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Lipids from attenuated and virulent *Babesia bovis* strains induce differential TLR2-mediated macrophage activation

G. Gimenez[a,*], K.G. Magalhães[b], M.L. Belaunzarán[a], C.V. Poncini[a], E.M. Lammel[a], S.M. Gonzalez Cappa[a], P.T. Bozza[b], E.L.D. Isola[a]

[a] Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina
[b] Laboratório de Imunofarmacologia, Instituto Oswaldo Cruz – Fundaçao Oswaldo Cruz, Rio de Janeiro, Brazil

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ABSTRACT

Babesia bovis is an intraerythrocytic apicomplexan protozoa of cattle that causes an acute infection with parasite persistence. Babesiosis limitation depends on macrophages, essential effector cells of the host innate defense, which generate inflammatory cytokines and nitric oxide. Herein, we report quantitative differences in the lipid composition of merozoites from two *B. bovis* strains with polar behaviour: attenuated R1A and virulent S2P. Accordingly, we observed a distinct inflammatory response induced by the total lipids of R1A (L_A) and S2P (L_V) in murine peritoneal macrophages. L_A and particularly its fractions phosphatidic acid and phosphatidylserine + phosphatidylinositol (PS + PI), produced a strong activation of these cells with lipid body formation, cyclooxygenase-2 expression and pro-inflammatory TNFα, IL-6 and KC secretion. Although L_V did not activate these cells, the corresponding PS + PI fraction induced TNFα, IL-6 and KC release. Therefore, these facts might be suggesting the presence of an inhibitor in L_V. Furthermore, the employment of wild type and toll like receptor 2 knockout (TLR2KO) mice allowed us to demonstrate that macrophage activation by the stimulating lipid fractions was mediated through TLR2. Interestingly, only L_A activated the extracellular signal-regulated kinases 1 and 2 (ERK1/2). Inhibitory studies employing UO126, indicated that the ERK pathway was required for TNFα, IL-6 and KC release. In conclusion, the absence of inflammatory response observed with the lipids of S2P virulent strain could constitute an evasion mechanism of the innate immune response enabling parasite establishment in the host.

1. Introduction

Bovine babesiosis is an economically important tick-borne disease of cattle caused by the hemoparasite *Babesia bovis*. This intraerythrocytic apicomplexan parasite is prevalent in tropical and subtropical regions worldwide and is poorly controlled by existing chemotherapy and immunoprophylaxis. *B. bovis*-infected erythrocytes undergo sequestration by attachment to capillary endothelium, resulting in organ damage, cerebral dysfunction and pulmonary edema, similar to the most severe form of human malaria, caused by the apicomplexa *Plasmodium falciparum* (Wright et al., 1988).

Innate immune mechanisms are hypothesized to be important for the resolution of acute infection produced by *B. bovis*, whereas acquired immunity is likely more important for resistance to parasite challenge (Fell and Smith, 1998; Brown and Palmer, 1999). In this sense, it has been reported that limitation of acute infection with this protozoan is dependent on innate immune products derived from activated macrophages such as nitric oxide and inflammatory cytokines (Shoda et al., 2000).

There is a growing body of evidence pointing to an important modulatory role of microorganisms' lipids in the immune system (Schofield and Hackett, 1993; Roach and Schorey, 2002; Quesniaux et al., 2004). Different members of the TLR family, including TLR2, TLR4, TLR6 and TLR1, which dimerize with TLR2, have been implicated in the recognition of molecules with a lipid component from a broad spectrum of organisms ranging from viruses up to parasites (Brightbill et al., 1999; Means et al., 1999; Underhill et al., 1999; Akira, 2003). As regards *B. bovis* lipids, it has been reported that this protozoa possesses a high rate of phosphatidylcholine (PC)

Abbreviations: WT, wild type; TLR2KO, toll like receptor 2 knockout; L_A, total lipids of *B. bovis* attenuated strain; L_V, total lipids of *B. bovis* virulent strain; SM, sphingomyelin; LPC, lysophosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PA, phosphatidic acid; CHO, cholesterol; DG, diacylglycerol; FFA, free fatty acids; COX-2, cyclooxygenase; PGE_2, prostaglandin E_2; ERK1/2, extracellular signal-regulated kinases 1/2.

* Corresponding author at: Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155 piso 13, 1121 Ciudad Autónoma de Buenos Aires, Argentina.
Tel.: +54 11 5950 9500x2191; fax: +54 11 5950 9577.
E-mail address: paradife@fmed.uba.ar (G. Gimenez).

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and phosphatidic acid (PA) biosynthesis (Florin-Christensen et al., 2000).

TLRs modulate the expression of hundreds of host genes through a complex network of signalling events that allow for the appropriate response to a microbial pathogen (Lasker and Nair, 2006). The mitogen activated-protein kinase (MAPK) pathways are crucially involved in macrophage stimulation by TLRs, leading to the activation of transcription factors related to inflammatory cytokines, among others. Accordingly, it has been described that the extracellular signal-regulated kinases 1/2 (ERK1/2) are involved in the expression of the pro-inflammatory cytokine TNFα in human macrophages infected with *Mycobacteria* spp. (Reiling et al., 2001; Surewicz et al., 2004; Yadav et al., 2004).

Lipid bodies, hydrophobic and osmiophilic organelles that store intracellular lipids in different cell types are involved in signalling processes and inflammatory mediators' production (Murphy, 2001; Van Meer, 2001; Tauchi-Sato et al., 2002; Martin and Parton, 2006; Bozza et al., 2009). Leukocytes have virtually no lipid bodies under resting conditions, however, the increase of lipid body numbers produced during infections correlates with the generation of eicosanoids and lipid body localization of eicosanoid-forming enzymes like cyclooxygenase-2 (COX-2) (Dvorak et al., 1993; Bozza et al., 1997; Melo et al., 2003; Pacheco et al., 2002; D'Avila et al., 2006). The latter is a regulatory enzyme in the production of prostaglandins and thromboxane A_2, modulators of inflammation (Pawlowski et al., 1983; Tsatsanis et al., 2006).

Regarding lipids as mediators of the immunity against *B. bovis*, it has been previously reported that naive cattle vaccinated with a chloroform extract from *B. bovis*-infected erythrocytes developed delayed and decreased parasitaemias when challenged with a virulent strain of *B. bovis* (Goodger et al., 1990). Furthermore, lipids from erythrocytes infected with *B. bovis* Mexican strain stimulated inducible nitric oxide synthase and nitric oxide production by bovine macrophages in the presence of gamma interferon (INFγ) (Shoda et al., 2000).

