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Potential of KM+ lectin in immunization against *Leishmania amazonensis* infection

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Abstract

In the present study we evaluated *Canavalia brasiliensis* (ConBr), *Pisum arvense* (PAA) and *Artocarpus integrifolia* (KM+) lectins as immunostimulatory molecules in vaccination against *Leishmania amazonensis* infection. Although they induced IFN-γ production, the combination of the lectins with SLA antigen did not lead to lesion reduction. However, parasite load was largely reduced in mice immunized with KM+ lectin and SLA. KM+ induced a smaller inflammatory reaction in the air pouch model and was able to inhibit differentiation of dendritic cells (BMDC), but to induce maturation by enhancing the expression of MHC II, CD80 and CD86. These observations indicate the modulatory role of plant lectins in leishmaniasis vaccination may be related to their action on the initial innate response.
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1. Introduction

Leishmania parasites are intracellular protozoa that cause a variety of disease that can range from a self-healing cutaneous lesion to a visceral disseminated disease. Cutaneous leishmaniasis is the most common form that can be caused by *L. amazonensis* and *L. braziliensis* in South America, including Brazil [1]. In experimental cutaneous leishmaniasis, it has been clear that host protection depends on the expansion of Th1 CD4+T cells and suppression of Th2 CD4+T cells [2–6]. Lymphocytes committed to a Th1 profile are classically known to secrete mainly IFN-γ, a major macrophage activator factor, in response to IL-12 stimulation. Although, the role of Th1 cells is clear, the mechanisms governing the expansion of Th1 and Th2 cells in vivo are currently unexplained. During these early events, the role of antigen presenting cells (APC) is critical to direct the response to a specific phenotype. Among this cells, dendritic cells (DC) are known as professional APC because of their natural potential to process and present antigens. The early production of some cytokines during this initial step is important to define a Th1 or Th2 response.

Many molecules have been tested in order to drive a protective Th1 response. Lectins are proteins with the capacity to bind specifically to carbohydrates [7]. They have been isolated from many different sources including higher plant seeds, animal tissues and algae. Plant lectins have been described for their immunostimulatory potential to cell proliferation and lymphokine production. Lectin from

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Canavalia brasiliensis (ConBr) is able to induce IFN-γ production by human lymphocytes [8]. ConBr and the lectin from *Pisum arvense* (PAA) were able to directly stimulate murine macrophages and lymphocytes both in vitro and in vivo to produce NO. In addition, animals treated with this lectins were able to maintain NO production ex vivo [9]. NO is the major molecule that mediates *Leishmania* killing and its production is induced by IFN-γ [10]. A lectin from *Artocarpus integrifolia* (KM+) induced IL-12p40 production by macrophages, which then stimulates IFN-γ production by lymphocytes. Moreover, immunization of BALB/c mice with KM+ lectin and SLA antigen from *Leishmania major* resulted in protection against further challenge [11].

In the present report we explore the immunostimulatory potential of three plant lectins, *Canavalia brasiliensis* (ConBr), *Pisum arvense* (PAA) or *Artocarpus integrifolia* (KM+) in the immunization against *Leishmania*. Our results indicate that only mice immunized with KM+ lectin were able to control *Leishmania* infection probably due to the capacity of KM+ to act on the initial innate response by modifying inflammatory and dendritic cell responses.

2. Materials and methods

2.1. Lectins

Lectin from *Canavalia ensiformis* (ConA) was obtained from Sigma (Sigma/St. Louis, EUA). Lectins from *Canavalia brasiliensis* (ConBr) and *Pisum arvense* (PAA) and *Artocarpus integrifolia* lectin (KM+) were obtained according to methods previously described [12-14]. The concentrations of the lectins were determined based on previous work [8,9,11].

All lectins preparations used were free of bacterial endotoxin.

2.2. Mice, parasites and preparation of SLA antigen

Inbred BALB/c of both sexes, 8–12 weeks of age were obtained from the central animal facility of Centro de Pesquisas Gonçalo Moniz (CPqGM-FIOCRUZ). Males and females animals were not mixed in the experiments. *Leishmania amazonensis* BA125 strain (MHOM/BR/87/BA125) were maintained at 28 °C in NNN plus DMEM (Gibco BRL, Life Technologies, Grand Island, NY) biphasic medium supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL, Life Technologies, Gaithersburg, MD). Details of isolation, maintenance and course of disease in mice have been reported elsewhere [15]. SLA was prepared from log-phase *L. amazonensis* by sonication and ultracentrifugation as previously described [16].

