

## The contribution of Toll-like receptor 2 to the innate recognition of a *Leishmania infantum* silent information regulator 2 protein

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### Summary

We have characterized a *Leishmania* protein belonging to the silent information regulator 2 (SIR2) family [SIR2 related protein 1 (SIR2RP1)] that might play an immunoregulatory role during infection through its capacity to trigger B-cell effector functions. We report here that SIR2RP1 leads to the proliferation of activated B cells, causing increased expression of major histocompatibility complex (MHC) II and the costimulatory molecules CD40 and CD86, which are critical ligands for T-cell cross-talk during the development of adaptive immune responses. In contrast, B cells isolated from Toll-like receptor 2 (TLR2) knockout mice were unable to respond to the SIR2RP1 stimulus. Similarly, SIR2RP1 induced the maturation of dendritic cells (DCs) in a TLR2-dependent manner with the secretion of pro-inflammatory cytokines [interleukin (IL)-12 and tumour necrosis factor (TNF)- $\alpha$ ] and enhanced the costimulatory properties of DCs. Nevertheless, immunization assays demonstrated that TLR2-deficient mice were able to mount a specific humoral response to SIR2RP1. Interestingly, further investigations showed that macrophages were activated by SIR2RP1 even in the absence of TLR2. Therefore, a different type of interplay between SIR2RP1 and the major antigen-presenting cells *in vivo* could explain the immune response observed in TLR2-deficient mice. Together, these results demonstrate that TLR2 signalling contributes to SIR2RP1 recognition by innate immune host cells.

**Keywords:** B cells; cytokines; innate immunity; *Leishmania* spp. (leishmaniasis); Toll receptors/Toll-like receptors

### Introduction

Toll-like receptors (TLRs) are key components of effective innate immunity. They perform a vital role in mediating the innate recognition of pathogens and orchestrating the acquired immune response to bacteria, viruses and parasites. The family of TLRs is highly expressed in cells of the innate immune system, such as macrophages, dendritic cells (DCs), B cells and natural killer (NK) cells, and is responsible for recognizing conserved motifs, termed pathogen-associated molecular patterns (PAMPs), that are unique to invasive pathogens and not normally found in the host cells.<sup>1,2</sup>

All the members of the Toll family are transmembrane proteins containing an extracellular domain composed of

leucine-rich repeats and a cytoplasmic domain homologous to the cytoplasmic region of the interleukin (IL)-1 receptor, known as the Toll/Interleukin-1 receptor (TIR) domain, which is required for downstream signalling.<sup>3</sup> The identification of mammalian TLRs represented an important advance in our understanding of innate immunity to pathogenic micro-organisms. To date, 11 human and 13 mouse TLRs have been identified,<sup>4</sup> and each TLR appears to respond to distinct PAMPs, leading to the activation of specific signalling pathways.<sup>5</sup> TLR2 recognizes a myriad of unrelated molecules, including lipopeptides,<sup>6</sup> peptidoglycans,<sup>7</sup> outer membrane proteins,<sup>8</sup> a *Trypanosoma cruzi* protein belonging to the thiol-disulfide oxidoreductase family<sup>9</sup> and porins from a broad spectrum of Gram-negative bacteria.<sup>10–12</sup> This diversity is a result of

Abbreviations: SI, stimulatory index; SIR2RP1, silent information regulator 2 related protein 1; TDR1, thiol-dependent reductase; VL, visceral leishmaniasis.

heterodimerization with TLR1 or TLR6<sup>13</sup> and/or accessory molecules, such as CD14<sup>14</sup> and CD36.<sup>15</sup> The activation of TLR2 by ligands triggers several intracellular signalling responses, including the activation of nuclear factor (NF)- $\kappa$ B and the induction of pro- and anti-inflammatory cytokines.<sup>16</sup> One of the most extensively studied pathways involves signalling through MyD88.<sup>17</sup> In fact, MyD88 is involved in NF- $\kappa$ B activation by every TLR with the exception of TLR3.<sup>18</sup> Engagement of TLRs by PAMPs mediates downstream signalling that leads to up-regulation of both major histocompatibility complex (MHC) and costimulatory molecules, such as CD80 and CD86, which are involved in the optimal activation of naïve T cells, strengthening the adaptive immune response.<sup>19</sup>

Although the majority of experimental studies have indicated an essential role for MyD88 signaling in resistance against several intracellular infections,<sup>20–26</sup> the role of TLR2 is still controversial. A protective role for TLR2 has been described for several infections, such as infections with *Staphylococcus aureus*,<sup>27</sup> *Streptococcus pneumoniae*,<sup>28,29</sup> *Toxoplasma gondii*,<sup>30</sup> *Mycobacterium bovis*,<sup>31</sup> *Mycobacterium avium*<sup>32,33</sup> and *Mycobacterium tuberculosis*.<sup>34,35</sup> However, other studies have suggested that TLR2-dependent mechanisms might also contribute to the evasion or inhibition of an effective immune response in organisms such as *Yersinia enterocolitica*, *Aspergillus fumigatus* and *Candida albicans*.<sup>36–39</sup> Although TLR2 has been proposed to play a role during infection, as it is one of the molecules involved in *Leishmania* phagocytosis,<sup>40</sup> the absence of TLR2 did not modify the course of visceral leishmaniasis (VL) in either the spleen or the liver.<sup>41</sup> Nevertheless, very few studies have so far explored the interaction of *Leishmania* constituents with the family of Toll-like receptors. Lipophosphoglycan (LPG), a major surface promastigote phosphoglycan, can be considered as an exception, as it was demonstrated to behave as a TLR2 agonist activating mouse macrophage and human NK cells.<sup>22,42</sup> This highlights the need for a thorough dissection of the parasite factors involved in TLR activation. In addition, TLR-based therapeutic or prophylactic strategies are currently measures used to fight infectious diseases. Indeed, TLRs have been implicated in the mechanism of adjuvanticity of many immunostimulants used in clinical or experimental vaccination. In this context, several bacterial proteins have recently been reported to mediate adjuvanticity by activating antigen-presenting cells (APCs) via TLR2. This has led to the assumption of multicomponent vaccines containing TLR2 agonists.<sup>43</sup>

Human leishmaniasis and experimental murine leishmaniasis have clearly demonstrated a central role for T lymphocytes in the immunological mechanisms of resistance against leishmaniasis. The contribution of B cells during the infectious process, either as APCs or as immunoglobulin-secreting cells, is more controversial.

Some reports dissected the role of B cells and antibodies (Abs) in assisting cell-mediated responses during host defence against leishmaniasis. Although some reports failed to show evidence of a contribution of B cells to the development of polarized T-cell responses,<sup>44–46</sup> others demonstrated a correlation between B-cell depletion and enhanced resistance to the disease.<sup>47,48</sup> In this context, B lymphocytes were shown to contribute to susceptibility to *Leishmania donovani* infection unrelated to the presence or absence of immunoglobulin.<sup>49</sup> Nevertheless, antigen-specific as well as natural immunoglobulin G (IgG) molecules are responsible for the opsonization of the parasite, so that components of the innate immune system will recognize the parasites as foreign.<sup>50</sup> Recently, Woelbing *et al.*<sup>51</sup> have reaffirmed the role of B cell-derived Abs in the development of adaptive immune responses during leishmaniasis. They reported that resistant mice without B cells or deficient in the Fc $\gamma$  receptor became susceptible to disease. This was attributed to decreased numbers of infected CD11c<sup>+</sup> DCs *in vivo*, which led to impaired T-cell priming and dramatically reduced interferon (IFN)- $\gamma$  production. These observations suggest that parasite-reactive IgG and DC Fc $\gamma$  receptors I and III are essential for optimal development of protective immunity.<sup>51</sup> Overall, we face the challenge to identify parasite antigens that could be involved in such mechanisms.

The protein encoded by the *SIR2* gene belongs to a highly conserved family of proteins, with NAD-dependent class III deacetylases, found in organisms from bacteria to humans and named homologous to SIR two (Hst) proteins or sirTuins.<sup>52</sup> Several roles have been attributed to this protein family, including cell cycle progression and chromosome stability,<sup>53</sup> DNA damage repair,<sup>54</sup> and life-span extension in yeast<sup>55</sup> and in *Caenorhabditis elegans*.<sup>56</sup> We have detected the presence of a SIR2-like gene in *Leishmania major* and *Leishmania infantum* termed SIR2RP1.<sup>57,58</sup> In addition, two other homologous sequences (SIR2RP2 and 3) can be found in the *Leishmania* genome database. Immunological investigations suggested an immunoregulatory role for SIR2RP1 during infection through its capacity to trigger specifically B-cell effector functions.<sup>59</sup> In an attempt to unravel the molecular mechanism of its activity, we investigated the direct effect of SIR2RP1 on the spleen B-lymphocyte population. Our results showed that SIR2RP1 activates B cells via a TLR2-dependent mechanism. The activated B cells up-regulated expression of both MHC II and costimulatory molecules CD40 and CD86, and secreted tumour necrosis factor (TNF)- $\alpha$ . Similarly, SIR2RP1 induced the maturation of bone marrow-derived dendritic cells (BMDCs). Nevertheless, TLR2-deficient mice were capable of secreting Ab specific to SIR2RP1 after a classical immunization procedure. This could be explained by the interaction of SIR2RP1 with macrophages even in the absence of TLR2. Overall, the data presented in this study clearly

demonstrate the importance of TLR2 in SIR2RP1 recognition by the innate immune system.