The aim of this study was to investigate the effect of the lipids purified from merozoites of two *B. bovis* strains with polar behaviour, attenuated and virulent, on the induction of an inflammatory response and the involvement of TLR2 in this process. With this purpose, we analyzed cytokine/chemokine and lipid body induction, COX-2 expression and prostaglandin E_2 (PGE_2) release in wild type (WT) and toll like receptor 2 knockout (TLR2KO) mice as well as the participation of ERK signalling pathway in cytokine/chemokine release.

2. Materials and methods

2.1. Reagents and antibodies

Silica gel 60 thin layer chromatography plates, organic solvents and osmium tetroxide were purchased from Merck (Darmstadt, Germany). Lipid standards, peroxidase labelled (HRP) rabbit polyclonal anti-goat IgG, LPS from *Escherichia coli* (serotype O127:b8) and U0126 (1,4-diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto) butadiene) were obtained from Sigma–Aldrich Co. (St Louis, MO, USA). IL-4, IL-10, TNFα, KC and PGE_2 enzyme immunoassay kits were from Cayman Chemical (Ann Arbor, MI, USA). IL-6 enzyme-linked immunoassay kit was obtained from R&D Systems (Minneapolis, MN, USA). Goat polyclonal anti-mouse COX-2, rabbit polyclonal anti-mouse β-actin and peroxidase labelled (HRP) goat anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit anti-phospho ERK and anti-total ERK antibodies were obtained from Cell Signalling Technology, Inc. (Danvers, MA, USA). Nitrocellulose membrane and auto radiographic films were from GE Healthcare. Supersignal® West Pico Chemiluminescent substrate was purchased from Pierce (Rockford, IL, USA).

2.2. Parasites

Merozoites from two strains of *B. bovis* with polar behaviour were employed: R1A, attenuated and S2P, pathogenic. R1A strain was isolated from a bovine during a babesiosis outbreak in Santa Fe, Argentina, and attenuated by several passages through splenectomised cattle (Anziani et al., 1993). S2P strain was isolated from a bovine during a babesiosis outbreak in Salta, Argentina (Mangold et al., 1993). Parasites from both strains were cultured in bovine erythrocytes under microaerophilic conditions (Levy and Ristic, 1980). In order to isolate R1A and S2P merozoites, cultures were left overnight at 4 °C to favour parasite release from bovine erythrocytes to the extracellular media. Cultures were then centrifuged at 400 × *g* for 10 min at 4 °C and parasites contained in the resulting supernatants were further separated by centrifugation at 12,800 × *g* for 20 min at 4 °C. Pellets were stored at −20 °C until used for lipid extraction.

2.3. Animals

C57BL/6 mice were obtained from the Fundação Oswaldo Cruz (Rio de Janeiro, Brazil) breeding unit. TLR2 knockout (TLR2KO) mice in a homogeneous C57BL/6 background (Takeuchi et al., 1999) were kindly donated by Dr. Shizuo Akira (Osaka University, Japan). Animals were bred and maintained under standard conditions with free access to food and water in a room at 22–24 °C and a 12-h light/dark cycle until used. All protocols were approved by the Fundaçao Oswaldo Cruz welfare committee.

2.4. Preparation of total lipid extracts from R1A and S2P B. bovis merozoites

The method of Bligh and Dyer (1959) was used for lipid extraction in all cases. The solvents were evaporated under nitrogen to a constant weight and lipids from *B. bovis* merozoites were then resuspended in chloroform for lipid analysis or ethanol, for macrophage stimulation assays. Lipid extracts were stored at −20 °C until used.

2.5. Analysis of total lipid extracts from R1A and S2P B. bovis merozoites

Equal amounts of lipids from R1A and S2P *B. bovis* merozoites (L_A and L_V respectively) were separated by thin layer chromatography (TLC) on silica gel plates. A double solvent system was employed: chloroform/methanol/acetic acid/water (65:35:6:2, v/v) up to the middle of the plate to separate phospholipids and hexane/diethylether/acetic acid (90:10:1, v/v) up to the top to separate neutral lipids (Florin-Christensen et al., 2000). For mass determination, plates were sprayed with 10% $CuSO_4$ in 8% H_3PO_4 and charred by exposure to 150 °C for 13 min (Baron et al., 1984). Lipids were identified by comparison with authentic standards. Densitometric analysis was performed with Gel-Pro® Analyzer 4.0 software (Media Cybernetics, Inc., Silver Spring, MD, USA).

2.6. Preparation of lipid fractions from L_A and L_V

Chloroform lipid extracts from both strains were separated by TLC as described above. Lipids were identified by comparison with authentic standards and the following fractions: SM + LPC, PS + PI, PA and FFA were eluted from the TLC plates with two 2 ml portions of chloroform/methanol (1:1, v/v). The solvents were evaporated

under nitrogen to a constant weight and lipids were then resuspended in ethanol for macrophage stimulation assays.

2.7. Stimulation of WT and TLR2KO murine peritoneal macrophages with L_A and L_V

Peritoneal cells from WT and TLR2KO mice were harvested by lavage with sterile RPMI 1640 medium. Macrophages were adhered in cover slides within 24-well plates (1×10^6 cells/well) and 6-well plates (4×10^6 cells/well) overnight with RPMI 1640 + 2% foetal calf serum. The non-adherent cells were then removed after vigorous PBS wash (twice) and macrophages were stimulated with ethanol suspensions of L_A or L_V (50 μg/ml) for 24 h at 37 °C, in 5% CO_2. For controls, cells were incubated with culture medium or vehicle (ethanol) or with LPS (1.5 ng/ml) as positive control for the TLR knockout assays, since this compound is a TLR4 ligand. Supernatants from the 24-well plates were then collected and stored at −20 °C until used for cytokine/chemokine and PGE_2 measurements. Macrophages adhered to the slides in these plates were used for the lipid body staining assays. Cells adhered to the 6-well plates were resuspended in Laemmli's sample buffer, boiled and stored at −20 °C until used for COX-2 immunoblot analysis.