2.3. Mice spleen cells stimulation with lectins

BALB/c mice were sacrificed and their spleen removed. Fivex10^5 spleen cells/well were cultured in RPMI medium (Gibco-BRL Life Technologies, Grand Island, NY) supplemented with heat-inactivated 10% fetal calf serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 5×10^{-5} M of 2-mercaptoethanol (all from Sigma) in the presence of 20 μl of ConA (10 μg/ml) (Sigma/St. Louis, EUA), ConBr (50 μg/ml), PAA (50 μg/ml) or KM+ (1.0 μg/ml) in 96-well flat bottom culture plates (Corning, Cell Wells, New York) in humidified atmosphere (37 °C, 5% CO_2). After 48 h the supernatants were collected and analyzed for IFN-γ.

2.4. Immunization and infection of mice

BALB/c mice (seven per group) were injected with 100 μl of saline, SLA (10 μg), lectin (0.5 or 50 μg) or SLA (10 μg) plus lectin (0.5 or 50 μg) subcutaneously at the tail. After 15 and 30 days, mice were boosted subcutaneously at the same place with the same preparations. Fifteen days after the last boosting, animals were inoculated in one of the hind footpads with 1×10^6 infective-stage promastigotes of *L. amazonensis* from stationary culture. The evolution of the lesion was monitored by measuring footpad thickness using a metric caliper.

2.5. ELISA-based IFN-γ and IL12p70 detection assay

Measurements of IFN-γ and IL-12p70 in culture supernatants were performed using specific solid-phase sandwich ELISA. Capture and detection mAbs pairs and recombinant cytokines used were purchased from Pharmingen (San Diego, CA) using the procedure recommended by the manufacturer. Microtiter plates were coated with capture monoclonal IFN-γ mAb (RA-642 clone) or IL12p70 mAb (9A5 clone). Detection was carried out with a cytokine-specific byotinilated monoclonal anti-IFN-γ (XMG1.2 clone) or anti-IL12p70 (C17.8 clone) from Pharmingen (San Diego, CA) and streptoavidin peroxidase from Genzyme (Cambridge, MA). The reaction was developed using TMB from Sigma (St. Louis, MO) as substrate. Standard curves were prepared with rmIFN-γ or rmIL-12p70 from Pharmingen (San Diego, CA).

2.6. Parasite burden

Parasite burdens were determined using the quantitative limiting dilution assay as previously described and were analyzed by the ELIDA® software [17,18]. Briefly, draining infected footpads were aseptically excised and homogenized with a tissue glass grinder in 2 ml of Schneider's medium (Sigma, St. Louis, MA). After removal of debris by sedimentation for 5 min, the homogenates were serially diluted in Schneider's medium supplemented with antibiotics, 10% heat-inactivated fetal calf serum and 2% sterile human urine in 96-well plates containing blood agar slants. Twelve replicates of each dilution (100 μl/well) were incubated at 25 °C for 3 weeks and wells were examined for motile parasites at 3-day intervals using an inverted microscope.

2.7. Air pouch and leukocyte migration

Air pouches were prepared by injecting 3 ml of air into the dorsum of BALB/c mice, as described elsewhere [19,20]. Briefly, 100 μl of ConBr, PAA (50 μg) or KM+ (5 or 50 μg) lectins were injected in 1 ml of endotoxin-free saline immediately into the air pouches following the air injection. Control mice were injected with endotoxin-free saline (negative control) and LPS (20 μg/ml; positive control) from Calbiochem (La Jolla, CA). At 6, 12, 24 and 48 h after intrapouch inoculation, three to five animals per experimental group were lethally anesthetized, and the pouch contents were washed with a total of 5 ml of endotoxin-free saline to collect leukocytes of the exudates. Lavage fluids were centrifuged at $100 \times g$ for 10 min at 4 °C and pellets resuspended in saline, stained in Turk's solution, and counted in a Neubauer hemocytometer, and recruited leukocyte populations were identified microscopically on hematoxilin and eosin-stained cytospin preparations.