## Materials and methods

### *Mice and immunization protocol*

Male BALB/c and wild-type C57BL/6 mice were obtained from Harlan Iberica (Barcelona, Spain). TLR2-deficient mice with a C57BL/6 background<sup>60</sup> were obtained from Dr S. Akira (Osaka University, Japan) via Dr Salomé Gomes (IBMC, Porto University, Portugal). Animal experiments were carried out in the approved facilities of Instituto de Biologia Molecular e Celular (IBMC) (licence no. 34-18 and 06016 to A. Ouaiissi and A. Cordeiro-da-Silva, respectively). Defined groups of each strain of mice were injected three times intraperitoneally at 7-day intervals with 50 µg of SIR2RP1 in 300 µl of phosphate-buffered saline (PBS). As a control, mice received 300 µl of PBS. The sera were collected 2 weeks after the final immunization.

### *Purification of SIR2RP1 and thiol-dependent reductase 1 (TDR1)*

The *L. infantum* SIR2RP1 and TDR1 proteins were obtained as recombinant proteins containing six-histidine residues at their N-terminals, as previously described.<sup>61</sup> The purity of SIR2RP1 and TDR1 was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie blue. The proteins were dialysed against PBS using PD10 desalting columns (Amersham, Carnaxide, Portugal). To eliminate endotoxins, the recombinant proteins were passed through an EndoTrap<sup>®</sup> red column (Profos, Regensburg, Germany) following the manufacturer's instructions. The concentration of both recombinant proteins was determined using the Folin procedure.

### *B-cell isolation, T-cell enrichment and generation of BMDCs and macrophages*

After cervical dislocation, the spleens were removed and homogenized in Petri dishes to obtain a single-cell suspension. After two washes in RPMI-1640 culture medium (Cambrex, East Rutherford, NJ, USA), the cell concentrations were adjusted to 10<sup>7</sup> cells/ml in complete culture medium (RPMIc medium), consisting of RPMI-1640 culture medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 mM HEPES and 0.05 µM β-mercaptoethanol. Spleen B-cell isolation was carried out using the B-Cell Isolation Kit MACS<sup>®</sup> (Miltenyi Biotec, Bergisch Gladbach, Germany) as described by the manufacturer. The effectiveness of B-cell purification was determined by double labelling with specific mAbs (anti-µ<sup>+</sup>, anti-CD4<sup>+</sup>

and anti-CD8<sup>+</sup>) and further fluorescence-activated cell sorter (FACS) analysis. B-cell purity above 97% was obtained after the purification process. An enriched T-cell population was obtained from the spleen suspension depleted of B cells. Hence, the B cell depleted spleen cell suspension was left in a six-well flat-bottomed plate for 8 hr at 37° for macrophage and DC adherence. The non-adherent cells were then recovered and FACS analysis was performed, indicating T-cell enrichment between 85 and 90% in all cell preparations from BALB/c as well as C57BL/6 and TLR2<sup>(-/-)</sup> mice, with a B-cell percentage below 0.5%. BMDCs and bone marrow-derived macrophages (BMMφ) were generated as described in previous reports.<sup>62–64</sup> Briefly, bone marrow cells were obtained by flushing the femurs of the mice with RPMI medium. The resulting cell suspension was centrifuged and the cells were re-suspended in RPMI (for BMDCs) or Dulbecco's modified Eagle's minimal essential medium (DMEM) (for BMMφ) supplemented with 10 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 10% FCS. BMDCs and BMMφ were obtained by culturing bone marrow cells from each mouse strain with recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) (Pepro- tech, Rocky Hill, NJ, USA) for 14 days or with L929 supernatant as source of macrophage colony-stimulating factor (M-CSF) for 10 days, respectively. After 14 days of culture, BMDCs were submitted to flow cytometry expression sorting. DCs were > 95% pure based on flow cytometry analysis of the CD11c integrin. Mφ were phenotypically characterized as F4/80<sup>+</sup> and CD11b<sup>+</sup> (Mac1<sup>+</sup>).

### *RNA isolation and semiquantitative reverse transcription–polymerase chain reaction (RT-PCR)*

Isolated spleen B cells were stimulated or not with SIR2RP1 (10 µg/ml) and incubated at 37° in 5% CO<sub>2</sub>. After 20 hr of stimulation, total RNA (from 4 × 10<sup>6</sup> cells per stimulus) was isolated using a guanidium thiocyanate–phenol–chloroform single-step method.<sup>65</sup> Reverse transcription was performed with equal amounts of total extracted RNA (1 µg) obtained from cells treated with or without SIR2RP1 using Superscript II RT (Gibco BRL, Grand Island, NY, USA) and random primers (Promega, Charbonnières, France). An aliquot of the first strand was amplified by PCR in a thermocycler using a Taq polymerase (Gibco BRL). Specific primers for TLR1 (forward: 5'-ACATCAAGTGTGTGCTTGAA-3'; reverse: 5'-CCCCATC ACTGTACCTTAGA-3') (GenBank accession number AF316985), TLR2 (forward: 5'-ACAGCTACTGTGTGA CTCTCCGCC-3'; reverse: 5'-GGTCTTGGTGTTTCATTAT CTTGCGC-3') (GenBank accession number AF124741), TLR4 (forward: 5'-GACCTCAGCTTCAATGGTGC-3'; reverse: 5'-TATCAGAAATGCTACAGTGGATAACC-3') (GenBank accession number AF095353), TLR6 (forward: 5'-ACATCTCTTGCTGCCCTATG-3'; reverse: 5'-GAGG

AACACTTGGTTTTTAC-3') (GenBank accession number AF314636), MyD88 (forward: 5'-CAC CTGTGTCTGGTCCATT-3'; reverse: 5'-CGCAGGATACT GGGAAAGT-3') (GenBank accession number MMU 84409), NF- $\kappa$ B (forward: 5'-TGGCTACTATGAGGCT-GACC-3'; reverse: 5'-GTTGATGGTGCTGAGGGAT-3') (GenBank accession number AF199371) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5'-CCATGGAGAAGGCTGGGG-3'; reverse: 5'-CAAAGTT GTCATGGATGACC-3') were used for amplification. After a hot start at 94° for 3 min, a PCR cycle consisted of a denaturation step at 94° for 30 seconds, an annealing step at 55° for GAPDH, 56° for TLR1, 57° for TLR6 and NF- $\kappa$ B and 59° for TLR2, TLR4 and MyD88 for 30 seconds, and an elongation step for 72° for 30 seconds. For the last cycle, the elongation step was extended to 10 min at 72°. Reactions were carried out for 25–30 cycles in a thermocycler. PCR products were analysed by electrophoresis on a 1.0% agarose gel and visualized and photographed on an ultraviolet (UV) transilluminator (Vilber Lourmat, Eberhardzell, Germany). PCR band densities were determined using the BIO-PROFIL BIO-ID software (Vilber Lourmat).

#### Measurement of cellular proliferation

Single-cell suspensions ( $2 \times 10^5$  cells/well) of spleen cells or isolated B cells from normal BALB/c, C57BL/6 wild-type or TLR2 knockout mice were cultured in triplicate in 96-well flat-bottom plates in a final volume of 200  $\mu$ l. Cells were stimulated with lipopolysaccharide (LPS; 10  $\mu$ g/ml) or SIR2RP1 (0.1, 1, 10 or 50  $\mu$ g/ml) and incubated at 37° in 5% CO<sub>2</sub>. After 72 hr of incubation, [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) (Amersham Corp, Arlington, IL) was added for the last 8 hr. Pulsed cells were harvested on a glass filter using an automated multiple-sample harvester and dried. Incorporation of radioactive thymidine was then determined by liquid scintillation as specified in the standard protocol. Results are expressed as mean counts per minute (c.p.m.) for stimulated cells subtracted from background values for non-stimulated cells cultured in RPMIc medium.

#### Flow cytometry determinations

SIR2RP1 or TDR1 stimulated and non-stimulated cells (total spleen and isolated spleen B cells, BMDCs and BMM $\phi$ ) from BALB/c, C57BL/6 wild-type or TLR2 knockout mice were washed by centrifugation and re-suspended in PBS supplemented with 2% FCS. For CD69 labelling, a total of  $2 \times 10^5$  viable cells were incubated for 30 min at 4° with saturating concentrations of phycoerythrin (PE)-conjugated mAb to CD69 plus fluorescein isothiocyanate (FITC)-conjugated mAbs to CD4, CD8 $\alpha$  or IgM (anti- $\mu$ ) from BD Pharmingen (Le Pont de Claix, France). For other labelling, a total of  $10^6$  viable cells were incubated for

30 min at 4° in the dark with saturating concentrations of FITC-conjugated mAbs to CD40, CD80, CD86, I-A<sup>b</sup> or I-A<sup>d</sup> (both MHC class II) from BD Pharmingen. The mouse isotype controls IgG1 (FITC) and IgG2a (FITC) were purchased from Immunotools (Friesoythe, Germany). After two washing steps with PBS–2% FCS, the cells were analysed by flow cytometry in a FACS Scan equipped with CELLQUEST PRO software (Becton Dickinson, Pont-de-Claix, France). Lymphocytes were selected on the basis of forward-scatter/side-scatter values and dead cells were excluded from all samples by propidium iodide labelling.