2.8. Stimulation of WT and TLR2KO murine peritoneal macrophages with lipid fractions from L_A and L_V

Peritoneal WT and TLR2KO macrophages were obtained and left to adhere in 24- and 6-well plates, as described above. Macrophages were then stimulated 24 h with ethanol suspensions of: SM + LPC (7.5 μg/ml), PS + PI (3.25 μg/ml), PA (1.35 μg/ml) and FFA (2 μg/ml) from R1A attenuated strain or SM + LPC (6.5 μg/ml), PS + PI (1.65 μg/ml), PA (0.65 μg/ml) and FFA (2 μg/ml) from S2P virulent strain. The concentrations of every fraction here employed were selected taking in consideration the percentage of each fraction in their corresponding total extract. Supernatants from the 24-well plates were collected and stored at −20 °C until used for TNFα, IL-6 and KC measurements. Cells adhered to the 6-well plates were resuspended in Laemmli's sample buffer, boiled and stored at −20 °C until used for COX-2 immunoblot analysis.

2.9. Lipid body staining and quantification

Slides with WT and TLR2KO macrophages stimulated with L_A or L_V, while still moist, were fixed in 3.7% formaldehyde in Ca^{2+}/Mg^{2+} free Hanks' balanced salt solution (pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4) stained in 1.5% OsO_4 (30 min), rinsed in H_2O, immersed in 1.0% thiocarbohydraxide (5 min), rinsed in 0.1 M cacodylate buffer, restained in 1.5% OsO_4 (3 min), rinsed in H_2O, dried and mounted (D'Avila et al., 2006). Fixed cells morphology was observed and lipid bodies were enumerated in 50 consecutively scanned macrophages, using phase contrast microscopy with 100× objective lens (BX51 Olympus, Optical Co., Japan). Images were obtained using CoolSNAP-Pro CF digital camera with Image-Pro Plus version 4.5.1.3 software (Media Cybernetics).

2.10. COX-2 immunoblotting

Protein samples from peritoneal macrophages stimulated with L_A, L_V or the lipid fractions from the attenuated strain were separated in a 10% SDS-PAGE and transferred to nitrocellulose membranes using a Tran-blot semi dry transfer cell (Biorad, CA, USA). Membranes were blocked with Tris buffered saline + 0.05% Tween 20 (TBST) + 5% skimmed milk (blocking solution) for 2 h at room temperature. After three washes with TBST, the membranes were incubated with goat IgG anti-COX-2 1:100 (v/v) in TBST overnight at 4 °C. Membranes were then washed thrice with TBST, incubated with rabbit polyclonal anti-goat IgG-HRP 1:5000 (v/v) 1 h at room temperature and washed thrice before detection. HRP-labelled conjugate was detected using Supersignal® West Pico chemiluminescent substrate, membranes were exposed to auto radiographic films and scanned using an hp scanjet 2400 apparatus.

For loading control, detection of β-actin was performed. The same membranes were incubated with blocking solution 1 h at room temperature and then incubated with rabbit polyclonal anti-mouse β-actin 1:5000 (v/v) overnight at 4 °C. After washing the membranes thrice, they were incubated with goat polyclonal anti-rabbit IgG-HRP 1:5000 (v/v) 1 h at room temperature and washed thrice; HRP-labelled conjugate was detected as described above. The intensity of the signal of the COX-2 band was quantified by densitometry using Gel-Pro® Analyzer 4.0 software and normalized to the intensity of the corresponding β-actin band.

2.11. PGE_2 measurement

PGE_2 levels were determined by competitive enzyme immunoassay directly in the 24-h supernatants from WT and TLR2KO macrophages stimulated with L_A or L_V, according to the manufacturer's instructions (Cayman Chemical). The time condition chosen for PGE_2 measurement corresponds to that previously reported for lipid body formation and COX-2 induction (D'Avila et al., 2006).

2.12. Cytokine and chemokine analysis

TNFα, IL-4, IL-6, IL-10 and KC levels were measured by ELISA in the supernatants from WT and TLR2KO macrophages stimulated with L_A, L_V or the lipid fractions from both strains, according to the manufacturer's instructions (Cayman Chemical and R&D Systems).

2.13. Phospho ERK immunoblotting

WT macrophages were obtained and left to adhere in 6-well culture plates (4×10^6 cells/well) as described above. Macrophages were stimulated with L_A or L_V (50 μg/ml) for 5, 15, 30 and 60 min at 37 °C, in 5% CO_2. Control cells were incubated with culture medium or vehicle (ethanol) at the same time points. Proteins from cells adhered to the 6-well plates were resuspended in Laemmli's sample buffer, boiled and stored at −20 °C until used for phospho ERK immunoblot analysis. Samples were separated in a 10% SDS-PAGE, transferred to nitrocellulose membranes and blocked as described above. After three washes with TBST, the membranes were incubated with rabbit anti-phospho ERK antibody 1:1000 (v/v) in TBST overnight at 4 °C. Membranes were then washed thrice with TBST, incubated with goat polyclonal anti-rabbit IgG-HRP 1:2000 (v/v) 1 h at room temperature and washed thrice before detection. Images were revealed by chemiluminescence as previously described.

For loading control, detection of total ERK was performed. The same membranes were blocked and incubated with rabbit anti-total ERK antibody 1:1000 (v/v) overnight at 4 °C. After three washes membranes were incubated with goat polyclonal anti-rabbit-HRP 1:2000 (v/v) 1 h at room temperature and washed thrice; HRP-labelled conjugate was detected as described above. The intensity of the signal of phospho ERK1/2 bands were quantified by densitometry using Gel-Pro® Analyzer 4.0 software and normalized to the intensity of the corresponding ERK band.