2.8. Generation of bone marrow-derived DCs (BMDC)

BM-derived DCs were generated as previously described with some modifications [21]. Briefly, BM cells were removed from femurs and tibias of mice and cultured in 24 well-culture plates at a concentration of 2.5×10^6 cells per well in 800 μL of RPMI-1640 (Gibco-BRL Life Technologies, Grand Island, NY) supplemented with heat-inactivated 10% fetal calf serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 5×10^{-5} M of 2-mercaptoethanol (all from Sigma) plus GM-CSF (25 ng/mL) and IL-4 (10 ng/mL). On days 3, 6 and 9 the supernatant was gently removed and replaced with the same volume of the supplemented medium. On day 9 of culture approximately 80% of them were CD11c+ DCs. For the differentiation assays, BM-derived DCs were cultured under the same conditions as described above, in the presence or absence of different concentrations of KM+ lectin (0.1, 1.0 or 10 μg/mL). The differentiated cells were harvested on days 3, 6 and 9 of culture and analyzed the population of CD11c+CD11b+. For the maturation assays, the cells were differentiated in absence of KM+ lectin. At day 9, the cells were activated by the addition of LPS (1 μg/mL; Calbiochem, La Jolla, CA) or KM+ (0.1, 1.0 or 10 μg/mL). Cells cultured with supplemented medium plus the recombinant cytokines were considered immature DCs (C, controls). After 48 h of incubation, the cells were recovered and labeled with specific mAbs. The mean fluorescence intensity value (MFI) for the CD11c+ cells expressing CD80, CD86, CD40 and MHC II molecules was analyzed by FACS.

2.9. Statistical analysis

Data were reported as the mean ± S.E.M. and were analyzed statistically by means of Kruskal–Wallis test and Dunn's post test with the level of significance at $p < 0.05$. All experiments were performed at least three times.

3. Results

3.1. Immunization of BALB/c mice with ConBr, PAA or KM+ lectins

BALB/c mice were immunized with SLA (10 μg/ml) in combination or not with ConBr, PAA or KM+ and challenged 15 days after the last booster with *Leishmania amazonensis* (BA125). The immunization with ConBr or PAA lectins alone or in combination with SLA did not result in lesion size reduction (Fig. 1A and B), while immunization with KM+ lectin alone resulted in significant lesion size reduction ($p = 0.0049$) when compared to the non-immunized control (Fig. 1C). Since lesion size not always correlates to *Leishmania* numbers, we also determined the parasite load in footpads by a limiting dilution essay. Although no reduction in parasite number was observed with ConBr lectin alone, the parasite load was reduced when associated to SLA (from 9.28×10^7 in unimmunized mice to 5.2×10^7 in ConBr + SLA immunized mice with 44% reduction). Immunization with KM+ lectin lead to reduced parasite numbers (4.78×10^7 with 48% reduction) with a much stronger and significant reduction ($p = 0.0366$) when associated to SLA (1.85×10^7 with 80% reduction) (Fig. 2).

3.2. Induction of IFN-γ production in vitro by ConBr, PAA and KM+ lectins

When splenic cells from BALB/c mice were stimulated with ConBr, PAA, KM+ or ConA lectins IFN-γ was produced (Fig. 3). The results show that all tested lectins are able to induce similar levels of IFN-γ. KM+ was able to induce the same production of IFN-γ when much lower concentrations (1.0 μg/ml) were used as compared to ConBr or PAA lectins (50 μg/ml). Control (unstimulated) cultures produced undetectable levels of IFN-γ.

3.3. Inflammatory response induced by ConBr, PAA and KM+ lectins

Since we observed a difference between lesion size and parasite burden, we evaluated the inflammatory potential of the three lectins. Air pouches were raised on the backs of BALB/c mice and 100 μl of LPS (20 μg/ml), saline, ConBr, PAA (50 μg) or KM+ (5 or 50 μg) lectins were inoculated into the pouches. ConBr and PAA lectins were able to induce a higher and significant inflammatory response (39.17×10^4 ml^{-1} and 36.83×10^4 ml^{-1}, respectively) when compared to saline control (6.3×10^4 ml^{-1}) reaching a peak after 24 h ($p = 0.0357$). The stimulation with 5 μg of KM+ lectin, however, did not induce a relevant cell infiltration in any of the time points evaluated (Fig. 4A). Interestingly, even if an increased concentration of KM+ is used (50 μg) the number of cells recruited at 24 h were significantly reduced ($p = 0.0357$) when compared to ConBr and PAA with the same concentration (Fig. 4B).