#### Enzyme-linked immunosorbent assay (ELISA) for immunoglobulins

Sera from all of the immunized groups of mice were analysed by ELISA for the presence of anti-SIR2RP1 specific Abs. The ELISA technique used was adapted from the protocol described elsewhere.<sup>59</sup> Briefly, 96-well flat-bottomed microtitre plates (Grainer, Courtaboeuf, France) were coated overnight at 4° with the recombinant SIR2RP1 (5  $\mu$ g/ml) in 0.01 M carbonate/bicarbonate buffer, pH 8.5. After blocking and several washing steps, the plates were incubated at 37° with serial dilutions of each serum for 2 hr, followed by 30 min of incubation with peroxidase-labelled goat anti-mouse immunoglobulin isotypes (anti-IgG, anti-IgG1 and anti-IgG2a). The reactions were developed with the addition of 0.5 mg/ml of *o*-phenylenediamine dihydrochloride (OPD; Sigma, Sintra, Portugal) with 10  $\mu$ l of H<sub>2</sub>O<sub>2</sub> in citrate buffer. Absorbance values were read at 492 nm in an automatic ELISA reader (Power Wave XS; Bio-Tek, Winooski, VT, USA).

#### Cytokine ELISA

Isolated spleen B cells, BMDCs and BMM $\phi$  from BALB/c, C57BL/6 wild-type or TLR2 knockout mice were stimulated or not with LPS (1 or 10  $\mu$ g/ml for APCs or B cells, respectively), SIR2RP1 (1, 5 or 10  $\mu$ g/ml) or TDR1 (10  $\mu$ g/ml) for 24 hr (BMDCs and BMM $\phi$ ) or 72 hr (B cells). The levels of IL-10, IL-12p40, IL-12p70 and TNF- $\alpha$  were measured in the culture supernatants by ELISA following the manufacturer's recommendations (BD Pharmingen for IL-10 and IL-12p70; BioLegend (San Diego, CA, USA) for IL12p40 and TNF- $\alpha$ ). Minimum detection levels were 7.8 pg/ml for IL-12p40 and TNF- $\alpha$ , 31.3 pg/ml for IL-10 and 62.5 pg/ml for IL-12p70. Samples were assayed in triplicate, and the data are expressed as the mean and standard deviation (SD) for each cytokine examined.

#### Primary allogenic mixed lymphocyte reaction (MLR)

Immature DCs generated from mouse C57BL/6 WT or C57BL/6 TLR2<sup>(-/-)</sup> bone marrow cells were stimulated or not with SIR2RP1 (10  $\mu$ g/ml) or LPS (1  $\mu$ g/ml). After

4 days, DCs were washed twice with RPMIc, treated with mitomycin C (Sigma) for 20 min at 37°, washed three times with RPMIc and cultured in quadruplicate at  $5 \times 10^3$  or  $2 \times 10^4$  cells/200  $\mu$ l/well in 96-well flat-bottomed cultured plates with  $2 \times 10^5$  allogeneic T cells. Proliferation of T cells in the allogeneic MLR was measured by [ $^3$ H]thymidine incorporation for the final 16 hr of the culture period of 5 days. Results are expressed as the mean ( $\pm$  SD) counts per minute (c.p.m.) of quadruplicate values.

### Statistical analysis

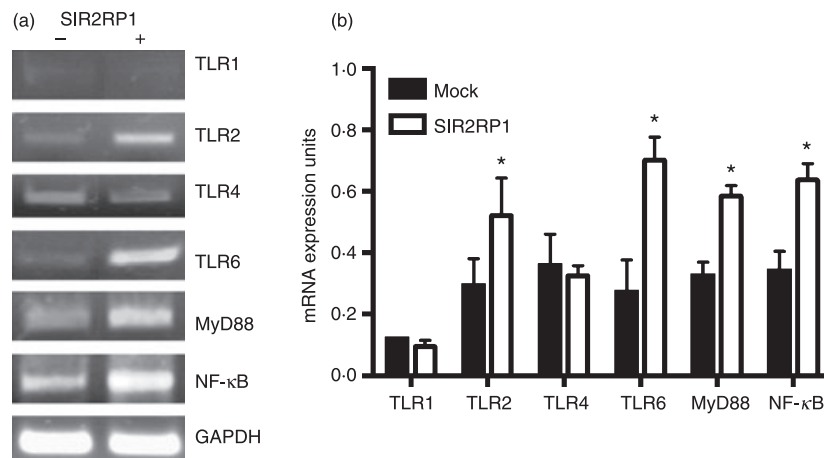
Statistical analysis were performed using GRAPH PAD PRISM 5 (GraphPad Software Inc., La Jolla, CA, USA). The data were analysed using two-sided unpaired Student's *t*-test. A one-way analysis of variance (ANOVA), with a Bonferroni multiple comparison post-test, was used to assess the significance of the differences among various groups. A *P*-value < 0.05 was considered significant.

## Results

### SIR2RP1 treatment of B cells led to up-regulation of TLR2, TLR6, MyD88 and NF- $\kappa$ B gene transcription

In previous studies, we characterized the effect of SIR2 proteins from *L. major*<sup>59</sup> and *L. infantum* (unpublished observations) on the spleen lymphocyte population. These proteins were found to trigger specifically B-cell effector functions, as demonstrated by CD69 up-regulation on the B-cell surface and secretion of significant levels of specific antibodies. Thus, it was of major relevance to explore the molecular basis of the SIR2RP1/B-cell interaction.

B cells possess functional characteristics of innate immune cells, as they can present antigen to T cells and can be stimulated with microbial molecules such as TLR ligands. Indeed, innate cell activation by pathogens often involves members of the TLR family.<sup>66</sup> Therefore, we tested the involvement of these signalling molecules in SIR2RP1-mediated B-cell activation. Total RNA was isolated from purified spleen B cells cultured for 20 hr in the presence or absence of SIR2RP1 (10  $\mu$ g/ml). The reverse-transcribed cDNA was submitted to PCR analysis using specific primers for TLRs, the accessory adaptor protein MyD88 and the transcription factor NF- $\kappa$ B (Fig. 1a). SIR2RP1 stimulus induced increased TLR2 mRNA expression compared with the unstimulated cells (Fig. 1b). As TLR2 has a unique ability to heterodimerize with TLR1 or TLR6,<sup>13</sup> we further explored the expression of these signalling molecules following SIR2RP1 stimulus. As shown in Fig. 1b, increased TLR6 mRNA expression was observed after 20 hr of SIR2RP1 stimulus compared with unstimulated cells. However, SIR2RP1 had no effect on the expression of TLR1. Also, TLR4, which is known to recognize primarily LPS from Gram-negative bacteria,<sup>67</sup> remained unaffected after SIR2RP1 stimulus. Recently, it has been proposed that the activation of membrane-bound TLR2 is followed by different signalling pathways. Among the signalling pathways, MyD88, an adaptor molecule, was shown to be involved in this process and upon stimulation, NF- $\kappa$ B, which is present in an inactive heterodimeric form in the cytoplasm, is translocated to the nucleus where it interacts with  $\kappa$ B-responsive elements mediating transcriptional gene activation.<sup>68,69</sup> Isolated B cells cultured in the presence of SIR2RP1 showed increased MyD88 and NF- $\kappa$ B transcripts, respectively, compared with unstimulated cells (Fig. 1b).



**Figure 1.** The effect of SIR2RP1 on mRNA expression of Toll-like receptor and downstream signaling molecules. (a)  $2.5 \times 10^5$  spleen isolated B-cells were cultured with or without 2  $\mu$ g of SIR2RP1 for 20 hr. Total RNA was extracted from the B cells and reverse transcribed to cDNA. The cDNA sample was used as a template for 25–30 cycles of PCR using primers for TLR1, TLR2, TLR4, TLR6, MyD88 and NF- $\kappa$ B. GAPDH was used as control housekeeping gene to assure the samples homogeneity. Ethidium bromide-stained PCR products were photographed, and the images were digitized and analyzed. (b) The PCR products were quantified and expressed as the ratio of each product regarding the GAPDH band density (mRNA expression units). The data represent the mean  $\pm$  standard deviation of three independent experiments. \**P* < 0.05.

