

A *Brucella* Virulence Factor Targets Macrophages to Trigger B-cell Proliferation^{*S}

Received for publication, January 14, 2013, and in revised form, May 14, 2013. Published, JBC Papers in Press, May 29, 2013, DOI 10.1074/jbc.M113.453282

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Background: PrpA is a *Brucella* B-cell lymphoproliferative virulence factor.

Results: PrpA and its homologue in *Trypanosoma cruzi* bind to macrophages through nonmuscular myosin IIA (NMM-IIA) to trigger B-cell proliferation.

Conclusion: *Brucella* exploits macrophage functions to evade the immune response.

Significance: A bacterial and a protozoan pathogen share a similar immune evasion strategy during infection.

Brucella spp. and *Trypanosoma cruzi* are two intracellular pathogens that have no evolutionary common origins but share a similar lifestyle as they establish chronic infections for which they have to circumvent the host immune response. Both pathogens have a virulence factor (*prpA* in *Brucella* and *tcPrac* in *T. cruzi*) that induces B-cell proliferation and promotes the establishment of the chronic phase of the infectious process. We show here that, even though PrpA promotes B-cell proliferation, it targets macrophages *in vitro* and is translocated to the cytoplasm during the intracellular replication phase. We observed that PrpA-treated macrophages induce the secretion of a soluble factor responsible for B-cell proliferation and identified nonmuscular myosin IIA (NMM-IIA) as a receptor required for binding and function of this virulence factor. Finally, we show that the *Trypanosoma cruzi* homologue of PrpA also targets macrophages to induce B-cell proliferation through the same receptor, indicating that this virulence strategy is conserved between a bacterial and a protozoan pathogen.

Microbial pathogens with the ability to establish chronic infections have evolved sophisticated strategies to subvert the immune response and avoid destruction. Although the number of microorganisms that hamper or avoid the immune system is extremely vast, there are, to date, few examples of how different pathogens (bacterial, viral, or protozoan) achieve this task (1). An accepted framework is that pathogens have evolved strategies to subvert the immune response tipping the equilibrium

between “response” and “nonresponse” of the immune system. This hypothesis proposes that pathogens have achieved a balance consistent with the survival of both the microbe and its infected host by fine-tuning the homeostasis of the latter with no major disturbances (2, 3).

Brucella spp. are Gram-negative facultative intracellular bacteria that cause brucellosis, a worldwide-distributed zoonosis affecting a broad range of mammals including humans. Brucellosis remains a serious problem in many developing countries, causing important economic losses and human health problems (4). The infection, characterized by an initial acute phase with flu-like symptoms, if not treated during this phase, can become chronic and persist over the life span of the host causing a broad range of disorders, in particular osteoarticular complications (5). Because of its lifestyle and its capacity to establish efficient chronic infections, *Brucella* is an interesting model to study immune evasion by a bacterial pathogen. *Trypanosoma cruzi*, the etiological agent of Chagas disease, is a protozoan pathogen belonging to the order Kinetoplastida, that affects more than 10 million humans world-wide and remains the third public health problem in Latin America, especially in developing countries (6). As with *Brucella*, the infection is characterized by an acute phase with vague symptoms and a chronic phase that can appear many years after the primo-infection, causing digestive, neurological, cardiological disorders and even death. Because of their infection strategies, it can be hypothesized that *Brucella* and *T. cruzi* share a common scheme in their infectious strategy: an initial immune evasion phase that allows them to reach secure niches in the host where they can establish long lasting chronic infections.

We have previously described a B-lymphocyte mitogen in *Brucella abortus* (PrpA,⁴ for proline racemase protein A) that induces a transient nonresponsive state of splenocytes, acts as a potent IL-10 inducer, and participates in the efficient establishment of a chronic infection in mice (7). This protein has a homologue in *T. cruzi* that also acts as a T-cell independent B-lymphocyte mitogen required for virulence (8, 9). Both viru-

* This work was supported, in whole or in part, by National Institutes of Health Grant AI078891 from the NIAID (to J. E. U.). This work was also supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) Grant PICT-PRH08-230 (to J. E. U.).

This article is dedicated to the memory of Rodolfo A. Ugalde.

^S This article contains supplemental Figs. 1–5.

¹ Supported by fellowships from the National Research Council of Argentina (CONICET).

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⁴ The abbreviations used are: PrpA, proline racemase protein A; NMM-IIA, nonmuscular myosin IIA.

lence factors are hypothesized to act during the acute phase of the infectious process, inducing a transient nonresponsive state of the immune system which delays or hampers the immune response facilitating the establishment of a chronic infection (7, 10). We report here that PrpA targets macrophages and that it binds to nonmuscular myosin IIA (NMM-IIA) *in vitro* and during the infection of cells and that this binding triggers B-cell proliferation via a yet unidentified soluble factor. Finally we demonstrate that the *T. cruzi* homologue (TcPRAC) also exploits NMM-IIA to bind to macrophages and trigger lymphoproliferation. Altogether, these results indicate that *Brucella* exploits the B-cell response in its own benefit and that a protozoan and a bacterial pathogen target the same protein in macrophages and share a common strategy to subvert the immune response.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*Escherichia coli* strains were grown at 37 °C in LB broth or Terrific broth (11). *Brucella* strains were grown at 37 °C in Bacto Tryptic soy broth (BD Biosciences). When necessary, media were supplemented with the appropriated antibiotics: ampicillin at 100 µg/ml for *E. coli* and 50 µg/ml for *B. abortus* or gentamicin at 4 µg/ml.