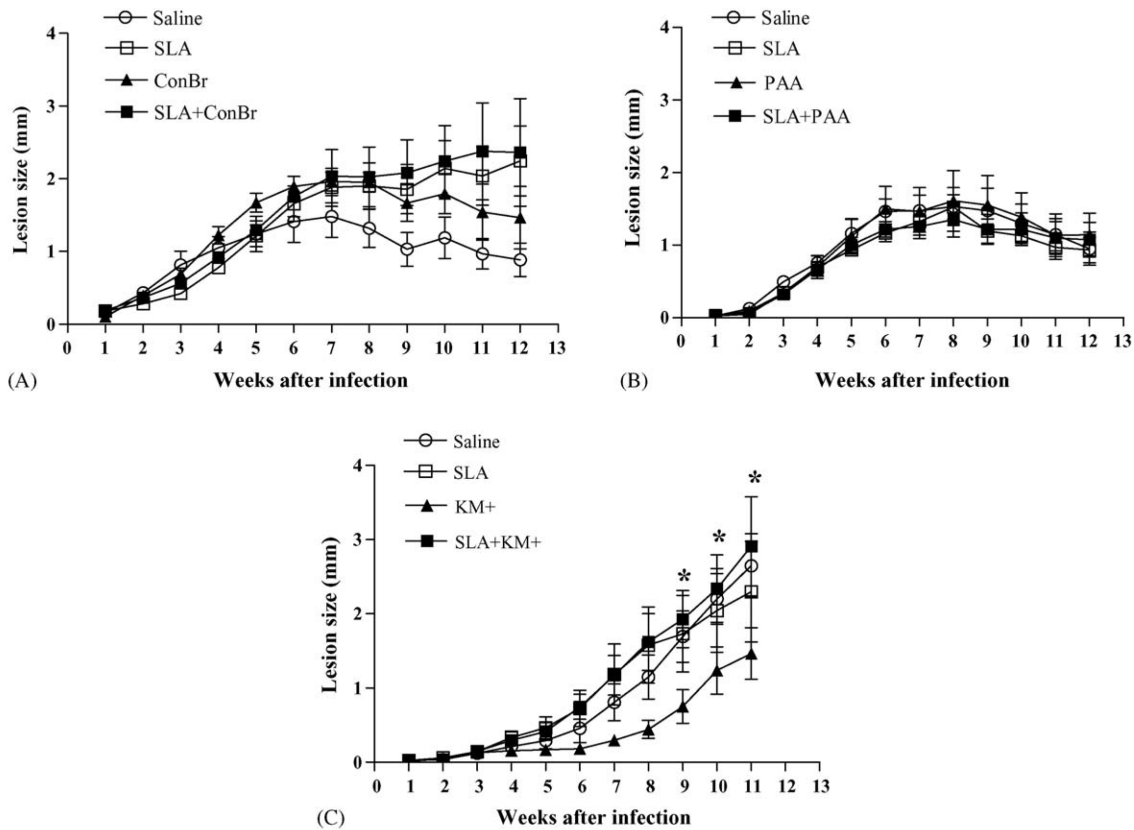


Fig. 1. Time course of lesion development in BALB/c mice ($n = 9$) immunized with different lectins and infected in the hind footpad with 1×10^6 infective-stage promastigotes of *L. amazonensis* after immunization. BALB/c mice were immunized with ConBr (A), PAA (B) or KM+ (C) lectins (lectin, filled triangles) in combination (SLA+ lectin, filled squares) or not with SLA (SLA, open squares). Saline was used as a negative control of the immunization (saline, open circles). Points represent the mean ± S.E.M. size (mm) of footpad lesions. Asterisk indicates significant difference ($*p = 0.0049$) from value for nonimmunized (saline) group and KM+ immunized-group.

Analysing the constitution of the 24 h peak we could observe that both ConBr and PAA were potent recruiters for all leukocyte populations compared to KM+. KM+ induced eosinophil ($6.08 \times 10^4\,\mathrm{ml}^{-1}$) and neutrophil ($4.94 \times 10^4\,\mathrm{ml}^{-1}$) recruitment but produced a modest infiltration of macrophages ($1.13 \times 10^4\,\mathrm{ml}^{-1}$) and lymphocytes ($0.73 \times 10^4\,\mathrm{ml}^{-1}$) to the pouch (Fig. 4C) compared to ConBr and PAA ($p = 0.0357$). Few cells were found in the pouch exudate when endotoxin-free saline was injected alone.