2.14. Effect of the ERK inhibitor UO126 on cytokine/chemokine release

WT macrophages were obtained and left to adhere in 24-well plates (1×10^6 cells/well) as previously described. Macrophages were incubated with 10 μM UO126 (ERK specific inhibitor) or 0.1%

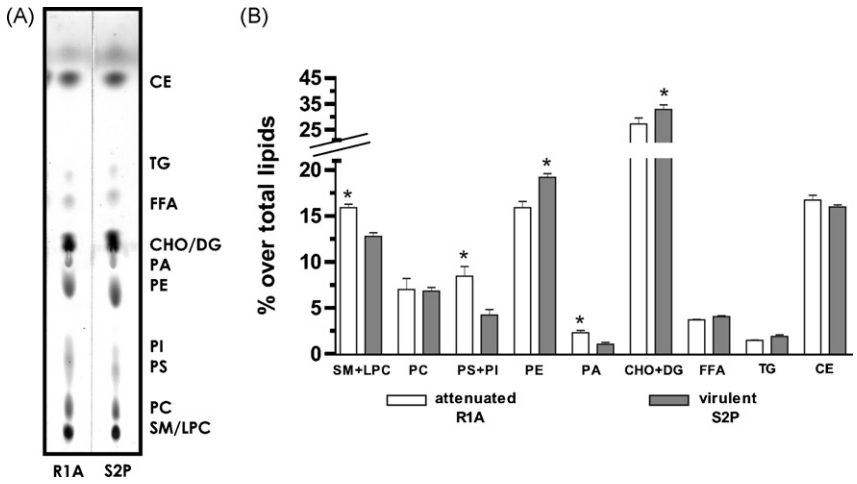

Fig. 1. Analysis of the lipid composition of R1A and S2P *B. bovis* merozoites. (A) Lipids from both *B. bovis* strains were extracted and separated by TLC using a double development system of solvents. For mass determination, TLC plates were sprayed with 10% $CuSO_4$ in 8% H_3PO_4, exposed to 150 °C for 13 min. Lipids were identified by comparison with authentic standards. The TLC plate is representative of three independent experiments. (B) Densitometric analysis of TLC plates was performed using Gel-Pro® Analyzer 4.0 software. The percentage of each lipid fraction was determined with respect to the total lipids of each strain. SM: sphingomyelin; LPC: lysophosphatidylcholine; PC: phosphatidylcholine; PS: phosphatidylserine; PI: phosphatidylinositol; PE: phosphatidylethanolamine; PA: phosphatidic acid; CHO: cholesterol; DG: diacylglycerol; FFA: free fatty acids; TG: triacylglycerol; CE: cholesteryl esters. *Statistically significant ($p < 0.05$).

DMSO (vehicle) 30 min at 37 °C, in 5% CO_2. After this treatment, cells were stimulated with L_A or L_V (50 μg/ml) for 24 h at 37 °C, in 5% CO_2. Supernatants were then collected for TNFα, IL-6 and KC measurements by ELISA and stored at −20 °C until analysis.

2.15. Statistical analysis

Results were expressed as mean ± SEM. Unpaired Student's *t*-test was used to compare between groups, using GraphPad Prism 4 software for Windows. An analysis of variance (ANOVA) was used for multi-group analysis. Values of $p < 0.05$ were considered significant.

3. Results

3.1. B. bovis merozoites from R1A and S2P strains presented quantitative differences in their lipid composition

Thin layer chromatographic analysis revealed striking differences in the lipid composition of the two *B. bovis* strains with polar behaviour, attenuated R1A and virulent S2P (Fig. 1A). We found that R1A presented significantly higher amounts of the following fractions: sphingomyelin + lysophosphatidylcholine (SM + LPC), phosphatidylserine + phosphatidylinositol (PS + PI, approximately 2-fold higher) and phosphatidic acid (PA, approximately 2-fold higher) compared to S2P, whereas the latter presented higher levels in the cholesterol + diacylglycerol (CHO + DG) and phosphatidylethanolamine (PE) fractions (Fig. 1B).

3.2. L_A induced higher increase in lipid body numbers than L_V through a TLR2 dependent pathway

We next studied if the lipids of the two *B. bovis* strains could induce differential lipid body formation. We found that both L_A and L_V induced a higher response in WT macrophages compared to control (Fig. 2A). Remarkably, L_A produced a significantly higher lipid body number than L_V (Fig. 2A and B). The induction of lipid body formation by L_A and L_V was inhibited in TLR2KO macrophages with respect to WT cells (Fig. 2B). LPS is a TLR4 ligand, therefore cells stimulated with this compound were used as positive controls to show that TLR2KO macrophages remain functional. The higher lipid body formation by L_A indicated that the lipids of the attenuated *B. bovis* strain generated a stronger inflammatory response. A requisite role for TLR2 in *B. bovis* lipids recognition to induce lipid body formation was also demonstrated.

3.3. L_A induced COX-2 expression in WT macrophages via TLR2

Since lipid bodies contain eicosanoid-forming enzymes, we investigated if the induction of lipid bodies by L_A and L_V would lead to enhanced COX-2 expression. We determined that L_A induced COX-2 expression in WT macrophages (Fig. 3A, lane 5), whereas L_V did not (Fig. 3A, lane 7). The densitometric analysis of immunoblots corroborated these data (data not shown). Considering the results obtained with L_A, we deepened this study by testing SM + LPC, PA and PS + PI from the attenuated strain, that presented quantitative differences with respect to the virulent strain and free fatty acids (FFA), with similar amounts in both cases (Fig. 1). Fig. 3B shows that PA and PS + PI stimulated COX-2 expression in WT macrophages (lanes 5 and 7), while SM + LPC and FFA did not (lanes 1 and 3). These results were verified by densitometric analysis (data not shown), highlighting the significant inflammatory response produced by L_A. Noteworthy, COX-2 expression was not induced in TLR2KO cells stimulated with L_A, L_V or the lipid fractions mentioned above, evidencing the involvement of TLR2 in this process (Fig. 3A and B). LPS stimulated cells were used as a positive control, showing that TLR2KO cells retained the capacity to induce COX-2 expression (Fig. 3A, lanes 3 and 4).

3.4. L_A induced PGE_2 release in WT macrophages

PGE_2, one of the products of COX-2 pathway, was determined in the supernatants of WT and TLR2KO cells stimulated either with L_A or L_V. As shown in Fig. 4, when WT cells were incubated with L_A, higher levels of PGE_2 were observed with respect to control cells, whereas in TLR2KO cells this increase was not detected. In the case of L_V, PGE_2 increase was not detected either in WT or in TLR2KO cells (Fig. 4), in accordance with COX-2 assays (Fig. 3A).