Expression of Recombinant PrpA and TcPRAC Proteins—A PCR product encoding the *B. abortus* 2308 *prpA* gene was cloned in-frame from its second codon with an N-terminal His₆ tag and a 3×FLAG epitope into the pQE30 expression vector (Qiagen). The resulting plasmid was named pQE-PrpA-FLAG. TcPRAC from *T. cruzi* was synthetically produced from its second codon with an N-terminal His₆ tag and a 3×FLAG epitope (GenScript) and cloned into the pQE30 expression vector (pQE3×FLAG-TcPRAC). Soluble recombinant proteins were produced in *E. coli* M15 (Qiagen), induced with isopropyl 1-thio-β-D-galactopyranoside (Sigma) and purified to homogeneity by metal affinity chromatography through Ni²⁺ Hi-Trap chelating columns (Amersham Biosciences). After purification, PrpA and TcPRAC were sterilized by filtration through a 0.22-µm membrane, and the protein concentration was determined by the Bradford method (12).

Cell Preparations—For proliferation assays or binding assays, splenocytes were obtained from naïve BALB/c 8–10-week-old females. B- or T-lymphocyte cell suspensions were prepared by depleting total splenocytes with the corresponding Dyna-beads Mouse Pan T (Thy 1.2) or Pan B (B220) monoclonal immunomagnetic kit (DynaL Biotech, Oslo, Norway), and adherent cells were depleted by incubation at 37 °C for 2 h in plastic dishes. Splenic macrophages or dendritic cells were purified using MicroBeads CD11b⁺ or CD11c⁺ (Miltenyi), respectively, according to the manufacturer's instructions. In all cases depletion success was measured by flow cytometry, and presence of the depleted population was <3%. Adherent and nonadherent cell fractions were purified by a 2-h incubation at 37 °C in plastic dishes. Platelet-enriched fractions were obtained by two sequential blood centrifugation cycles at 300 and 900 × g and PBS washings.

Proliferation Assays—Splenocytes from naive mice were subjected to proliferation assays as described by Reina-San-Martin *et al.* (8). Cell suspensions (50 µl) were exposed to 50 µl of

different concentrations of PrpA or TcPRAC for 48 h. Concanavalin A (10 µg/ml) was included as a positive control. For the inhibition of proliferation, cells were incubated for 4 h with 160 or 400 µg/ml anti-NMM-IIA (Biomedical Technologies) or with the same concentration of an isotype-control antibody (rabbit-anti-VP1) prior to addition of PrpA or TcPRAC. After incubation, cells were pulsed for 18 h with 1 µCi/well [³H]thymidine, and [³H]thymidine uptake was determined in a liquid scintillation counter (LKB-Wallac). Heat inactivation of PrpA was performed at 80 °C for 15 min. All determinations were performed in triplicate and expressed in cpm. The corresponding S.D. values were calculated.

Immunoprecipitations—J774 A.1 cells were treated with RPMI 1640 medium or PrpA (50 µg/ml) for 24 h (samples). Cells were then washed, lysed in radioimmune precipitation assay buffer, immunoprecipitated with 5 µg of a monoclonal M2-anti-FLAG (Sigma), resuspended in Laemmli buffer, subjected to SDS-PAGE, and silver stained. Differential bands were subjected to MS²-peptide identification and analyzed using MASCOT database search algorithm. For the reverse assay, samples were immunoprecipitated with 5 µg of polyclonal anti-NMM-IIA and immunoblotted with M2-anti-FLAG to detect PrpA. For the *in vivo* immunoprecipitations, J774 A.1 cells were infected with *B. abortus* 2308 or *B. abortus prpA-flag* for 48 h (multiplicity of infection of 1:1500), then lysed, filtered to discard bacteria, immunoprecipitated with 5 µg of a monoclonal M2-anti-FLAG, and subjected to Western blotting using a monoclonal anti-NMM-IIA antibody (Abnova).

Binding Assays—Cells were stimulated for 2, 4, or 24 h with PrpA or TcPRAC (5 µg/ml), washed three times with PBS, resuspended in Laemmli buffer, and subjected to SDS-PAGE. For inhibition of binding, cells were pretreated with 25 or 125 µg/ml anti-NMM-IIA or the same concentration of an isotype control antibody for 2 h, prior to the addition of PrpA or TcPRAC. Proteins were transferred onto nitrocellulose membranes using semi-dry transfer. Immunoblotting was performed using monoclonal M2-anti-FLAG antibodies.

