).

levels. Interestingly, LB group showed increased levels of IFN- γ at 48 h. These results reinforce that both antigen and vaccines groups can produce key cytokines responsible for lymphocyte activation. Thus, our results suggest that migration and activation of cells in the draining lymph node, such as dendritic cells, macrophages, and T and B lymphocytes was accompanied by production of cytokines associated with each cell type. Similar findings were described by [36], that assessed the cytokine profile of immunized mice with different adjuvant as MPL and Saponin plus freeze thawed promastigotes of *L. donovani*. They found that Type 1 cytokines may enhance mice protection after challenge. It is important to note that IFN-γ production has a major role in eliciting anti-parasite macrophage responses, notably it induces production of reactive oxygen species (ROS) and induction of nitric oxide synthase (iNOS), which are required for intracellular *Leishmania* killing [37].

In conclusion, this study shows that different adjuvants may promote changes on the kinetics of cell migration and the innate immune response and these substances contribute in recruiting different cell types and cytokines, important in the formation of a microenvironment favorable for the antigen processing and presentation. However, while these adjuvants apparently mimic the ability of pathogens to activate the innate immune system, the complexity of non-specific signals generated with many overlapping and redundant mechanisms hinder the understanding of antigen-specific immune response. Further analyses from this study should determine the impact of adjuvants on the quality of these responses after an immunization protocol vaccine and experimental challenge. Taken together, the results presented by this study will provide important information about the initiation of the local responses promoted by adjuvants that could be administered to develop more effective and safer vaccines. More than that, it will open frontiers for studies of new vaccine technological approaches with adjuvant associations.

Financial support

This study was funded by Brazilian agencies: Fundação de Amparo a Pesquisa de Minas Gerais – FAPEMIG, Brazil (grants ID: APQ-01373-14, APQ-02638-17), Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, Brazil (grant ID: 435224/2018-2), Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais – INCT-DT, Brazil and Universidade Federal de Ouro Preto (UFOP), Brazil. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil – Finance Code 001.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

AT-C, CMC, RCG, RTF, BMR, FASM and AR are grateful to CNPq, Brazil for fellowships. RCFB is grateful to CAPES, Brazil for fellowship. The authors are also grateful for the use of the facilities at Animal Center Science/UFOP, Brazil.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.09.067.

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