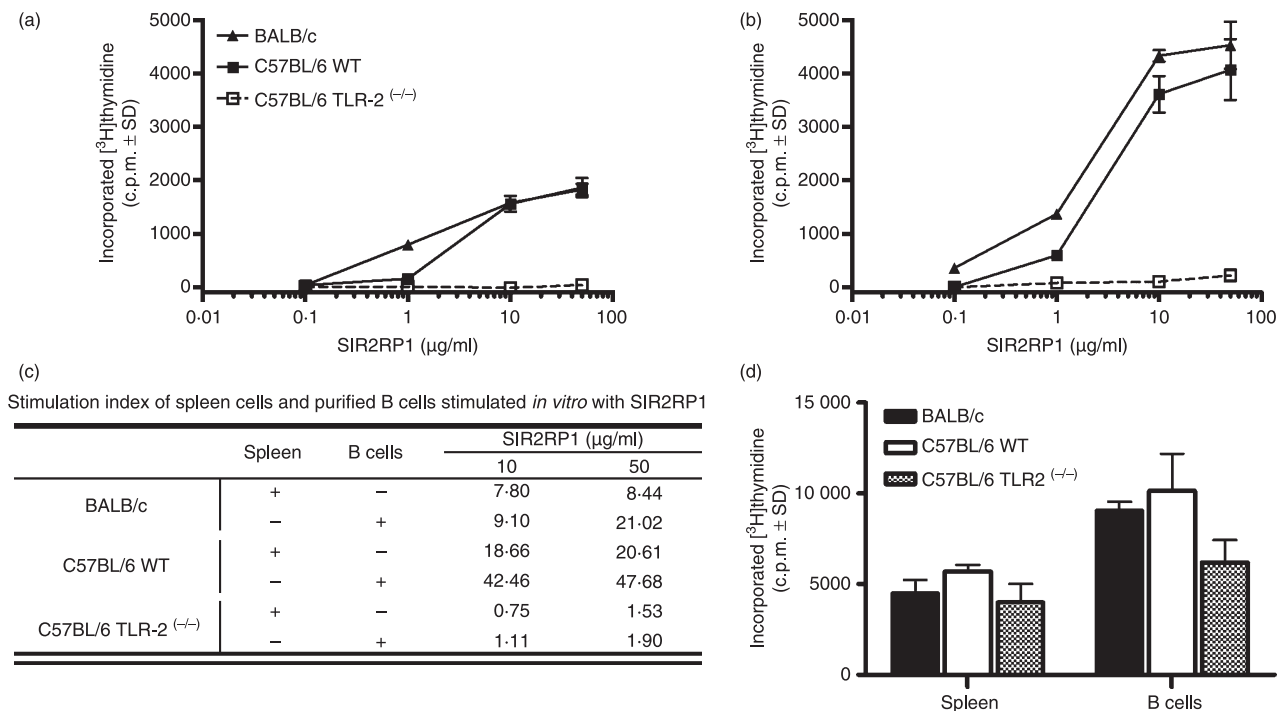
Taken together, these data indicate that TLR2 and TLR6, but not TLR1, are co-expressed and might work in tandem with MyD88 and NF- $\kappa$ B to mediate SIR2RP1-dependent signal transduction.

**Proliferation of B cells in response to SIR2RP1 depends on TLR2 expression**

We had previously observed that SIR2RP1 triggers the direct activation of BALB/c B cells, as shown by up-regulation of the CD69 marker.<sup>59</sup> The above data confirm and further characterize SIR2RP1-induced B-cell activation. To further examine the stimulatory activity of SIR2RP1 on the spleen cell populations, we measured proliferation by the incorporation of [<sup>3</sup>H]thymidine in total spleen and isolated B cells, when submitted to an *in vitro* SIR2RP1 stimulus. As shown in Fig. 2a, SIR2RP1 stimulated [<sup>3</sup>H]thymidine incorporation in spleen cells from BALB/c or C57BL/6 mice in a dose-dependent manner. The proliferating effect of SIR2RP1 was already evident at 1  $\mu$ g/ml and reached a plateau at a concentration of 10  $\mu$ g/ml. Interestingly, spleen cells from C57BL/6 TLR2<sup>(-/-)</sup> mice did not respond to any concentration tested. Complementary experiments were conducted to determine whether the SIR2RP1 proliferative effect on B cells was induced directly or, instead, if the presence of accessory

cells, such as macrophages and DCs present in total spleen cell culture, was required. To this end, the proliferative capacity of isolated B cells purified from spleens was assessed. Similarly to total spleen cell cultures, a dose-dependent proliferative effect on B cells was observed upon SIR2RP1 treatment (Fig. 2b). Stimulation with 10 or 50  $\mu$ g/ml of SIR2RP1 resulted in a significant proliferative response, when compared with SIR2RP1-stimulated total spleen cell cultures, as confirmed by an increase in the stimulatory index (SI) (using 50  $\mu$ g/ml of SIR2RP1 and a 72-hr incubation period the SI reached 21.02 and 47.68 in isolated B cells versus 8.44 and 20.61 in total spleen cell cultures of BALB/c and C57BL/6 mice, respectively) (Fig. 2c). In order to rule out the possibility that LPS contaminating the SIR2RP1 preparation was responsible, at least in part, for the cell stimulation, isolated B cells from C3H/HeJ mice (LPS non-responder)<sup>67</sup> were cultured with or without SIR2RP1. The proliferation of C3H/HeJ isolated B cells induced by the SIR2RP1 stimulus reached similar levels to that observed with BALB/c isolated B cells, as revealed by the corresponding SIs: 6.79 and 8.09 for C3H/HeJ mice and 7.80 and 8.44 for BALB/c mice, after 48 hr of stimulation with 10 and 50  $\mu$ g/ml of SIR2RP1, respectively.

The above observations suggest that B cells are the major targets of SIR2RP1. However, this does not necessarily rule

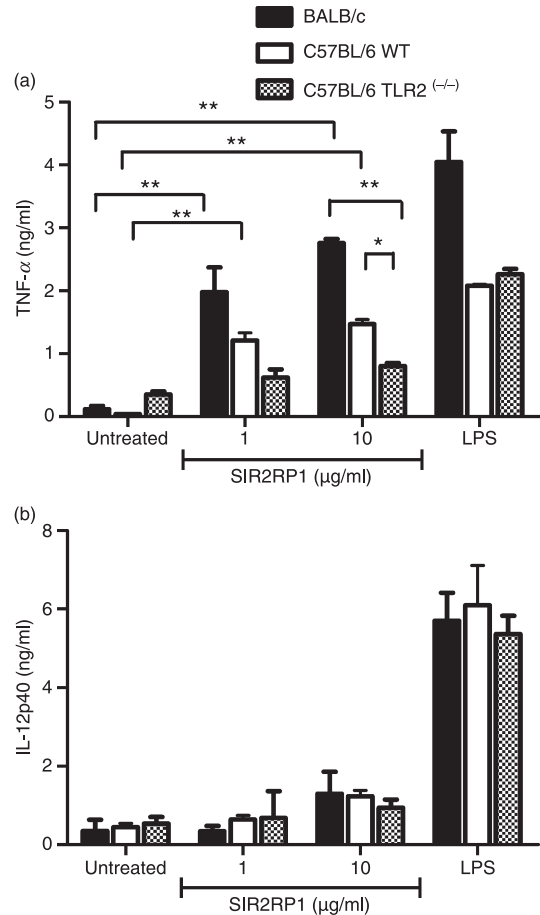


**Figure 2.** Cell proliferation responses upon SIR2RP1 treatment. Total spleen cells (a) or purified spleen B-cells (b)  $2.5 \times 10^5$  cells per well from BALB/c, C57BL/6 WT and C57BL/6 TLR2<sup>(-/-)</sup> mice were incubated with increasing concentrations of SIR2RP1 (0.02, 0.2, 2, 10  $\mu$ g per well) for 72 hr. The corresponding stimulation index was determined by dividing the c.p.m. value for a given set of SIR2RP1-stimulated cells by the c.p.m. of the associated SIR2RP1-untreated cell (c) Proliferative response was also assessed using a LPS stimulus (d) Proliferation was assessed by [<sup>3</sup>H]-thymidine as described in *Material and Methods* section. Data are presented as mean c.p.m. and standard deviations subtracted from background values from unstimulated cells. The data are representative of three independent experiments.

out a potential role for accessory cells *in vivo*. In addition, isolated B cells from TLR2-deficient C57BL/6 mice have been shown to be unresponsive to SIR2RP1 stimulation. Given that the TLR2 knockout mice are known to respond similarly to LPS as C57BL/6 WT, in terms of both proliferating capacity and secretion of cytokines,<sup>60</sup> we conducted *in vitro* studies using LPS as a triggering agent. As shown in Fig. 2d, C57BL/6 TLR2<sup>(-/-)</sup> mice responded in a similar manner to LPS as C57BL/6 WT or BALB/c mice, suggesting that the direct proliferative effect of SIR2RP1 on B cells specifically depends on TLR2, reinforcing the notion that SIR2RP1 is free of endotoxin contaminants.