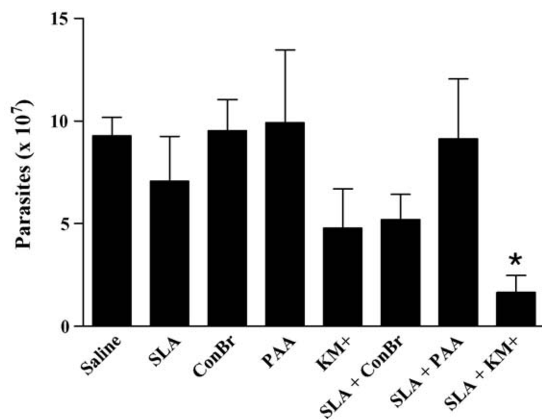


Fig. 2. Parasite numbers in lesions of immunized BALB/c mice 7 weeks after *L. amazonensis* challenge. Parasite numbers determined by limiting dilution and expressed as number of viable parasites per footpad ± S.E.M. Each value represents the mean of nine individuals. Asterisk stands for difference between control and SLA+ KM+ groups. Asterisk indicates significant difference ($*p = 0.0366$) from value for nonimmunized (saline) group and SLA+ KM+ immunized-group.

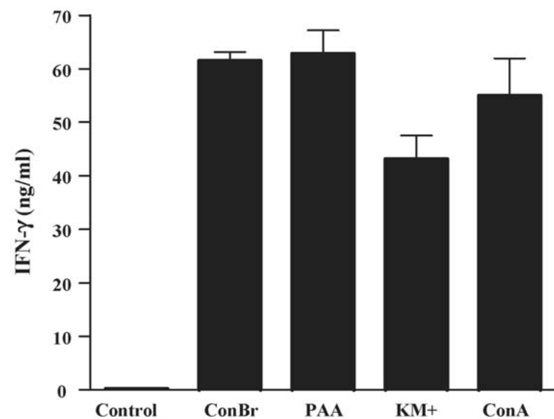
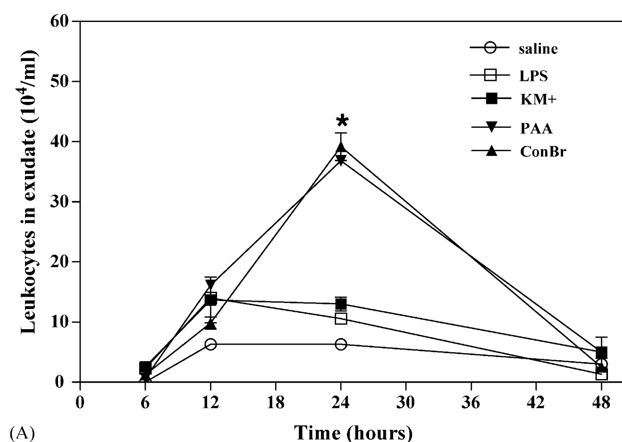
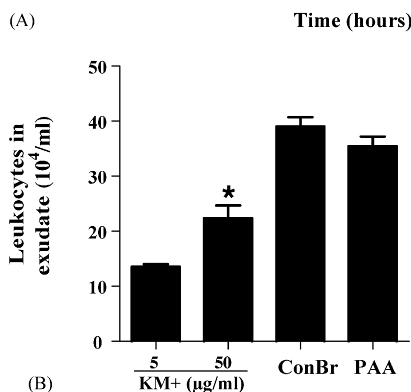


Fig. 3. ConBr, PAA and KM+ lectins were capable of stimulating IFN-γ production in vitro. Mice splenic cells were stimulated in vitro with ConBr, PAA (50 μg/ml), KM+ (1.0 μg/ml) or ConA (5.0 μg/ml) lectins. After 48 h supernatants were collected and IFN-γ was deteced by ELISA. The results are representative of at least three experiments ± S.E.M.