Immunofluorescence Assays for Microscopy—10⁵ J774 A.1 cells were plated on coverslips for 24 h in 500 µl of RPMI 1640 medium supplemented with 5% FBS (Invitrogen). 500 µl of 50 µg/ml PrpA was added to the cells for 5 min and washed with PBS, and the cells were fixed for 15 min in 3% paraformaldehyde (pH 7.4) at 37 °C. Coverslips were then processed for immunofluorescence labeling with a mouse M2-anti-FLAG and rabbit-anti-NMM-IIA as primary antibodies. Anti-mouse Alexa Fluor 488 (green) and anti-rabbit Alexa Fluor 568 (red) were used as secondary antibodies. For DNA staining, Hoechst dye at 2 µg/ml (final concentration) was used. After immunofluorescence labeling, the coverslips were mounted onto slides with FluorSave (Calbiochem). Samples were examined on a Nikon microscope (TE 2000) for image acquisition. Images of 1024 × 1024 pixels were then assembled using Adobe Photoshop CS. For cytometry 10⁶ cells were blocked 30 min in PBS with 10% horse serum in 100 µl on ice. Antibodies were added in concentrations described by manufacturers for 30 min on ice. Anti-mouse F4/80-Alexa Fluor 488, anti-mouse CD11b-PE, anti-mouse CD86-PE, and isotype controls were purchased at Biolegend. Cells were then washed with cold PBS and fixed in

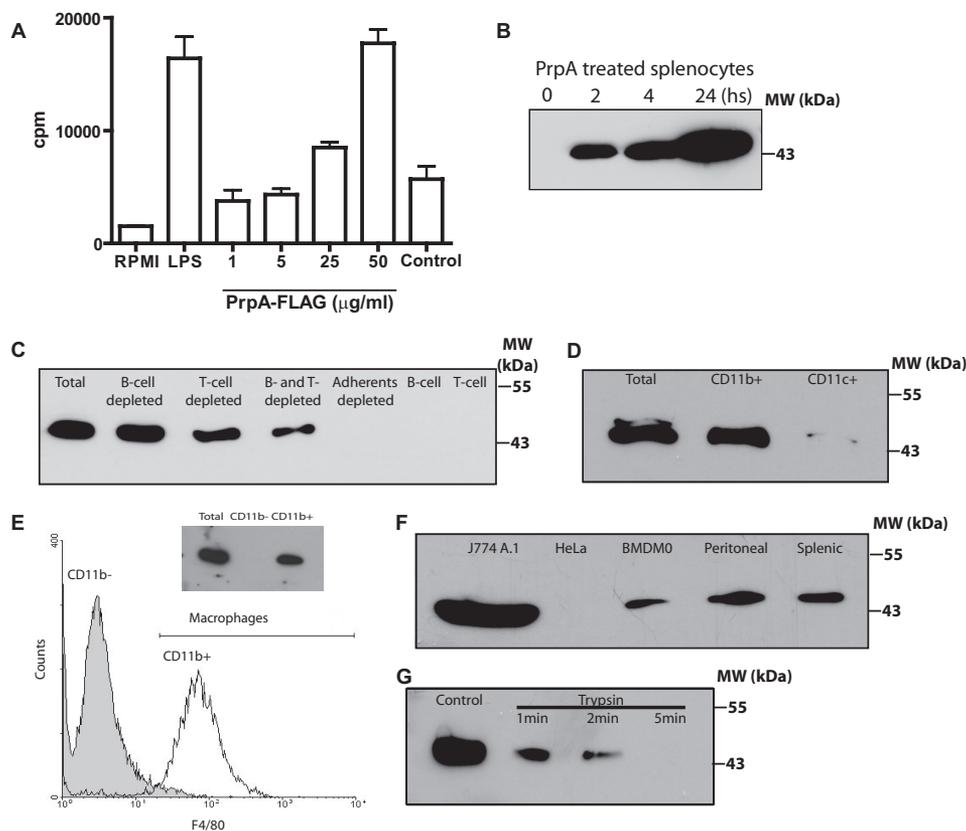


FIGURE 1. PrpA binds to macrophages. *A*, lymphoproliferative activity of the FLAG-tagged version of PrpA. Total mice splenocytes were incubated with increasing amounts of the recombinant protein, and proliferation was measured by [3 H]thymidine incorporation as described in Ref. 7. *Control*, heat-inactivated PrpA-FLAG. A one-way analysis of variance was performed to compare columns treated with 5, 25, and 50 μ g of PrpA, $p < 0.001$. *B*, binding of PrpA to total splenocytes at 2, 4, and 24 h after addition. Total mice splenocytes were incubated with 5 μ g/ml PrpA; and at 2, 4, or 24 h cells were pelleted, extensively washed, submitted to SDS-PAGE, and Western blotting was performed with a monoclonal anti-FLAG. *Lane 0*, no PrpA added. *C*, PrpA binding assay using total splenocytes, B-cell depleted splenocytes, T-cell depleted splenocytes, B- and T-cell depleted splenocytes, adherent depleted splenocytes, or purified B or T lymphocytes. *D*, PrpA binding assay using total splenocytes or purified CD11b $^+$ or CD11c $^+$ cells. *E*, cells extracted from the peritoneum and treated with PrpA. The CD11b $^+$ cells were purified with a column, and the flow-through and the retained cells were stained with an anti-F4/80 antibody and analyzed by flow cytometry. The *inset* shows a Western blot with an anti-FLAG of both CD11b $^+$ and CD11b $^-$ populations. *F*, PrpA binding assay on J774 A.1 cells, HeLa cells, bone marrow-derived macrophages, peritoneal adherent cells, or splenic adherent cells. *G*, J774 A.1 cells incubated with PrpA for 24 h followed by incubation with trypsin for 1, 2, and 5 min. All assays were developed by Western blotting using a monoclonal anti-FLAG antibody (see "Experimental Procedures").

3% paraformaldehyde and analyzed by FACS in a CyFlow Space-Partec (Germany).