3.5. L_A and L_V differentially induced macrophage cytokine/chemokine release

Cytokines IL-4, IL-6, IL-10 and TNFα and keratinocyte chemokine (KC) were determined in the supernatants of WT and

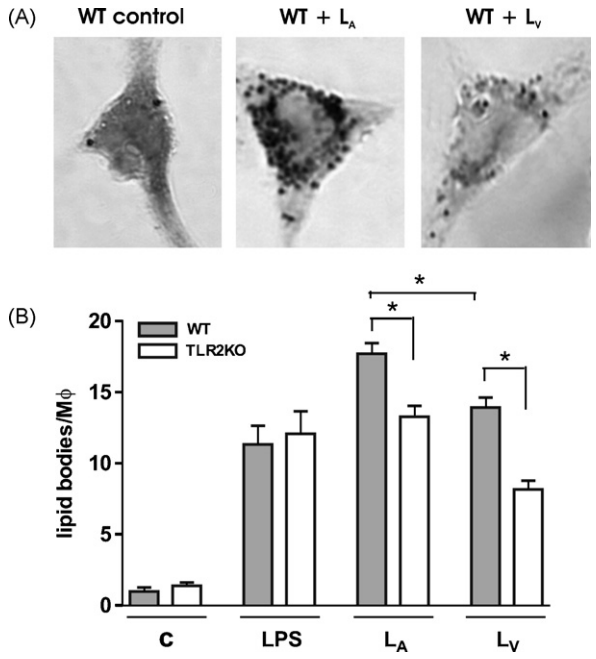

Fig. 2. L_A induced higher increase in lipid body numbers than L_V through a TLR2 dependent pathway. WT and TLR2KO macrophages were stimulated with L_A or L_V (50 μg/ml) 24 h at 37 °C. Cells were incubated with culture medium or vehicle (control) or with LPS (positive control). Lipid bodies were visualized after osmium staining. (A) Lipid bodies in WT macrophages stimulated with L_A or L_V and control cells. (B) Quantification of macrophage lipid bodies in WT and TLR2KO cells. Lipid bodies were enumerated after osmium staining. Each bar represents the mean ± SEM from 50 consecutively counted macrophages of three independent pools (ten animals each). WT: wild type; TLR2KO: toll like receptor 2 knockout; L_A: total lipids of *B. bovis* attenuated strain; L_V: total lipids of *B. bovis* virulent strain; C: control. *Statistically significant ($p < 0.05$).

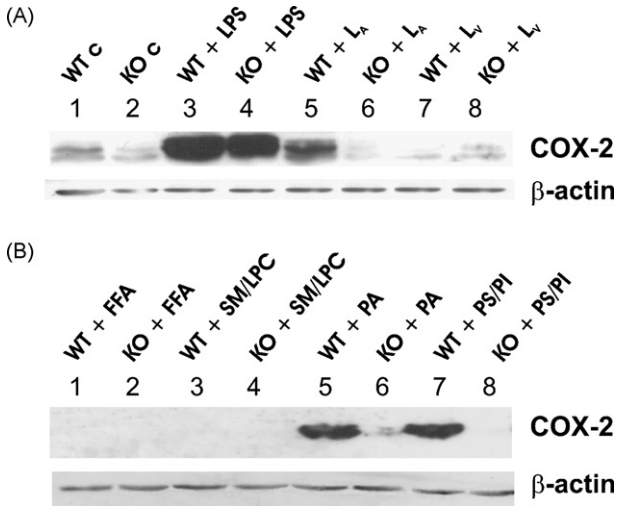

Fig. 3. L_A induced COX-2 expression in WT macrophages via TLR2. Immunoblot analysis of COX-2 induction of WT and TLR2KO macrophages after stimulation with: (A) L_A or L_V (50 μg/ml) or (B) FFA (2 μg/ml), SM + LPC (7.5 μg/ml), PS + PI (3.25 μg/ml) or PA (1.35 μg/ml) from R1A attenuated strain, 24 h at 37 °C. Cells were incubated with culture medium or vehicle (control) or LPS (positive control). Blots were reproved for β-actin as loading control. Blots are representative of three independent assays from three independent pools (ten animals each). WT: wild type; KO: toll like receptor 2 knockout; C: control; L_A: total lipids of *B. bovis* attenuated strain; L_V: total lipids of *B. bovis* virulent strain; FFA: free fatty acids; SM/LPC: sphyngomyelin + lysophosphatidylcholine; PA: phosphatidic acid; PS/PI: phosphatidylserine + phosphatidylinositol.

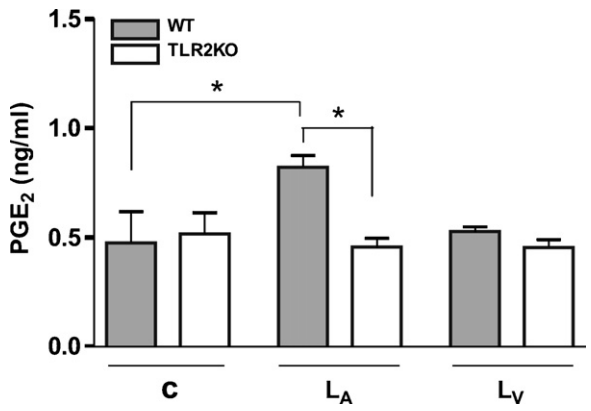

Fig. 4. L_A induced PGE_2 release in WT macrophages. PGE_2 was determined by competitive enzyme immunoassay in the supernatants of WT and TLR2KO macrophages stimulated with L_A or L_V (50 μg/ml), 24 h at 37 °C. Cells were incubated with culture medium or vehicle as control. Results represent the mean ± SEM of triplicate assays from two independent pools (ten animals each). WT: wild type; TLR2KO: toll like receptor 2 knockout; C: control; L_A: total lipids of *B. bovis* attenuated strain; L_V: total lipids of *B. bovis* virulent strain. *Statistically significant ($p < 0.05$).

TLR2KO cells stimulated with L_A, L_V or the lipid fractions mentioned above from both strains. As shown in Fig. 5A, TNFα release was significantly higher in WT cells when stimulated with L_A, whereas L_V-stimulated cells did not secrete significant levels of this cytokine, compared to control. No response was observed with each strain lipids in TLR2KO macrophages (Fig. 5A). Similar results were obtained for IL-6, another pro-inflammatory cytokine and KC, functionally similar to IL-8 and one of the major chemoattractants of neutrophils (Lee et al., 1995; Zlotnik and Yoshie, 2000), in both WT and TLR2KO cells (Fig. 5B and C). Regarding the lipid fractions, only PA from the attenuated strain and PS + PI from both strains generated significantly higher levels of TNFα, IL-6 and KC in WT macrophages, compared to control (Fig. 5D–F). As regards PS + PI, the response generated by this fraction from the attenuated strain was significantly higher than that produced by the virulent strain (Fig. 5D–F). No response was observed in WT macrophages stimulated with SM + LPC or FFA from each strain (data not shown). The present results show a correlation in the cytokine/chemokine response between L_A and its corresponding fractions. Noteworthy, when comparing the effect of L_V vs. PS + PI from the same source, the latter was significantly more stimulating than L_V, suggesting the presence of an inhibitor in this total lipid extract (Fig. 5). The fact that none of the stimulating lipid fractions from both strains were able to induce cytokine/chemokine release in TLR2KO macrophages, confirmed the requirement of TLR2 in this process (Fig. 5D–F). Concerning IL-10 and Th2 cytokine IL-4, L_A and L_V did not stimulate the release of these cytokines neither in WT nor in TLR2KO cells (data not shown).