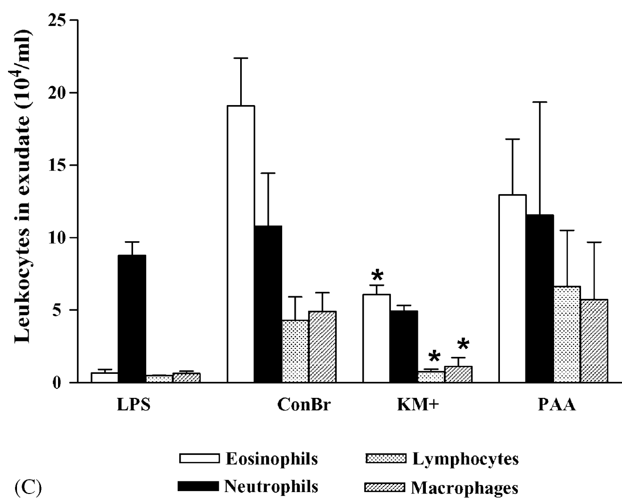


Fig. 4. Leukocytes recruitment in response to saline, LPS, ConBr, PAA or KM+ lectins. (A) Total leukocyte recruitment in air pouch exudate after stimulation with LPS (20 μg/ml), saline, ConBr, PAA (50 μg) or KM+ (5 μg) lectins. Asterisk indicates significant difference (* $p = 0.0357$) from value for ConBr and PAA leukocyte recruitment compared to the control group (saline). (B) Total leukocyte recruitment in air pouch exudate in response to ConBr, PAA (50 μg) or KM+ (5 or 50 μg) lectins at 24 h. Asterisk indicates significant difference (* $p = 0.0357$) from value for KM+ (50 μg) recruitment compared to ConBr and PAA (50 μg). (C) Distribution of cell populations recruited in air pouch exudates based on differential counts at 24 h. Asterisk indicates significant difference (* $p = 0.0357$) from value of eosinophil, lymphocyte and macrophage recruitment in KM+ stimulated group compared to ConBr and PAA. Values are the mean ± S.E.M. of results from three different mice.

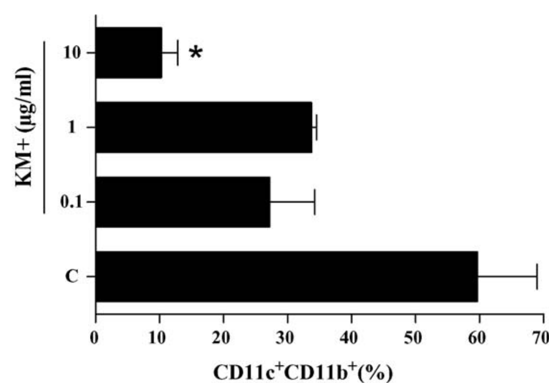


Fig. 5. KM+ inhibits differentiation of BM-derived DC. BM-derived cells from C57Bl/6 mice were cultured in 24-well plates (2.5×10^6 cells per well) with GM-CSF (25 ng/mL) and IL-4 (10 ng/mL) in the presence or absence (C, control) of variable concentrations of KM+ as indicated. At day 9 of culture, cells were harvested, labeled with the designated mAbs and analyzed by two-color flow cytometry. Results are shown as mean of percentage of cells CD11c+CD11b+ ± S.E.M. from duplicate wells. Data shown is representative of two experiments. Asterisk indicates significant difference (* $p = 0.0307$) from value for KM+ at 10 μg/ml compared to the control (C).

3.4. *Effect of KM+ lectin on BM-derived dendritic cells*

Since only KM+ combined with SLA was able to induce a significant reduction on parasite load we decided to further explore the possible effect on dendritic cells (DC) differentiation and maturation. Dendritic cells rather than macrophages and monocytes, are more likely to be responsible for the initiation of T cell-mediated immunity.

When BM cells were cultivated with different doses of KM+ lectin there was an inhibition of CD11c expression (Fig. 5), indicating that KM+ lectin inhibited dendritic cell differentiation from bone marrow (BM) cells. BM cells differentiated without KM+ had 60% of CD11c+CD11b+ cells while cells cultivated with KM+ lectin in different concentrations had a decreased number of differentiated cells and only 10% of CD11c+CD11b+ cells when 10 μg/ml of KM+ was used ($p = 0.0307$).

We also evaluated a possible effect of KM+ lectin on BMDC maturation. When KM+ was cultivated in different doses with differentiated BMDCs there was a dose-dependent and significant enhancement of MHC class II (1143 MFI with 0.1 μg/ml of KM+ as compared to 243 MFI of unstimulated cells, $p = 0.0102$), CD80 (186 MFI with 10 μg/ml of KM+ as compared to 51.1 MFI of unstimulated cells, $p = 0.0342$) and CD86 (564 MFI with 0.1 μg/ml of KM+ as compared to 46.33 MFI of unstimulated cells, $p = 0.0184$), but not of CD40 molecules expression in CD11b+CD11c+ cells similar to the LPS treated group indicating the phenotype of mature dendritic cells (Fig. 6).

3.5. *Induction of IL-12p70 production in vitro after KM+ stimulation*

IL-12 secreted by dendritic cells is responsible for the differentiation of helper T lymphocytes to a type 1 response.