Translocation Assay—Translocation of PrpA from the bacterial to the macrophagic cytoplasm was determined as described in (13). Briefly, the *cya* domain of *Bordetella pertussis* or fusions of *prpA* to *cya* in the N or C terminus were cloned in pDK51 (14) and introduced into *B. abortus* 2308. These strains were used to infect J774 A.1 cells, and, 5 h after infection, the intracellular levels of cAMP were determined by ELISA (direct cAMP enzyme immunoassay kit; Sigma-Aldrich) according to the manufacturer's specifications.

RESULTS

PrpA Binds to Macrophages Inducing the Secretion of Soluble Factors that Trigger B-cell Proliferation—We have previously demonstrated that upon treatment of splenocytes, PrpA induces B-cell proliferation (7). Although the B-cell population is the only one that proliferates upon PrpA treatment of total splenocytes we asked which cell types PrpA actually targets in the splenocyte population. To answer this we produced a carboxyl-terminal FLAG-His $_6$ -tagged version of PrpA and corroborated that this fusion protein was capable of inducing B-cell

proliferation (Fig. 1*A*). As described under "Experimental Procedures," we designed an assay to study PrpA binding kinetics and to determine its cell target. Total splenocytes were treated with PrpA (5 μ g/ml) for 2, 4, or 24 h, washed, and subjected to a SDS-PAGE and immunoblotting with an anti-FLAG monoclonal antibody. As observed in Fig. 1*B*, PrpA bound to total splenocytes in a time-dependent manner. Due to experimental practicality and sensitivity, we used a 24-h treatment for the following experiments. To determine which cell subpopulation PrpA targets, total splenocytes were depleted of B-cells, B- and T-cells or adherent cells, and the resulting cell populations were subjected to SDS-PAGE and immunoblotting with anti-FLAG. As observed in Fig. 1*C*, PrpA bound to total, B-cell depleted, T-cell depleted, or B- and T-cell depleted splenocytes but not to B- or T-cells alone, indicating that the mitogen target cell is not a lymphocyte. However, PrpA did not bind when splenocytes were depleted of adherent cells (dendritic cells and macrophages). To determine whether PrpA targets dendritic cells (CD11c $^+$) or macrophages (CD11b $^+$), adherent cells were treated for 24 h with PrpA (5 μ g/ml) and washed, and both cell populations were purified by immunomagnetic antibodies

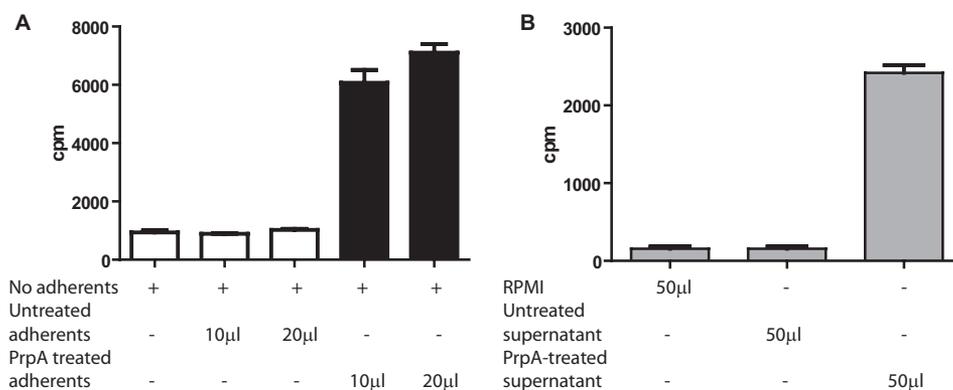


FIGURE 2. The PrpA-macrophage interaction triggers B-cell proliferation. *A*, proliferation assay of adherent depleted splenocytes (*No adherents*) co-cultured with adherent cells treated or not with PrpA. *B*, proliferation assays of purified splenic B-cells in the presence of filtered conditioned media of adherent cells treated or not with PrpA.

against their surface markers and submitted to a SDS-PAGE and immunoblotting with anti-FLAG. As shown in Fig. 1*D*, PrpA bound preferentially to CD11b⁺ cells, strongly suggesting that macrophages could be its target. To confirm this, we purified peritoneal CD11b⁺ cells and determined the presence of another macrophagic surface marker, F4/80, by flow cytometry. As can be seen in Fig. 1*E*, PrpA bound to the CD11b⁺ population extracted from the peritoneum, and this population was also F4/80-positive. Fig. 1*F* shows that PrpA was also capable of binding to cells from J774 A.1 macrophagic cell line, bone marrow-derived macrophages, primary splenic macrophages, but not to HeLa cells. **Supplemental Fig. 1** shows the surface expression of macrophagic markers CD11b and F4/80 on the bone marrow-derived macrophages.

To determine whether PrpA remains extracellularly bound to the membrane of treated cells, we added the protein to J774 A.1 cells, incubated them for 24 h, and washed and treated the cells with trypsin for 1, 2, and 5 min. As can be observed in Fig. 1*G*, PrpA remained bound to cells for 24 h and was degraded upon trypsin treatment, indicating that it was not internalized. This assay was also performed on total splenocytes with the same results (**supplemental Fig. 2**). Altogether these results demonstrate that PrpA binds to macrophages, suggesting that its mitogenic effect on B-cells might be indirect.