### SIR2RP1 up-regulates CD40, CD86 and MHC class II on isolated spleen B cells

To further characterize the stimulatory activity of SIR2RP1 on isolated B cells, we evaluated whether SIR2RP1 affects the expression pattern of costimulatory ligands which are critical for B-cell interaction with other immune cells, such as T lymphocytes. Therefore,  $2 \times 10^5$  isolated spleen B cells were stimulated with 2  $\mu$ g of SIR2RP1 for 20 hr and the expression of the accessory ligands was analysed by flow cytometry. Analysis of the relative fluorescence intensity demonstrated that SIR2RP1 only induced CD40, CD86 and class II MHC surface expression on B cells isolated from TLR2-competent mice (Table 1). The cell surface expression of CD80 (Table 1) and MHC I (data not shown) remained unaffected after SIR2RP1 stimulation. As a positive control, LPS was used, which induced up-regulation of all costimulatory molecules. The above data underline the essential role of TLR2 in regulating B-cell proliferative responses to SIR2RP1. Taken together, these observations indicate that the TLR2-dependent stimulatory activity of SIR2RP1 on B cells induces the expression of costimulatory molecules,



**Figure 3.** TLR2-dependent secretion of interleukins by B cells. Purified spleen B-cells derived from BALB/c (black bars), C57BL/6 WT (white bars) or C57BL/6 TLR2<sup>(-/-)</sup> (dashed bars) mice were cultured in 96-well plates at  $1 \times 10^6$ /ml in the absence or the presence of LPS (1  $\mu$ g/ml) or SIR2RP1 (1 or 10  $\mu$ g/ml). After 72 hr of incubation, the levels of TNF- $\alpha$  (a) and IL-12p40 (b) were measured in the culture supernatants by ELISA. The results shown are one representative of two different experiments that yielded similar results. \* $P < 0.05$ , \*\* $P < 0.01$ .

**Table 1.** Increased expression of costimulatory molecules on isolated B cells and bone marrow-derived macrophages upon incubation with silent information regulator 2 related protein 1 (SIR2RP1)

Cell type	Assay	Unstimulated		SIR2RP1 (10 $\mu$ g/ml)		Lipopolysaccharide	
		C57BL/6 WT	C57BL/6 TLR2 <sup>(-/-)</sup>	C57BL/6 WT	C57BL/6 TLR2 <sup>(-/-)</sup>	C57BL/6 WT	C57BL/6 TLR2 <sup>(-/-)</sup>
B cells	CD40	76 $\pm$ 8	83 $\pm$ 12	98 $\pm$ 12*	86 $\pm$ 10	164 $\pm$ 21	183 $\pm$ 30
	CD80	10 $\pm$ 3	10 $\pm$ 1	9 $\pm$ 2	10 $\pm$ 3	26 $\pm$ 8	32 $\pm$ 11
	CD86	6 $\pm$ 1	9 $\pm$ 2	23 $\pm$ 8**	10 $\pm$ 3	42 $\pm$ 5	31 $\pm$ 10
	MHC class II (IAb)	18 $\pm$ 3	23 $\pm$ 7	76 $\pm$ 16**	31 $\pm$ 9	256 $\pm$ 32	182 $\pm$ 41
Macrophages	CD40	6 $\pm$ 1	5 $\pm$ 2	30 $\pm$ 8**	12 $\pm$ 3*	35 $\pm$ 5	26 $\pm$ 3
	CD80	15 $\pm$ 2	17 $\pm$ 3	18 $\pm$ 2	20 $\pm$ 4	20 $\pm$ 3	20 $\pm$ 2
	CD86	10 $\pm$ 2	9 $\pm$ 3	16 $\pm$ 3*	14 $\pm$ 2*	32 $\pm$ 7	32 $\pm$ 4
	MHC class II (IAb)	8 $\pm$ 2	7 $\pm$ 1	10 $\pm$ 4	9 $\pm$ 2	31 $\pm$ 9	28 $\pm$ 7

Results are expressed as mean fluorescence intensity (MFI); mean  $\pm$  standard deviation of three independent experiments is shown.

\* $P < 0.05$ ; \*\* $P < 0.01$ , for comparison between SIR2RP1-stimulated and unstimulated cells.

TLR, Toll-like receptor; WT, wild type.

which are known to play a crucial role in the initiation and maintenance of an immune response.

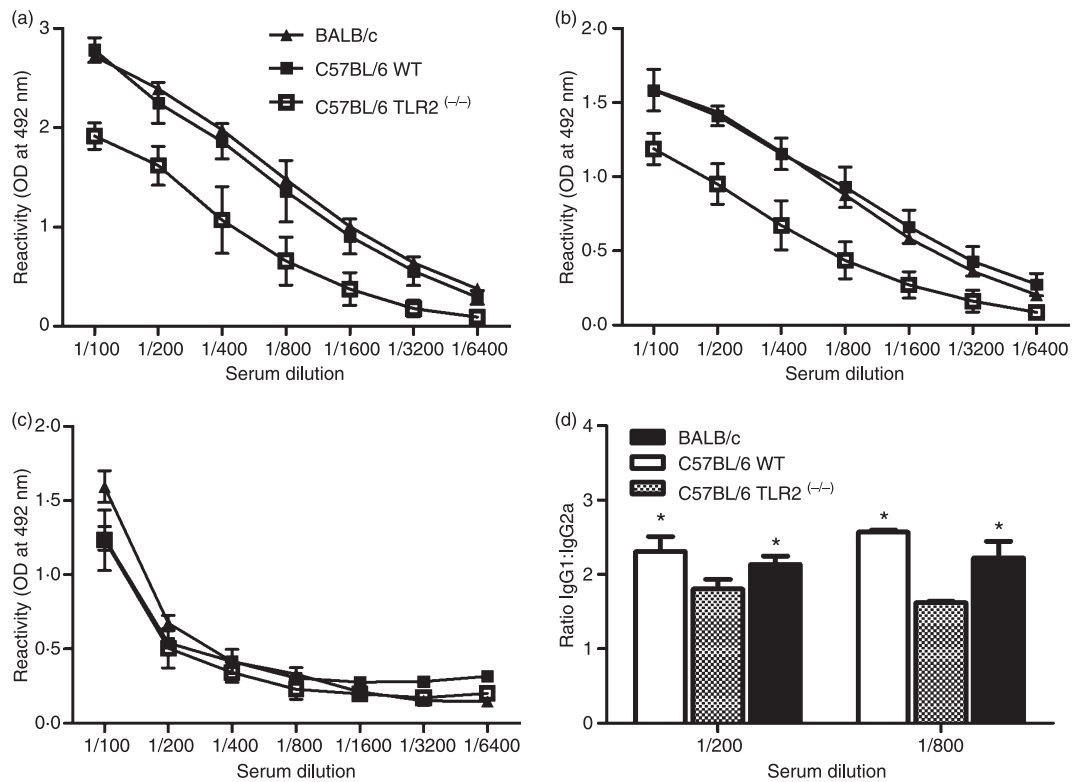
**B cells secrete TNF- $\alpha$  in response to an SIR2RP1 stimulus**

Complementary experiments were performed to further examine the effect of SIR2RP1 on the B-cell response, namely cytokine production. Hence, isolated B cells from BALB/c, C57BL/6 WT and C57BL/6 TLR2<sup>(-/-)</sup> mice were cultured for 72 hr in the presence or absence of SIR2RP1. As a positive control, isolated B cells were cultured with LPS (10  $\mu$ g/ml). SIR2RP1 induced the secretion of large amounts of TNF- $\alpha$  even at a low dose, although this induction was higher in BALB/c than in C57BL/6 mice (Fig. 3a;  $P < 0.01$ , for BALB/c and C57BL/6 mice with 1 or 10  $\mu$ g/ml). Although IL-10 production by B cells upon stimulation with SIR2RP1 has been investigated, the results obtained were not conclusive. No changes were seen in the pro-inflammatory cytokine IL-12p40 (Fig. 3b). In order to confirm the specificity of this response, we performed complementary experiments with another unrelated excreted/secreted *Leishmania* protein named

TDR1. Similar to SIR2RP1, TDR1 was obtained as an endotoxin-free recombinant protein produced in a bacterial system containing a six-histidine residue at its N-terminal. TDR1 is capable of activating B cells, but not CD4 or CD8 T cells, even in the absence of accessory cells (Fig. S1a,b). Nevertheless, the mechanism of B-cell activation was shown to be TLR2-independent, as isolated B cells purified from the spleens of C57BL/6 TLR2<sup>(-/-)</sup> mice were able to respond to TDR1 in a similar manner to TLR2-competent B cells, whatever the parameter considered: surface expression of the CD69 marker or pro-inflammatory cytokine production (IL-12p40 and TNF- $\alpha$ ) (Fig. S1b and Table S1). Taken together, these observations support the notion of TLR2 signalling being involved in SIR2RP1-induced B-cell activation.

**TLR2 knockout mice secrete specific anti-SIR2RP1 Abs after immunization**

We had previously demonstrated that immunization of TLR2-competent mice (BALB/c) with SIR2RP1 induced a strong humoral response with the production of specific antibodies.<sup>59</sup> To analyse *in vivo* the TLR2 dependence of



**Figure 4.** Immunization of C57BL/6 TLR2<sup>(-/-)</sup> mice with SIR2RP1 induced a decrease of IgG1/IgG2a ratio. Groups of 3 mice were immunized i.p. with SIR2RP1. Fifteen days after the last immunization, the levels of SIR2RP1-specific IgG (a) IgG1 (b) and IgG2 (c) antibodies were quantified in the sera of C57BL/6 WT, C57BL/6 TLR2<sup>(-/-)</sup>, and BALB/c mice by ELISA-limiting dilution. The ratio between SIR2RP1-specific IgG1 and IgG2a levels is shown for 1/200 and 1/800 sera dilutions (d). The asterisk indicates a statistically significant difference ( $P < 0.05$ ) between C57BL/6 WT or BALB/c mice and TLR2-deficient C57BL/6 mice. The data represent the mean and the standard deviation of three animals analyzed individually and representative of two independent experiments.