3.6. L_A induced macrophage TNFα, IL-6 and KC release through an ERK dependent pathway

It has been reported that ERK, member of MAPKs family, is involved in the signalling pathway of TNFα (Surewicz et al., 2004), therefore we evaluated ERK activation in WT macrophages stimulated with L_A or L_V. The immunoblot analysis showed that L_A induced a significant ERK phosphorylation, starting at 5 min with a peak at 30 min, remaining phosphorylated at least until 60 min (Fig. 6A). In contrast, L_V did not produce a significant ERK phosphorylation at the times assayed (Fig. 6A). The control represents the basal phosphorylation pattern induced by the vehicle (Fig. 6A)

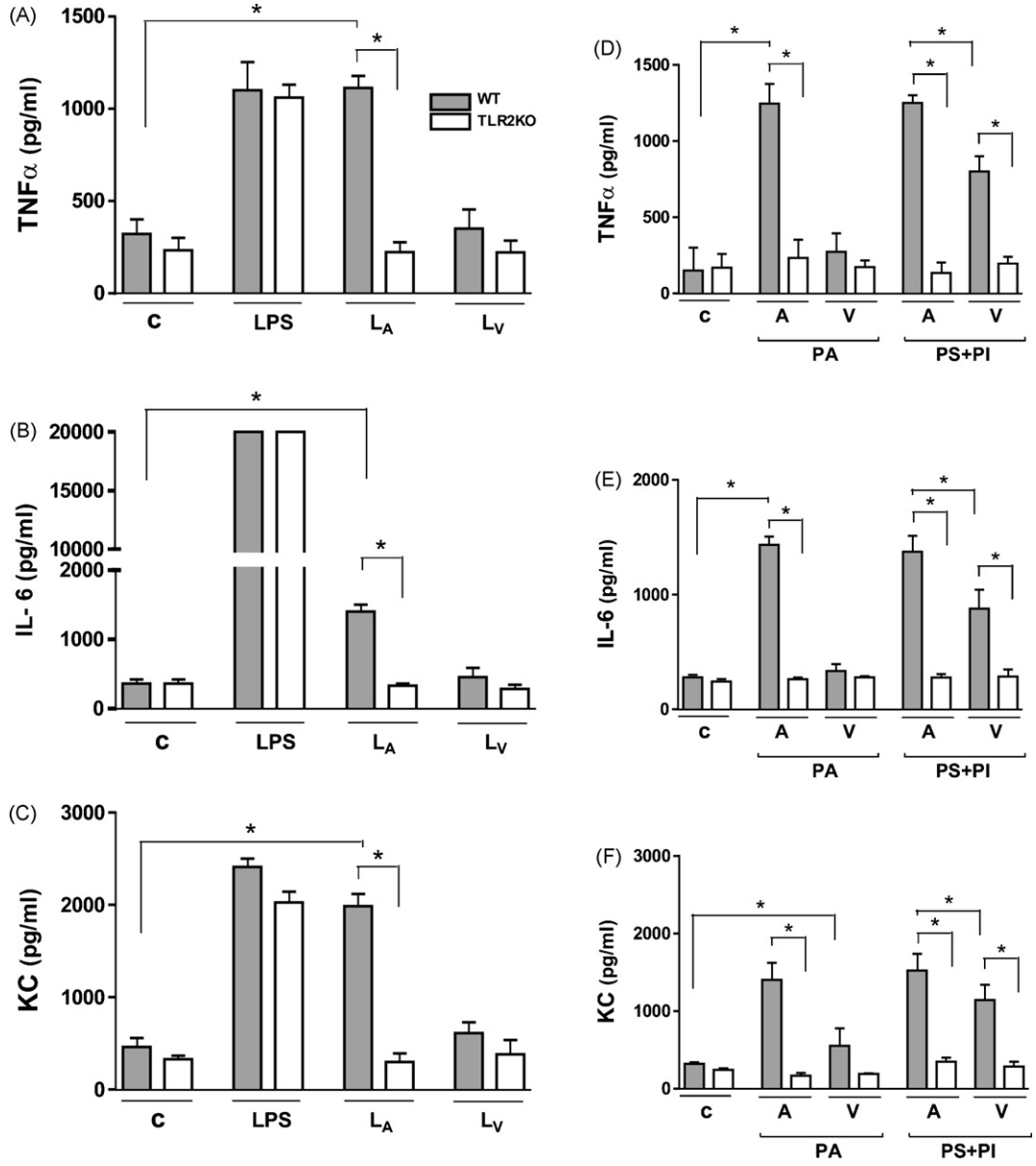

Fig. 5. L_A and L_V differentially induced macrophage cytokine/chemokine release. WT and TLR2KO peritoneal macrophages were stimulated with: L_A or L_V (50 μg/ml); SM + LPC (7.5 μg/ml), PS + PI (3.25 μg/ml), PA (1.35 μg/ml) and FFA (2 μg/ml) from the attenuated strain; or PS + PI (1.65 μg/ml) and PA (0.65 μg/ml) from the virulent strain, 24 h at 37 °C. Cells were incubated with culture medium or vehicle (control) or LPS (positive control). TNFα (A and D), IL-6 (B and E) and KC (C and F) were analyzed by ELISA in the culture supernatants. Results represent the mean ± SEM of triplicate assays from two independent pools (ten animals each). WT: wild type; TLR2KO: toll like receptor 2 knockout; L_A: total lipids of *B. bovis* attenuated strain; L_V: total lipids of *B. bovis* virulent strain; C: control; A: attenuated; V: virulent; PA: phosphatidic acid; PS + PI: phosphatidylserine+phosphatidylinositol. *Statistically significant ($p < 0.05$).

and no changes were observed at the different times assayed (data not shown). The densitometric analysis confirmed these results (Fig. 6B).