Binding of PrpA to macrophages could result in their activation. To test this, we analyzed the surface expression of CD86 as a marker for macrophage activation. Bone marrow-derived macrophages were treated with culture medium (RPMI 1640 medium), PrpA (50 μg/ml), *E. coli* LPS (2 μg/ml), or heat-inactivated PrpA (50 μg/ml) for 24 h, and CD86 surface expression was analyzed by flow cytometry. As shown in **supplemental Fig. 3**, PrpA and LPS induced similar levels of CD86 expression, suggesting that PrpA might be activating its target cell.

To determine whether the PrpA-treated macrophages were able to trigger B-cell proliferation, we separated adherent cells from total splenocytes, treated them with PrpA, and, after 24 h of incubation, washed, co-cultured with the nonadherent splenocyte fraction (lymphocytes), and measured proliferation. As shown in Fig. 2*A*, PrpA-treated adherent cells *per se* were able to induce lymphocyte proliferation. Because PrpA only binds to macrophages (Fig. 1), these results indicate that the signals necessary to trigger B-cell proliferation are originated

from macrophages. To determine whether signaling from the PrpA-treated macrophages to the B-cells requires contact between the two cell types or whether the signal is a soluble factor, we treated splenic adherent cells with PrpA for 24 h, collected and filtered the supernatant (conditioned media), treated purified B-cells for 24 h, and measured proliferation. As shown in Fig. 2*B*, the conditioned media induced B-cell proliferation, demonstrating that the signal is a soluble factor secreted by macrophages.

PrpA Interacts with NMM-IIA in Macrophages in Vitro and in Vivo—To gain further insight into the molecular mechanism used by PrpA to trigger B-cell proliferation, we went on to identify the molecular target of PrpA on the macrophages. Because PrpA binds to cells from the J774 A.1 macrophagic cell line, we treated them with the FLAG-tagged PrpA for 24 h and immunoprecipitated the protein with an anti-FLAG monoclonal antibody (see “Experimental Procedures”). The immunoprecipitated proteins were subjected to SDS-PAGE and silver staining. The differential protein bands compared with the nontreated negative control were identified by MALDI-TOF. As shown in Fig. 3*A*, a major band of ~225 kDa co-immunoprecipitated with PrpA and was identified as NMM-IIA. Additionally, a second protein of ~42 kDa was identified as β-actin. This is not surprising because the interaction between NMM-IIA and actin has been extensively described (15–19). To confirm the PrpA-NMM-IIA interaction, PrpA-treated J774 A.1 cells were immunoprecipitated using a commercial anti-NMM-IIA polyclonal antibody (Biomedical Technologies). As can be observed in Fig. 3*B*, PrpA co-immunoprecipitated with NMM-IIA, confirming that both proteins interact, although this did not indicate whether it occurred on the surface of the cell. To address this, we performed immunofluorescence staining of nonpermeabilized PrpA-treated J774 A.1 cells to determine the localization of NMM-IIA and PrpA. As shown in Fig. 3*D*, and consistent with the trypsin experiment, PrpA and NMM-IIA co-localized in the membrane of J774 A.1 cells.

Because platelets express high levels of NMM-IIA (20) we tested whether PrpA binds to them. For this, we isolated platelets from mice and performed a binding assay with 10⁵ cells. As observed in Fig. 3*C*, PrpA also bound to platelets, further supporting our previous results.