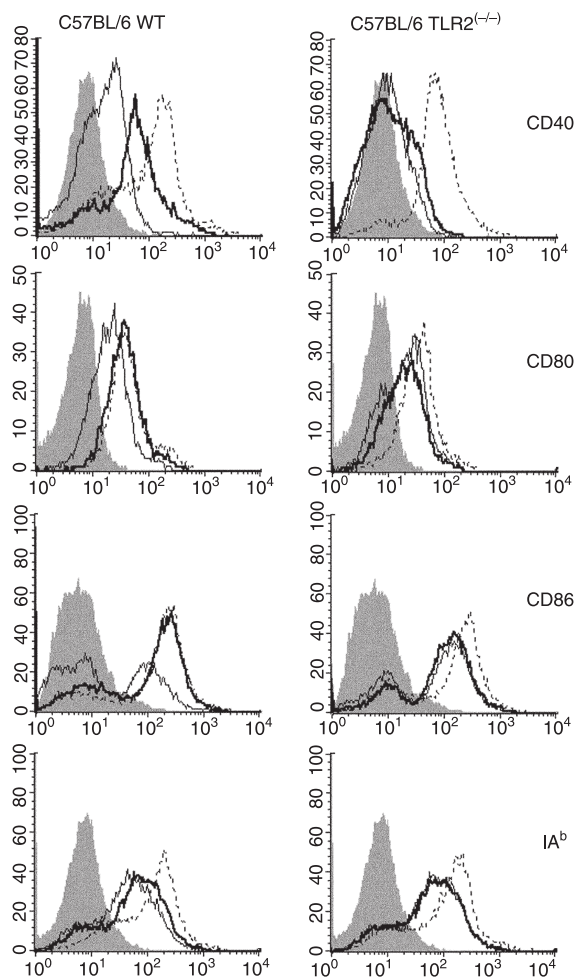
the secretion of antibodies, similar immunization experiments were performed in C57BL/6 TLR2<sup>(-/-)</sup> and respective control mice (C57BL/6). We determined the levels of specific IgG and different subclasses of IgG (IgG1 and IgG2a) in the sera of mice that received three i.p. injections of SIR2RP1 at 7-day intervals. Surprisingly, TLR2-deficient mice were still able to produce specific antibodies against SIR2RP1 (Fig. 4a). Nevertheless, the humoral response was found to be lower in C57BL/6 TLR2<sup>(-/-)</sup> mice than in TLR2-competent mice, which could in part be explained by the secretion of lower amounts of IgG1 (Fig. 4b), as the levels of specific IgG2a antibodies in the serum of TLR2-deficient or -competent mice were found to be similar (Fig. 4c). A careful analysis of the SIR2RP1-specific Ab response indicated a statisti-

cally significant decrease in the SIR2RP1-specific IgG1:IgG2a ratio in C57BL/6 TLR2<sup>(-/-)</sup> mice compared with TLR2-competent mice (Fig. 4d;  $P < 0.05$ ).

### SIR2RP1 induces DC maturation in a TLR2-dependent manner

The above data therefore led us to investigate whether other TLR2-presenting cells, such as DCs or macrophages, are among the potential targets for SIR2RP1 *in vivo*, which may suggest a wider role for the *Leishmania* SIR2RP1 during the infectious process. Complementary experiments were performed in order to elucidate the role of these cells, which form a bridge between the innate and adaptive immune systems, in the recognition and processing of *Leishmania* SIR2RP1.

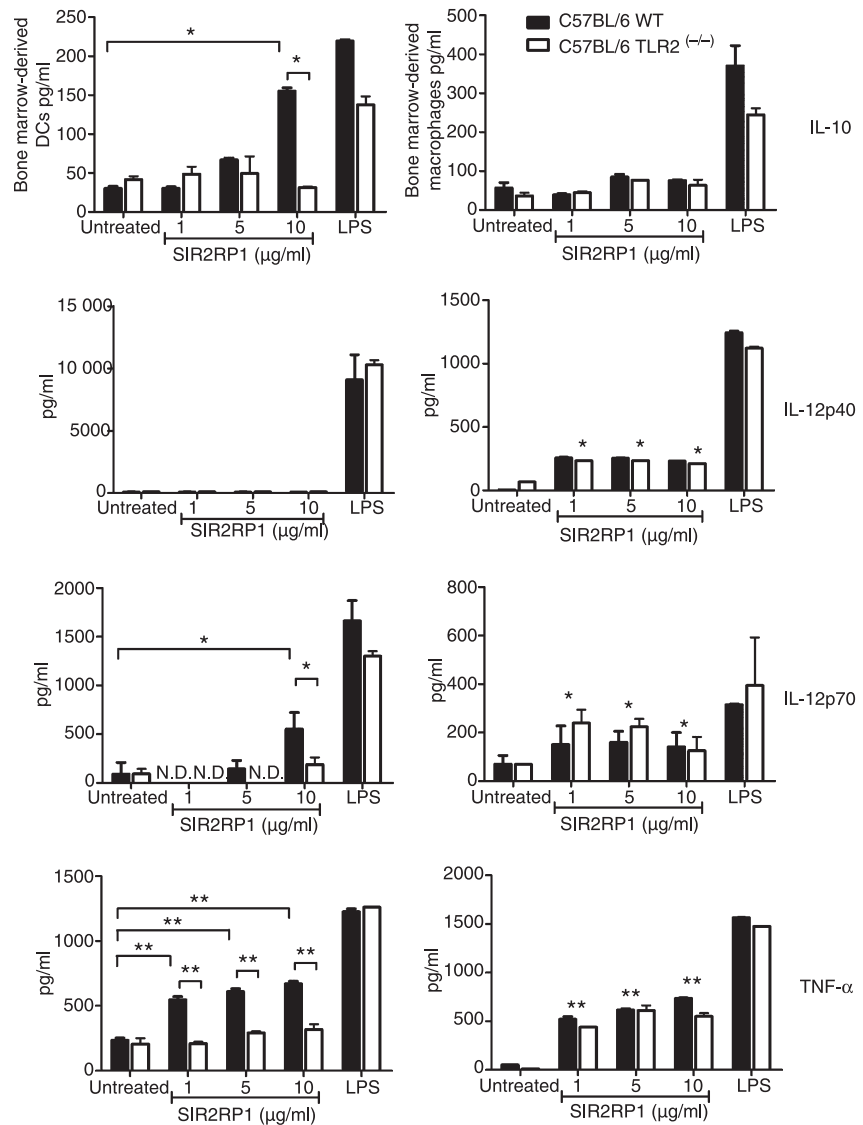
To evaluate the involvement of TLR2, BMDCs from TLR2-competent and TLR2-deficient C57BL/6 mice were stimulated for 24 hr with SIR2RP1 and the cell surface expression of activation markers was analysed by FACS. As shown in Fig. 5, SIR2RP1 induced up-regulation of surface CD40, CD80 and CD86 and a slight increase in class II MHC molecules in C57BL/6 mice. Moreover, stimulation of BMDCs with SIR2RP1 had no effect on class I MHC levels (data not shown). However, stimulation of BMDCs from TLR2-deficient mice with SIR2RP1 did not lead to up-regulation of any of these markers, although small increases were found in the CD40 molecule (Fig. 5). As a positive control, LPS induced the up-regulation of all evaluated markers in both TLR2-competent and -deficient mice. To further characterize the functionality of DCs induced to mature by SIR2RP1, we examined the ability of SIR2RP1 to induce the secretion of cytokines by DCs. In BMDCs, while LPS induced abundant secretion of both anti-inflammatory (IL-10) and pro-inflammatory (IL-12p40, IL-12p70 and TNF- $\alpha$ ) cytokines in the presence or absence of TLR2, SIR2RP1 induced barely detectable levels of IL-12p40 and low amounts of IL-10 and IL-12p70, but abundant levels of TNF- $\alpha$ , in a TLR2-dependent manner (Fig. 6). Interestingly, the profile of the secreted cytokines closely resembles those induced by SIR2RP1 on isolated B cells, which confirms the requirement of TLR2 signalling for SIR2RP1-induced BMDC and B-cell activation.



**Figure 5.** SIR2RP1 do not stimulate TLR2-deficient BM-DC cells. C57BL/6 WT and C57BL/6 TLR2<sup>(-/-)</sup> mice BM-DC were incubated for 24 hr with medium (thin solid line), 10  $\mu$ g/ml of SIR2RP1 (thick solid line) or 1  $\mu$ g/ml of LPS (dotted line) and the surface expression of CD40, CD80, CD86 and class II MHC was determined by flow cytometry analysis. The shaded line represents the correspondent isotypic control. Results are representative of one of three independent experiments.