We performed another experimental approach evaluating cytokine/chemokine release in the presence of UO126, specific inhibitor of ERK. Fig. 6C shows that L_A-stimulated macrophages treated with UO126 produced significantly lower levels of TNFα compared to non-inhibited cells. In the case of L_V, no differences were observed when WT macrophages were stimulated with these lipids in the presence of UO126, as expected. Similar results were obtained for IL-6 and KC release in the presence of ERK inhibitor (Fig. 6D and E). These findings confirmed the involvement of ERK in the signalling events triggered by L_A that promote pro-inflammatory cytokine/chemokine release.

4. Discussion

Herein, we report for the first time the involvement of lipids from merozoites of two *B. bovis* strains with polar behaviour, attenuated R1A and virulent S2P, in the modulation of the innate immune response, particularly in murine peritoneal macrophages. Besides, another novel contribution of this work is that the recognition of these compounds is mediated by TLR2.

Interestingly, we demonstrate that the lipid composition of R1A and S2P *B. bovis* strains showed quantitative differences that could account for the distinct inflammatory responses generated by each one. Moreover, we observed that lipids purified from *B. bovis* merozoites can function as relevant antigens, being recognized by TLR2. This receptor is activated by a broad spectrum of microbial molecules with a lipid component, such as:

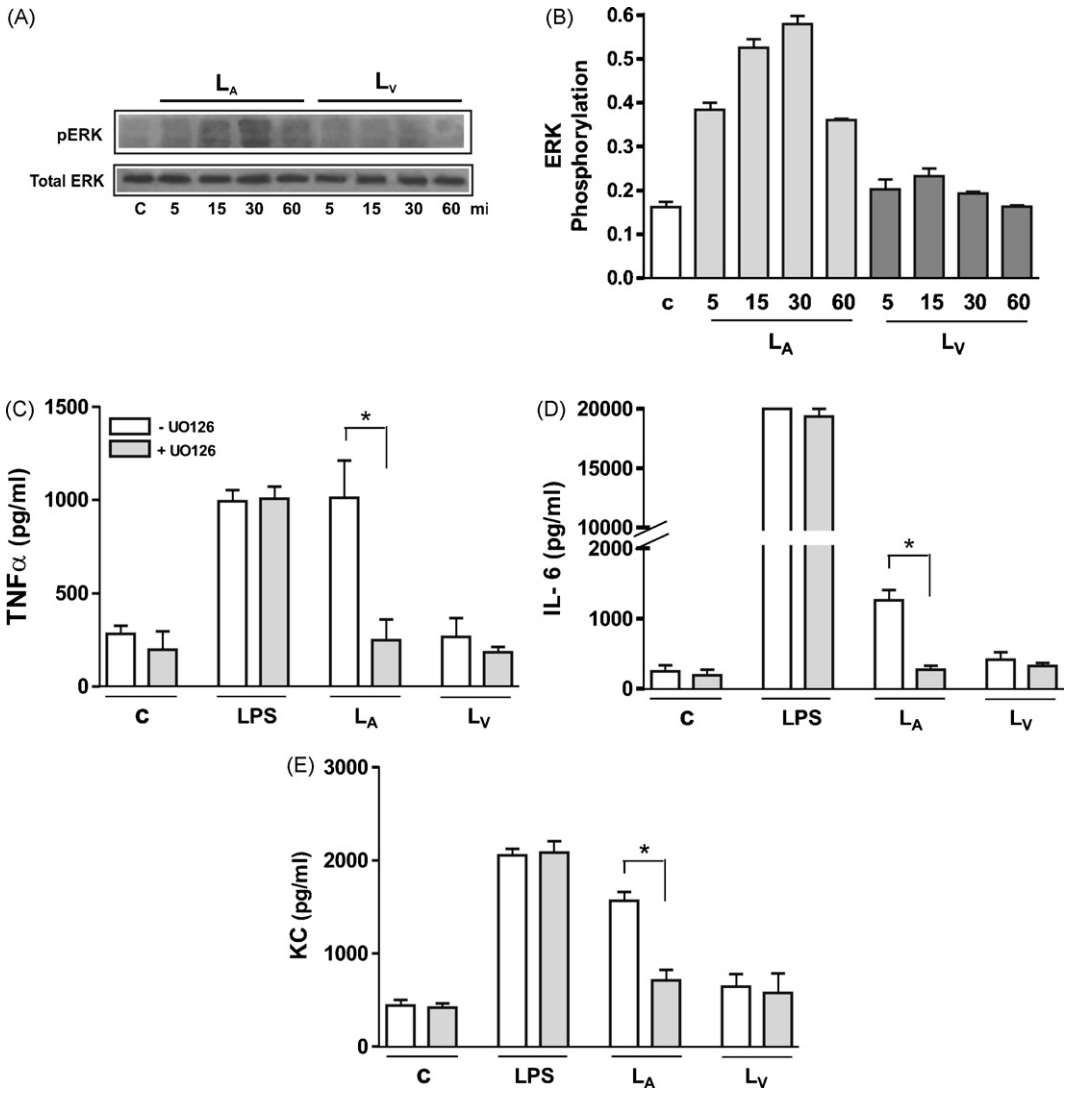

Fig. 6. L_A induced macrophage TNFα, IL-6 and KC release through an ERK dependent pathway. (A) Immunoblot analysis of ERK phosphorylation in WT macrophages treated with L_A or L_V (50 μg/ml) for 5, 15, 30 and 60 min at 37 °C. Blots were reproved for total ERK as loading control. Control cells show the basal phosphorylation profile. The blot is representative of three independent assays from two independent pools (ten animals each). (B) The intensity of the signal of ERK1/2 bands were quantified by densitometry using Gel-Pro® Analyzer 4.0 software and normalized to the intensity of the corresponding ERK band. (C, D and E) Effect of ERK inhibitor (UO126) on cytokines/chemokine release by WT macrophages stimulated with L_A or L_V. Peritoneal macrophages were pre-treated with 10 μM UO126 or 0.1% DMSO (vehicle), 30 min at 37 °C. Macrophages were then stimulated with L_A or L_V (50 μg/ml) for 24 h at 37 °C. TNFα (C), IL-6 (D) and KC (E) were measured in the resulting supernatants by ELISA. For controls, cells were incubated with culture medium or vehicle or LPS. The values are the mean ± SEM of triplicate assays from three independent pools (ten animals each). pERK: phospho ERK; L_A: total lipids of *B. bovis* attenuated strain; L_V: total lipids *B. bovis* virulent strain; C: control. *Statistically significant ($p < 0.05$).

lipoproteins and lipopeptides from cell walls of gram-negative bacteria, mycobacterial lipoarabinomannan and *P. falciparum* glycosylphosphatidylinositol, among others (Brightbill et al., 1999; Means et al., 1999; Nebl et al., 2005). Up to now, there has been no report about *B. bovis* purified lipids acting as TLRs stimulating antigens, as performed in the present work.