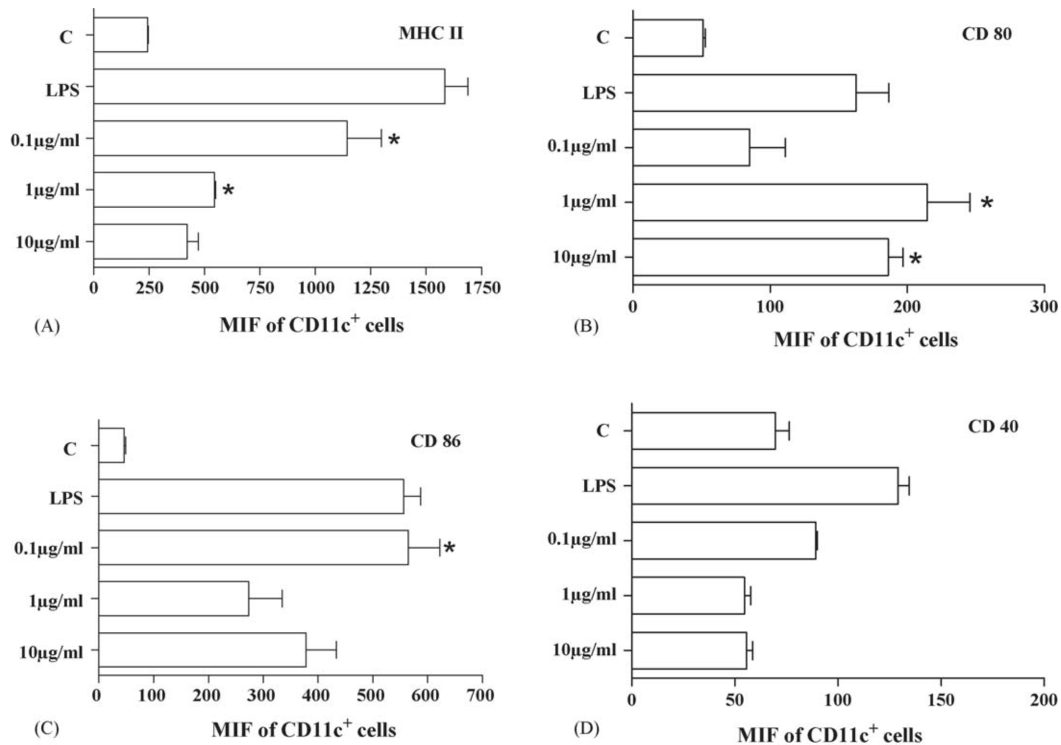


Fig. 6. KM+ induces DC maturation. BM-derived cells (2.5×10^6/well) from C57Bl/6 mice were differentiated for 9 days in the presence of GM-CSF (25 ng/mL) and IL-4 (10 ng/mL). At day 9, when cells were CD11c+, DC maturation was induced with the addition of LPS (1.0 μg/mL), KM+ (0.1, 1.0 or 10 μg/mL). Cells cultured with supplemented medium plus the recombinant cytokines were considered immature DCs (C, controls). Data in bar graphs are presented as the mean ± S.E.M. of mean fluorescence intensity (MFI) of CD11c+ cells expressing MHC class II (A), CD80 (B), CD86 (C) and CD40 (D) as indicated. Data is representative of three independent experiments performed in duplicate. Asterisk indicates significant difference ($*p = 0.0102$) from value for MHC II, CD80 ($*p = 0.0342$) and CD86 ($*p = 0.0184$) of KM+ stimulated groups compared to control (C).

Because of this decisive role, we investigated if KM+ was able to induce IL-12p70 production by BMDC. KM+ (10 μg/ml) induced significant IL-12p70 production by mature BMDC when compared to the non-stimulated group ($p = 0.0486$) (Fig. 7).

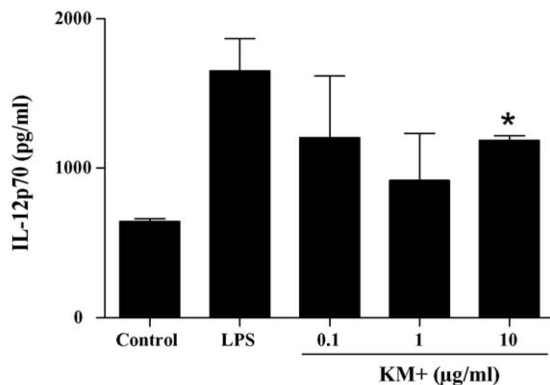


Fig. 7. IL-12p70 production by BMDC stimulated with KM+. Mice BMDC were stimulated in vitro with KM+ lectin (0.1, 1 and 10 μg/ml), LPS (1.0 μg/mL) or control (non-stimulated). After 48 h supernatants were collected and IL-12p70 was detected by ELISA. The results are representative of at least three experiments. Asterisk indicates significant difference ($*p = 0.0486$) from value for KM+ (10 μg/ml) stimulated group compared to control.