### SIR2RP1 enhances the T-cell stimulatory properties of DCs

DCs induced to mature by SIR2RP1 expressed high levels of accessory and costimulatory molecules and hence should be primed to induce activation of T cells. Therefore, we tested the allostimulatory activity of SIR2RP1-stimulated DCs. In primary MLR assays, an MHC-restricted BALB/c enriched T-cell fraction (H-2<sup>d</sup>) was co-cultured with SIR2RP1-stimulated DCs from C57BL/6



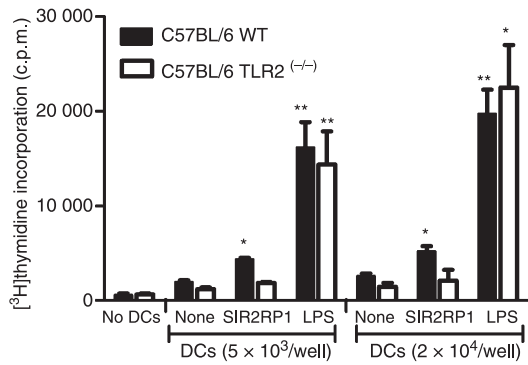
**Figure 6.** Different outcome in the SIR2RP1-APC interaction. BM-Dendritic cells and BM-Macrophage derived from C57BL/6 WT (black bars) or C57BL/6 TLR2<sup>-/-</sup> (white bars) mice were cultured in 24-well plates at  $1 \times 10^6$ /ml in the absence or the presence of LPS (1 μg/ml) or SIR2RP1 (1, 5 or 10 μg/ml). After 24 hr the levels of IL-10, IL-12p40, IL-12p70 and TNF-α were measured in the culture supernatants by ELISA. The results shown are one representative of three different experiments that yielded similar results. N.D. – below the detection limit; For BM-dendritic cells and BM-Macrophage, \* $P < 0.05$ , \*\* $P < 0.01$  between SIR2RP1 stimulated and unstimulated C57BL/6 WT or C57BL/6 TLR2<sup>-/-</sup> mice.

WT or C57BL/6 TLR2<sup>-/-</sup> (H-2<sup>b</sup>) mice, and the proliferation of alloreactive T cells was quantified by the incorporation of [<sup>3</sup>H]thymidine. As shown in Fig. 7, TLR2-competent BMDCs stimulated with SIR2RP1 exhibited enhanced allostimulatory capacity compared with unstimulated DCs (SI of  $2.1 \pm 0.2$ , with  $2 \times 10^4$  cells;  $n = 4$ ). As expected, BMDCs from C57BL/6 TLR2<sup>-/-</sup> mice presented low to zero levels of allostimulatory activity. As controls, BMDCs or T cells incubated alone showed an absence of proliferation and LPS-stimulated BMDCs induced high levels of proliferation of allogenic T cells.

**Macrophage activation by SIR2RP1 is TLR2-independent**

The above findings demonstrate the requirement for the TLR2-signalling pathway in SIR2RP1-induced B-cell and DC activation. Similar experiments were performed using

BMMφ. Thus,  $5 \times 10^5$  BMMφ were stimulated with 5 μg or 500 ng of SIR2RP1 or LPS, respectively. Analysis of relative fluorescence intensity after 24 hr of incubation demonstrated that SIR2RP1 induced increased expression of CD40 and CD86 compared with unstimulated cells (Table 1). In contrast, no significant increases in CD80 and class II MHC were observed. Surprisingly, TLR2-deficient BMMφ responded similarly to SIR2RP1 as TLR2-competent BMMφ. As a positive control, LPS induced up-regulation of all tested markers in both TLR2-competent and -deficient BMMφ. Moreover, cell-free supernatants of these cultures were harvested and examined by ELISA for cytokine production. SIR2RP1-induced BMMφ activation led to preferential secretion of the pro-inflammatory cytokines IL-12p40, IL-12p70 and TNF-α (Fig. 6). However, no significant differences were found in anti-inflammatory IL-10 secretion between SIR2RP1-stimulated and unstimulated cells. Interestingly, no dose-



**Figure 7.** Allostimulatory capacity of BM-DC cultured with SIR2RP1. Immature DCs generated from C57BL/6 WT or C57BL/6 TLR2<sup>-/-</sup> mice were stimulated with SIR2RP1 (10 µg/ml) or LPS (1 µg/ml). After 4 days, DC were treated with mitomycin C, washed, and then cultured with freshly isolated allogeneic T cells from BALB/c mice. Proliferation of T cells in the allogeneic MLR was measured by [<sup>3</sup>H]-thymidine incorporation on the final 16 hr of the culture period of 5 days. Results are expressed as mean c.p.m. of quadruplicate values. \**P* < 0.05 and \*\**P* < 0.01 compared to the unstimulated control.

dependent response was observed for any cytokine tested and the BMMφ recovered from C57BL/6 TLR2<sup>-/-</sup> mice showed similar levels of cytokine secretion to C57BL/6 WT BMMφ. As a control, TDR1 stimulation of both BMMφ cultures showed an overall increase in IL-12p40 and TNF-α in both TLR2-competent and -deficient mice (Table S1). Together, these data suggest that a different type of interplay between SIR2RP1 and the major APCs might occur *in vivo* and probably explain the antibody response observed in TLR2-deficient mice.

## Discussion

We previously reported a role for *Leishmania* SIR2RP1 in host B-cell activation and differentiation.<sup>59</sup> In an attempt to explore the molecular basis of this activity, we decided to evaluate the direct effect of SIR2RP1 on the spleen B-lymphocyte population. In this paper we demonstrated that SIR2RP1 induced TLR2-dependent B-lymphocyte activation and proliferation. The TLR signalling pathway is involved in the initial recognition of *Leishmania* parasites by the innate immune system of the host. It has been suggested that the activation of TLRs leads to early resistance to infection by inducing IFN-γ cellular mediated immunity and helps the development of acquired immunity.<sup>70</sup> In the past few years, several members of the TLR family and their respective adaptor molecules have been identified as key players in the resistance mechanism to a *Leishmania* spp. infection. However, the most convincing data indicating the importance of TLR in resistance to leishmaniasis are those obtained from infections with MyD88-deficient mice. Mice lacking the TLR adap-

tor protein MyD88 have increased susceptibility to infection in comparison with WT C57BL/6 mice, correlated with high levels of IL-4 and low levels of IFN-γ and IL-12.<sup>22</sup> Although the increased susceptibility of MyD88 knockout mice does not necessarily imply a role for TLRs, as members of the IL-1 receptor subfamily also signal through the same pathway, it strongly suggests a contribution of TLR to disease outcome. In this context, experiments with TLR4-deficient mice showed that lack of this receptor led to higher parasite burdens and less efficient resolution of cutaneous lesions caused by the *L. major* strain, which suggests a role for TLR4 in host defence against *Leishmania*.<sup>71</sup> Furthermore, using an RNA interference approach to eliminate the expression of TLRs, it was demonstrated that secretion of nitric oxide (NO) and TNF-α by infected macrophages is, at least in part, TLR2- and TLR3-dependent.<sup>40</sup> All these observations have driven researchers to identify parasite factors that interact with TLRs. De Veer *et al.* and Becker *et al.* have focused their efforts on one of the major constituents of the promastigote surface: LPG.<sup>22,42</sup> This molecule, but not other surface glycolipids, behaves as a TLR2 agonist capable of activating mouse macrophages and human NK cells, in a TLR2- and MyD88-dependent manner.

In several organisms, the role of TLRs in the innate and adaptive immune responses has been examined in detail. The activation of B cells, macrophages and DCs in response to different microbial products has been correlated to the presence of different TLRs on the cell surface, particularly TLR2 and TLR4. For example, structures such as *Neisseria* spp. and *Shigella dysenteriae* porins, macrophage-activating lipopeptide-2 (MALP-2) of *Mycoplasma fermentans* and *Staphylococcus aureus* protein A were shown to stimulate or sensitize B cells via TLR2.<sup>10,72–74</sup> In a general sense, TLR activation leads to the initiation of signalling pathways, which will affect the immune response via the nuclear translocation of the transcription factor NF-κB. The data presented in this study have demonstrated that the *in vitro* stimulation of isolated B cells with SIR2RP1 up-regulated the mRNA level of constitutively expressed TLR2 (Fig. 1a,b). In addition to TLR2, expression of TLR6 but not TLR1 or TLR4 was enhanced, suggesting that co-expression of TLR2 and TLR6 is essential as a combinatory repertoire to detect the SIR2RP1 protein (Fig. 1a,b). Also, the mRNA for MyD88, the major effector molecule associated with the TLR-mediated response in immune cells, and NF-κB were up-regulated in SIR2RP1-stimulated B cells (Fig. 1a,b). The analysis of mRNA levels in B cells after SIR2RP1 stimulation suggests that the signalling pathway may go from the surface receptor TLR2 through the adaptor molecule MyD88, mediating its effects via the transcription factor NF-κB.

Isolated spleen B cells proliferated upon SIR2RP1 stimulation, whereas isolated B cells from TLR2-deficient mice were unresponsive (Fig. 2b). Two major conclusions may

be drawn from this finding. First, SIR2RP1 directly stimulates the proliferation of B cells *in vitro* without the need for accessory cells. Secondly, TLR2 seems to be essential for SIR2RP1-induced B-cell activation. This conclusion was later confirmed by analysis of costimulatory molecule expression at the surface of B cells. The SIR2RP1 stimulus induced up-regulation of MHC II, CD40 and CD86 expression at the B-cell surface, which was abolished in the absence of TLR2 (Table 1). Interestingly, slight differences were observed in the proliferative responses and costimulatory molecule expression of isolated B cells from BALB/c and C57BL/6 mice over the range of LPS concentrations tested (Fig. 2d).