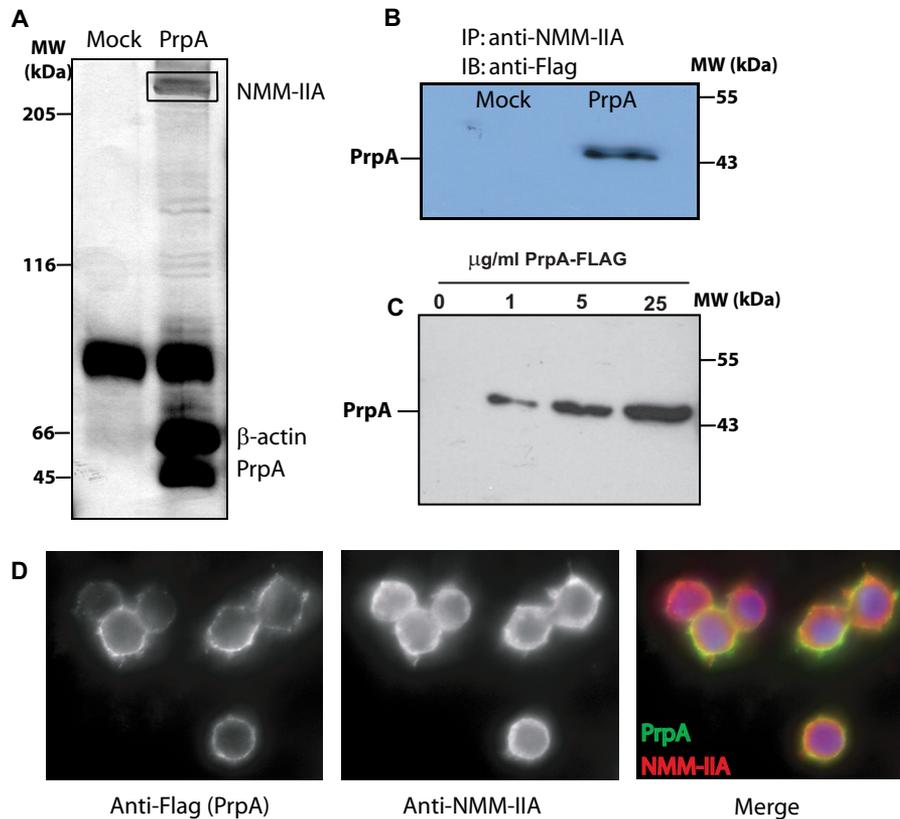


FIGURE 3. PrpA interacts with NMM-IIA on the membrane of macrophages. *A*, silver-stained SDS-PAGE of PrpA co-immunoprecipitated proteins of J774 A.1 cells treated with PrpA. The protein bands were identified by MALDI-TOF. *B*, Western blot (IB) with a monoclonal anti-FLAG antibody of PrpA-treated J774 A.1 cells immunoprecipitated (IP) with a polyclonal anti-NMM-IIA. *Control lane*, nontreated J774 A.1 cells. *C*, PrpA binding assay to platelets using 1, 5, and 25 μg of protein. *D*, immunofluorescence of nonpermeabilized PrpA-treated J774 A.1 cells. *Green*, anti-FLAG (PrpA); *red*, anti-NMM-IIA.

Although exogenously added PrpA binds to NMM-IIA in macrophages, it does not necessarily mean that they interact during the infectious process. Moreover, for this interaction to occur, PrpA has to be secreted from the bacteria and translocated into the host cells during infection. To determine this we used the Cya reporter fusion approach that has already been used for the identification of translocated effectors proteins in *B. abortus* (13) and in other systems (21, 22, 23). We constructed a reporter fusion of *prpA* to the *B. pertussis* adenylate cyclase (*cya*) in a plasmid, introduced it in *B. abortus* wild type and infected J774 A.1 cells. By measuring the total concentration of cyclic AMP (cAMP) in the cells infected with the strain carrying this construct in comparison with the one carrying only the *cya* domain, the system allows the detection of translocation events because the reporter is only active in the cytoplasm of the eukaryotic cell (21). As shown in Fig. 4A, the fusion of *prpA* to either the carboxyl or the amino terminus of *cya* promoted the translocation of its protein product into the host cells, demonstrating that *Brucella* secretes and translocates PrpA during the infectious process. Secretion from the bacteria and translocation into the host cells of PrpA does not mean that the protein will interact with NMM-IIA. To test whether both proteins interact during the intracellular stage of the infection, we infected J774 A.1 cells with the wild type *B. abortus* 2308 or a strain carrying a plasmid expressing a *prpA*-FLAG and, 48 h after infection, the cells were lysed, and PrpA was immunoprecipitated with a monoclonal anti-FLAG antibody. The immu-

noprecipitated proteins were subjected to SDS-PAGE, and Western blotting carried out with a monoclonal anti-NMM-IIA antibody. As can be observed in Fig. 4B, NMM-IIA immunoprecipitated only in the cells infected with the strain expressing the FLAG-tagged PrpA, demonstrating that both proteins interact also during the course of the infection.

Binding of PrpA to Macrophages and B-cell Proliferation Are Inhibited by NMM-IIA Neutralization—To determine whether the PrpA-NMM-IIA interaction plays a physiological role in the binding of the protein to macrophages, we initially tested whether neutralization of NMM-IIA with a polyclonal antibody could block binding of PrpA to J774 A.1 cells. To address this we performed a binding assay with cells pretreated with 25 and 125 $\mu\text{g}/\text{ml}$ polyclonal anti-NMM-IIA for 4 h or with the same concentrations of a nonspecific antiserum as a negative control. As can be observed in Fig. 5A, neutralization of NMM-IIA significantly reduced the attachment of PrpA in a dose-dependent manner, indicating that this interaction is required for an efficient binding. **Supplemental Fig. 4** shows that PrpA binding to platelets was also NMM-IIA-dependent. **Supplemental Fig. 5** shows that blockade of NMM-IIA in primary peritoneal macrophages also inhibited PrpA binding.

Because neutralization of NMM-IIA resulted in an inhibition of PrpA binding to macrophages, we asked whether this blockade could also inhibit the PrpA lymphoproliferative activity. As shown in Fig. 5B, pretreatment of mice splenocytes with 400 $\mu\text{g}/\text{ml}$ polyclonal anti-NMM-IIA for 4 h significantly reduced