4. Discussion

The search for immunostimulatory molecules and adjuvants in order to enhance or to direct an appropriate immune response against target immunogens has been a major and recurrent issue for vaccine development. In leishmaniasis, protection requires proper cellular immune response with the presence of specific CD4+ T cells and IFN-γ production and initial IL-12 production are elements required for protection. In fact, IL-12 used as an adjuvant to SLA conferred protection to mice against *L. major* infection [22].

Here we evaluate the immunostimulatory potential of three plant lectins in vitro and in vivo in a model of *Leishmania amazonensis* infection. All three lectins but KM+ were able to stimulate similar IFN-γ production by splenic cells. ConBr and PAA were induced IFN-γ production in the same level but KM+ achieved this effect at doses 50-times lower then ConBr and PAA. ConBr and PAA lectins did not induce IFN-γ production when tested at optimal KM+ concentrations (1 μg/ml; data not shown). Such high stimulatory capacity of KM+ may be related to the ability in driving DC maturation and inducing IL-12 production by these cells. Although immunization with ConBr combined with SLA reduced the number of parasites, lesion size was increased. Interestingly, KM+ alone was capable of reducing

lesion size and parasite load. Of note, despite reducing parasite load KM+ plus SLA did not lead to reduction in footpad thickness, usually taken as lesion size. These results reinforce that footpad thickness sometimes does not reflect the parasite numbers, and may be influenced by leukocyte infiltration in the infection site [23].

Since some lectins are able to induce macrophage stimulation and leukocyte accumulation [24,25], we investigated the inflammatory potential of the lectins using the air pouch model. All three lectins tested were able to induce leukocyte recruitment reaching a peak at 24 h. Interestingly, fewer leukocytes were recruited after KM+ stimulation compared to ConBr and PAA even if a higher concentration of KM+ was used.

ConBr or PAA lead to recruitment of polymorphonuclear and mononuclear cells to the pouch space whereas KM+ recruited polymorphonuclear cells with modest numbers of either lymphocytes or monocytes. The role of KM+ as a neutrophil migration inducer in vivo and in vitro has already been described in rats [14], mimicking IL-8 effect on neutrophils [25].

Since macrophages are the main host cell for Leishmania parasites it is interesting that ConBr and PAA are more potent macrophage recruiters then KM+ lectin. KM+ induced a reduced inflammatory response and consequently fewer macrophages were recruited. Macrophage recruitment may be related to higher susceptibility to Leishmania as recently recruited macrophages may play as "safe havens" for this parasite [26]. Therefore, a decreased number of host cells present in the initial hours after infection would be more challenging for a successful parasite establishment.

The capacity of macrophages to respond to Th1 activation signals against intracellular pathogens during the nonimmune early phases of infection is crucial for determining whether the invading organisms proliferate or are eliminated. These results suggest that not only the right production of Th1 cytokines but also the appropriate inflammatory response is important to drive immunity to a protective response. The fact that PAA and ConBr lectins were not able to induce protection can be partially explained by their inumerous effects that may exert a controversial response in the immune system. ConBr is able to induce IFN-γ production but also induces apoptosis and inflammation frequently associated with high endothelial venule necrosis [8]. It is also noteworthy that PAA lectin, described before as a potent stimulator of NO production [9] was not able to protect the immunized mice. However, NO may also play a role inhibiting proliferative responses that can result in immunosuppressive effects [27–29].

The production of Th1 CD4+T cells requires the action of antigen presenting cells (APC) that transport and process antigens to stimulate specific subsets of T cells. Dendritic cells (DC) are key cells in primary immune responses and thus fundamental in immune approaches aimed at vaccination. Since KM+ has been described to induce IL-12 production by macrophages [11] and showed here some degree of protection on immunized mice we investigated the possibility of KM+ to stimulate the maturation and differentiation of bone-marrow derived DC. As shown here KM+ lectin was able to induce maturation of BM-derived DC in a dose dependent manner characterized by a higher expression of MHC class II, CD80 and CD86 molecules in CD11c+ cells, a profile of mature DC more capable of priming T cells.

Thus, it is possible that a combined action of KM+ inducing maturation of DC present at sites of antigen delivery, coupled to stimulation of IL-12 production results in the higher capacity of this lectin in driving a protective response against Leishmania. Taken together these data reinforce the potential of lectins as immunostimulatory molecules but also lightened possible mechanisms evolved in a protective response against an intracellular parasite.

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