TLRs are also involved in the formation of lipid bodies, inflammatory organelles with roles in the innate immune response to infections (D'Avila et al., 2006, 2008). We found a higher induction of lipid bodies in macrophages stimulated with L_A, suggesting that lipids from attenuated R1A *B. bovis* are capable of generating a more important inflammatory effect than S2P strain. Moreover, our results showed that the induction of lipid body formation by L_A or L_V was significantly inhibited in TLR2KO macrophages, indicating the requirement of TLR2 in this process. In line with these findings, BCG or the purified cell component from mycobacteria, lipoarabinomannan, also failed to induce lipid body formation in TLR2KO cells (D'Avila et al., 2006; Almeida et al., 2009).

It is well known that COX-2, a key enzyme regulating the production of inflammatory mediators is localized in lipid bodies (Dvorak et al., 1993; Bozza et al., 1997, 1998). Only in WT macrophages stimulated with L_A, we observed the induction of COX-2 simultaneously with the increase in lipid bodies' number. This finding supports the fact that lipids from the attenuated strain generate a marked inflammatory response. Exclusively the lipid fractions PA and PS + PI from this strain, double in quantity with respect to the virulent strain, resembled the effect of L_A. Nevertheless, SM + LPC, also with a higher content in the attenuated strain and FFA, bioactive molecules with similar amounts in both strains, did not activate the expression of COX-2. Therefore, PA and PS + PI could be the main active components responsible for the inflammatory effect generated by L_A. Moreover, these data demonstrate again the recognition of lipids through a TLR2 pathway. The fact that L_V did not induce COX-2 in the conditions here employed, allows us to hypothesize either the absence of an inflammatory component or the presence of an inhibitor in this extract. In accordance, it has

been demonstrated that the pathogenic *Mycobacterium tuberculosis* possesses an inhibitory molecule, a mannosyl-lipoarabinomannan, that abrogates macrophage activation (Quesniaux et al., 2004).

On the other hand, cyclooxygenase may also produce certain anti-inflammatory prostaglandins including PGE_2 (Osterud and Bjorklid, 2003; Ramsay et al., 2003; Tsatsanis et al., 2006). Although we observed an increase of PGE_2 release in WT macrophages stimulated with L_A, these levels partially correlate with the high induction of lipid bodies and COX-2 detected in these cells. These results are consistent with the evidences presented above, where the response generated by L_A is predominantly pro-inflammatory. In this concern, it has been previously reported in monocytes that the prevailing product of cyclooxygenase metabolic pathway is the pro-inflammatory thromboxane A_2, with lesser amount of PGE_2 (Pawlowski et al., 1983).

Next, we investigated the release of cytokines and chemokines in the same experimental model. The fact that L_A-stimulated WT macrophages displayed a significantly higher TNFα, IL-6 and KC pro-inflammatory response compared to L_V, was totally compatible with the induction of lipid bodies and COX-2. Accordingly, other studies reported that TNFα levels were higher in macrophages infected with the non-pathogenic mycobacteria *Mycobacterium smegmatis* and *Mycobacterium phlei* compared to pathogenic *Mycobacterium avium* (Roach and Schorey, 2002). Concerning the lipid fractions, PA and PS + PI from the attenuated strain appeared again to be responsible for the strong inflammatory response generated by L_A. In agreement with our results regarding COX-2 induction, SM + LPC or FFA from both strains was not able to induce cytokine/chemokine release. Moreover, other studies reported that lipoarabinomannan isolated from non-pathogenic mycobacteria stimulates in macrophages a stronger cytokine response than does a modified molecule from pathogenic mycobacteria, mannose-capped lipoarabinomannan (Roach et al., 1993; Riedel and Kaufmann, 2000). Remarkably, in the case of the virulent strain, the cytokine response generated by PS + PI was stronger than that of the corresponding total extract, suggesting once again the presence of an inhibitor/blocker in L_V. In this regard, it has been described that *P. falciparum* possesses FFA that are responsible for the inhibitory effect on TNFα production induced by malaria glycosylphosphatidylinositols (Debierre-Grockiego et al., 2006). The fact that L_A and L_V did not stimulate the release of the anti-inflammatory IL-10 and Th2 cytokine IL-4 neither in WT nor in TLR2KO cells reinforces the pro-inflammatory profile here reported. It is noteworthy that the presence of TLR2 was necessary for TNFα, IL-6 and KC release by macrophages stimulated with the active lipid fractions.

ERK1/2 are members of the MAPK family. The signalling pathways of MAPKs can activate the expression of cytokine genes and have been implicated as important cellular targets for infectious organisms (Roach and Schorey, 2002). In the present work, ERK1/2 phosphorylation assays demonstrated that L_A triggered a transient signal in WT macrophages showing a significant activation of this pathway in contrast to those stimulated with L_V, which did not. In this respect, decreased MAPK activity has been reported for pathogenic relative to non-pathogenic mycobacteria-infected cells (Roach and Schorey, 2002). The use of the ERK inhibitor, UO126, markedly decreased TNFα, IL-6 and KC release in WT macrophages stimulated with L_A, supporting the involvement of this cascade in these events. In agreement, the pathogenic bacteria, *Yersinia enterocolítica*, suppress TNFα production by inhibiting ERK1/2, p38 and c-Jun kinase activities (Ruckdeschel et al., 1997). Moreover, ERK1/2 signalling is involved in increasing the expression of TNFα in human macrophages (Surewicz et al., 2004).

Collectively, all these results demonstrate that lipids from the attenuated *B. bovis* strain generate a strong inflammatory response, through a TLR2 dependent pathway, contributing to the control of acute infection and host survival. Therefore, these compounds might be included in immunoprophylactic strategies. Furthermore, the finding that lipids from the virulent strain inhibit/block the inflammatory response could constitute an evasion mechanism of the innate immune response, thus allowing the establishment of *B. bovis* in the host.

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