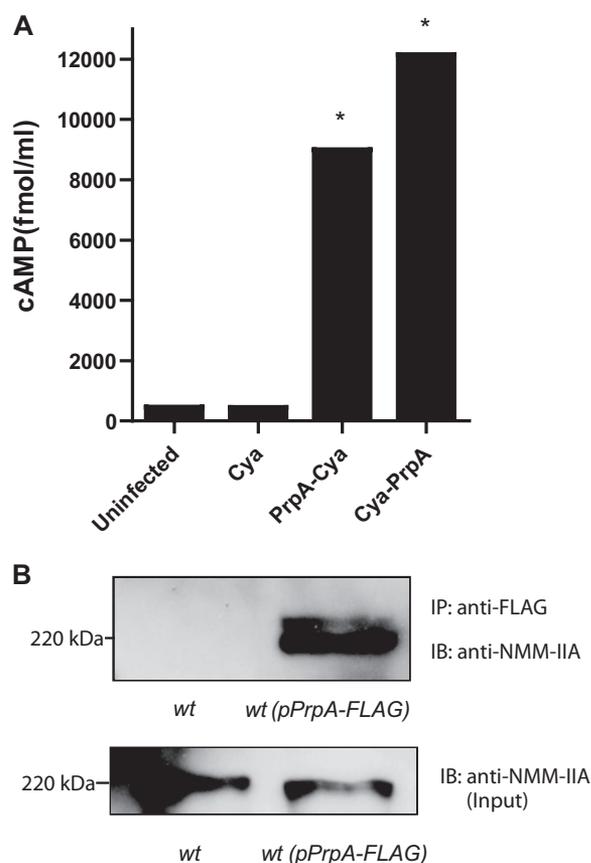


FIGURE 4. PrpA is translocated to the host cell during infection and interacts with NMM-IIA. *A*, cAMP determination of J774 A.1-infected cells with *B. abortus* wild type strains carrying a *prpA-cya*, a *cya-prpA* fusion, or *cya* alone cloned in pDK51 (see “Experimental Procedures”). The increase in the intracellular cAMP concentration indicates translocation of Cya into the cell cytoplasm. *B*, immunoprecipitation (IP) of PrpA-FLAG from infected J774 A.1 cells with either *B. abortus* carrying a plasmid coding a *prpA-FLAG* construct or with no plasmid. The immunoprecipitated proteins were subjected to a SDS-PAGE and Western blotting (IB) with a monoclonal anti-NMM-IIA antibody.

cellular proliferation. Altogether, these results demonstrate that PrpA-NMM-IIA interaction is necessary for the protein to bind to macrophages and to elicit its B-cell mitogenic activity, although the possibility that other proteins could also be necessary for these processes is not ruled out.

Trypanosoma cruzi prpA Homologue Binds to Macrophages and Triggers B-cell Polyclonal Proliferation in a NMM-IIA-dependent Manner—Because the homologue of PrpA in *T. cruzi* (TcPRAC) also induces T-cell independent B-cell proliferation (8), we asked whether the mechanisms used by both proteins could be conserved, this is, whether they both target the same cellular type and the same surface protein. To answer this, we produced the FLAG-tagged *T. cruzi* PrpA homologue recombinant protein (TcPRAC) and determined whether it was capable of binding to J774 A.1 cells in a NMM-IIA-dependent manner. As can be seen in Fig. 6A, TcPRAC was capable of binding to J774 A.1 cells, and this binding was inhibited in a dose-dependent manner by neutralization of NMM-IIA with a polyclonal antibody. To determine whether this inhibition also impaired the lymphoproliferative activity of TcPRAC, we performed a total splenocyte proliferation assay in the presence of the polyclonal anti-NMM-IIA. Fig. 6B shows that blockade of

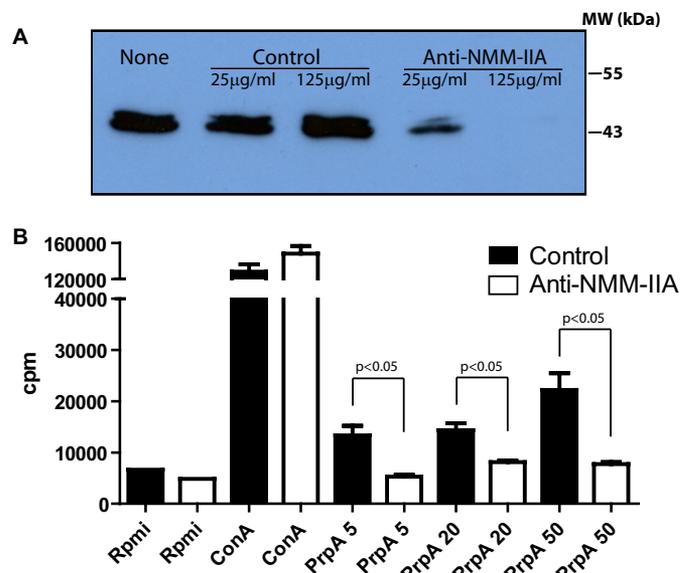


FIGURE 5. NMM-IIA neutralization inhibits PrpA binding to macrophages and B-cell proliferation. *A*, Western blot to determine PrpA bound to J774 A.1 cells after a 2-h preincubation with 25 and 125 μg/ml anti-NMM-IIA, the same concentrations of a rabbit hyperimmune serum against a nonrelated protein (control), or without preincubation (None). *B*, proliferation assays of total splenocytes with 5, 20, or 50 μg/ml PrpA preincubated with 400 μg/ml polyclonal anti-NMM-IIA or the same concentration of the control antibody. *Rpmi*, no PrpA added; *ConA*, concanavalin A.

NMM-IIA with polyclonal antibodies inhibited the TcPRAC mitogenic activity. These results demonstrate that, as with PrpA, protein-binding inhibition significantly reduced the proliferation activity, indicating that both proteins share a common mechanism.

To further confirm that PrpA and TcPRAC target the same surface structures we designed a competition assay between both proteins. For this, we treated J774 A.1 cells with 10 μg/ml of either PrpA-FLAG or TcPRAC for 2 h, washed and incubated with 10 or 50 μg/ml of the other protein for 4 h, washed again, and performed Western blotting with a monoclonal anti-FLAG antibody as previously described to determine which protein remained bound to the cells. Because both proteins have different molecular masses, they can be identified according to their mobility. Fig. 6C shows that PrpA and TcPRAC displace each other in these experiments, further confirming that they share the same receptors and additionally demonstrating that the binding of both proteins is reversible.