We have previously characterized the *Leishmania* SIR2RP1 as an antigen capable of playing a dual role *in vivo*, being able to act as a T-cell-independent or a T-cell-dependent antigen, which was suggested to contribute to its enhanced immunogenicity.<sup>59</sup> Nevertheless, the mechanism by which this protein is capable of inducing immunoglobulin isotype switching in the presence of T cells but not in their absence (BALB/c mice and BALB. nude mice, respectively) is unknown. Vos *et al.*<sup>75</sup> have proposed that, in immunocompetent mice, some T-cell-independent antigens require a 'second signal' for the development of an IgG response. This could be the case for the SIR2RP1 protein. Considering that TLR2 has an essential role in SIR2RP1 signalling, one might suggest that one or several of these antigen non-specific cells could be among the targets of SIR2RP1, and play a role in isotype switching. Indeed, DCs and macrophages have already been suggested to be involved in this process, through the release of cytokines after activation as well as through the mobilization of T cells and thereby their derived cytokines.<sup>76</sup> Also, the up-regulation of ligands such as B-cell activation factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL), which are expressed in macrophages, DCs and T cells, provides survival signals and also may contribute to class switch recombination in B cells activated by T-cell-independent antigens.<sup>77,78</sup> Indeed, and despite some controversy, TLRs were recently described as essential, in addition to CD4 T-cell help, for the generation of an optimal T-cell-dependent response of B cells to an antigen.<sup>79,80</sup> Therefore, on the one hand, the observed up-regulation of surface molecules, such as MHC class II, CD40 and CD86, on B cells after SIR2RP1 stimulus suggests an enhanced capacity of these cells to present antigen to T cells, which may explain the isotype switching described in immunocompetent mice. It is known that CD86 is expressed as an early response to antigen, well ahead of CD80 expression, which is involved in a delayed response.<sup>81</sup> On the other hand, the essential role of TLR2 in B-cell activation sheds some light on the potential contributions of other APCs, such as DCs and macrophages, either to class switch recombination or even to alternative biological roles of

SIR2RP1. These hypotheses were corroborated by the fact that intravenous injection of SIR2RP1 increases the total number of spleen cells in a greater quantity than that of B cells.<sup>59</sup> In addition, TLR2-deficient mice were able to mount a specific humoral response towards SIR2RP1 after immunization (Fig. 4a). Therefore, other cell populations, such as macrophages and DCs, may act as potential targets of SIR2RP1.

To investigate the possibility of an interaction between APCs and SIR2RP1, we performed complementary *in vitro* experiments with BMDCs and BMM $\phi$  from TLR2-competent (C57BL/6 WT) and -deficient (C57BL/6 TLR2<sup>(-/-)</sup>) mice. SIR2RP1 was capable of directly activating BMDCs, as shown by their up-regulation of costimulatory markers and cytokine secretion (Figs 5 and 6). Similar to B cells, the activation was dependent on the presence of TLR2, as BMDCs derived from TLR2-deficient mice were unresponsive to SIR2RP1 stimulus. Moreover, SIR2RP1 increased the T-cell costimulatory capacity of BMDCs (Fig. 7). In fact, SIR2RP1-activated BMDCs displayed increased allostimulatory activity and enhanced ability to induce the proliferation of naïve T cells in a TLR2-dependent manner. Hence, the stimulatory ability of SIR2RP1 in potentiating the immune functions of DCs can be a powerful tool for the extrinsic manipulation of immune responses. The choice of adjuvant is an important factor influencing the efficacy of any vaccine. In recent years, TLRs have been implicated in the mechanism of adjuvanticity. For example, well-known adjuvants such as monophosphoryl lipid A (MPL), imiquimod and CpG-containing oligodeoxynucleotides (CpG-ODN) produce their effects by mimicking TLR4, TLR7/8 and TLR9 agonists, respectively.<sup>82</sup> Recently, the role of TLR2 in the adaptive immune responses induced by potential vaccine adjuvants has also been directly demonstrated. The most remarkable examples are the *Neisseria* spp. and *S. dysenteriae* porins, which have been suggested to mediate adjuvanticity by activating APCs via TLR2.<sup>10,12,73</sup> In the majority of cases, the capacity to induce B-cell and DC activation via TLR2, along with up-regulation of cell surface costimulatory molecules, confirmed the role of TLR2 agonists as potent adjuvants.<sup>83</sup> These results suggest the use of TLR2 agonists in the improvement of vaccine formulations against intracellular pathogens. Indeed, Wang *et al.*,<sup>43</sup> using an *M. tuberculosis* model of infection, have suggested that the construction of fusion proteins consisting of well-characterized immunogenic proteins and a potent TLR2 agonist is a feasible approach that can be applied to many parasite proteins to provide cheap and effective new vaccines. We have previously demonstrated that an infectious *Leishmania* challenge after SIR2RP1 immunization results in decreased infectivity in the acute phase,<sup>84</sup> which was interpreted to be partially attributable to the secretion of lytic and neutralizing antibodies.<sup>59</sup> Here we have shown that *in vitro* SIR2RP1 stimulation induced several potent leishmanicidal

mechanisms, such as increased IL-12p70 and TNF- $\alpha$  secretion and activation of APCs with increased allostimulatory activity. In the light of these results, it is tempting to speculate about a possible contribution of SIR2RP1 as an adjuvant in a multicomponent vaccine.

The production of SIR2RP1-specific antibodies in C57BL/6 TLR2<sup>(-/-)</sup> mice was rather unexpected, given the dependence of SIR2RP1 activation of B cells and DCs on TLR2. Therefore, we extended our analysis to the macrophage population. BMM $\phi$  stimulated with SIR2RP1 displayed up-regulation of costimulatory molecules CD40 and CD86, but not CD80 or MHC class I or II (Table 1). Also, SIR2RP1 induced the release of pro-inflammatory cytokines, such as IL-12 (both p40 and p70) and TNF- $\alpha$  (Fig. 6). Remarkably, these modifications were independent of TLR2, as BMM $\phi$  recovered from C57BL/6 TLR2<sup>(-/-)</sup> mice showed a similar behaviour to TLR2-competent BMM $\phi$ . These observations might explain the fact that TLR2-deficient mice were able to mount a humoral response to SIR2RP1. Moreover, SIR2RP1-stimulated macrophages displayed a pro-inflammatory cytokine profile with increased IL-12 but no IL-10 secretion, which correlated with a decrease in the ratio of SIR2RP1-specific IgG1:IgG2a compared with TLR2-competent cells (Fig. 4d). It was shown that the ratio of IgG1:IgG2a Abs generally corresponded well with the ratio of Th2:Th1 cytokines, as IL-12<sup>(-/-)</sup> mice produced primarily IgG1 Abs.<sup>85</sup> Thus, the decrease in the IgG1:IgG2a ratio in TLR2-deficient mice could be evidence of SIR2RP1 processing by macrophages in these mice which induced the preferential secretion of IL-12. The different type of interplay found between SIR2RP1 and macrophages, when compared to DCs, suggests that receptors other than TLR2 are involved in SIR2RP1 sensing. In addition to TLRs, many other surface receptors have been proposed to participate in pathogen recognition by innate immune cells. Thus, TLR signalling is subjected to cross-talk from other signals, and such collaborative recognition of distinct pathogen components by different classes of innate immune receptors is crucial in orchestrating inflammatory responses.<sup>86,87</sup> The C-type lectin receptor dectin-1 has been described as an example of a TLR co-operative molecule that is expressed at low levels on macrophages and high levels on DCs.<sup>86</sup> Moreover, it is present on neutrophils, B cells and a subpopulation of T cells.<sup>88</sup> Further studies are needed to investigate the potential role of dectin-1 in the dual APC recognition of SIR2RP1.

In conclusion, all this evidence suggests that TLR2 plays a central but not essential role in SIR2RP1 recognition. This work demonstrates that SIR2RP1-induced proliferation and surface up-regulation of costimulatory molecules on B cells and DCs is TLR2-dependent. In contrast, SIR2RP1 sensing on macrophages is TLR2-independent, suggesting that more than one

surface receptor is involved in SIR2RP1 recognition. Overall, B cell and DC stimulation by TLR2 ligands such as SIR2RP1 seems to be a basic mechanism, which can be exploited to improve the immunogenicity of vaccine formulations.

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## Conflict of interest

The authors have no financial conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** B cells express CD69 in response to TDR1. Expression of CD69 activation marker in spleen cells from BALB/c (A) and purified spleen B cells isolated from BALB/c, C57BL/6 and C57BL/6 TLR2<sup>(-/-)</sup> mice (B) after culture with TDR1. A total of  $2.5 \times 10^5$  cells per well were cultured in the presence of TDR1 (10 µg/ml) or LPS (10 µg/ml) for 20 h. To determine the percentage of CD69 in B (■) cells or CD4 (●) or CD8 (▲) T cells, the different cell populations were positively gated. The results are from a representative experiment of two carried out independently.

**Table S1.** Effect of thiol-dependent reductase 1 (TDR1) on cytokine secretion by isolated spleen B cells, bone marrow-derived macrophages and bone marrow-derived dendritic cells.

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