DISCUSSION

Chronic pathogens such as *Mycobacterium*, *Leishmania* spp., trypanosomes, *Salmonella typhi*, HIV, and *Brucella* spp. are interesting models to study immune evasion mechanisms. Additionally, because many of these microorganisms have a profound health impact (usually in developing countries), the identification and characterization of their virulence strategies are central for the development of novel therapeutic tools that could alleviate the burden of these diseases.

Brucella and *T. cruzi* cause chronic infections that can persist in their hosts (mammals in both cases) for their remaining life span. This amazing ability to survive in the face of an active immune response shows the immune modulation capacity of

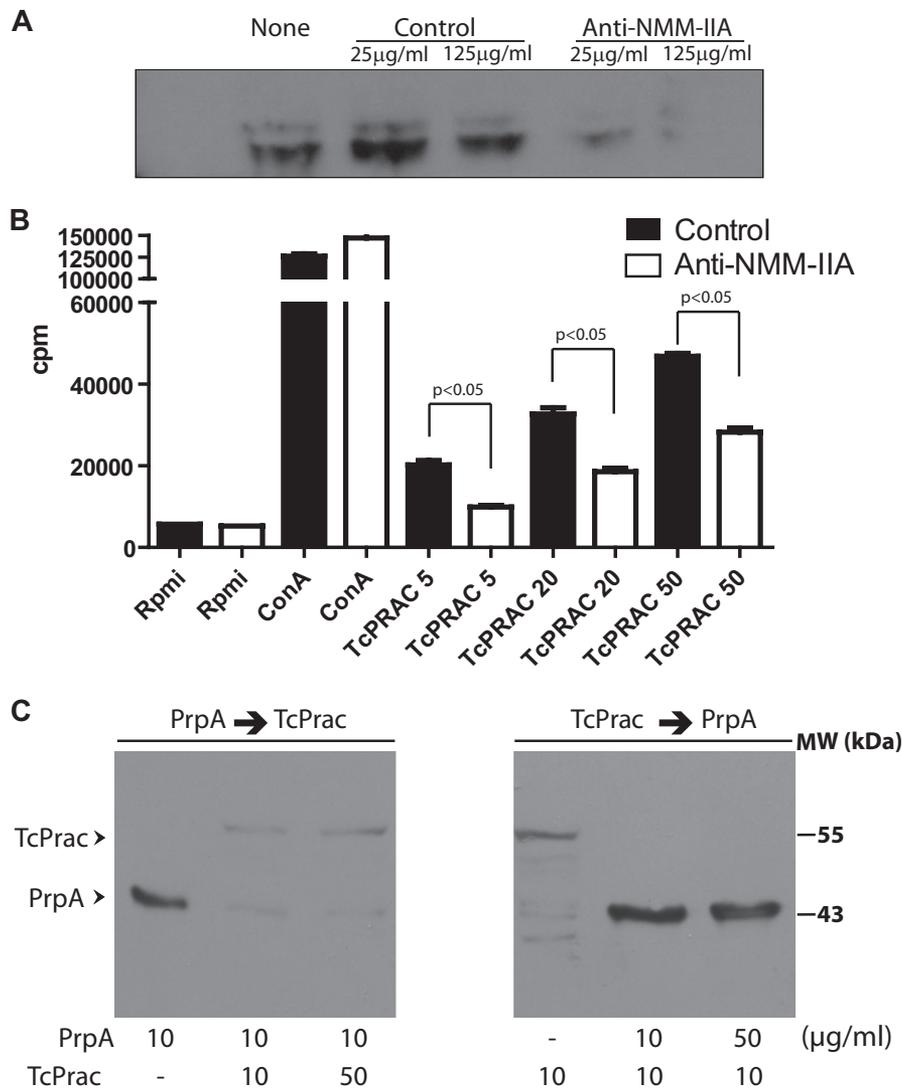


FIGURE 6. **NMM-IIA neutralization inhibits TcPRAC binding to macrophages and B-cell proliferation.** *A*, Western blot to determine TcPRAC bound to J774 A.1 cells after a 2-h preincubation with 25 and 125 μg/ml anti-NMM-IIA antibody, the same concentrations of a rabbit hyperimmune serum against a nonrelated protein (control), or without preincubation (*None*). *B*, proliferation assays of total splenocytes with 5, 20, or 50 μg/ml TcPRAC preincubated with 400 μg/ml polyclonal anti-NMM-IIA or the same concentration of the control antibody. *Rpmi*, no TcPRAC added; *ConA*, concanavalin A. *C*, PrpA and TcPRAC compete for the same receptor. Western blot with anti FLAG of J774 A.1 cells sequentially treated with PrpA-FLAG and TcPRAC-FLAG (or vice versa) is shown.

these pathogens and, although the general virulence properties of these microorganisms are completely different, allows us to speculate that some features of their immune evasion strategies might be conserved.

In this report we have further advanced in the molecular characterization of a virulence factor of *B. abortus* (PrpA) that induces a transient anergic state of the immune system and participates in the establishment of a chronic infection (7). This protein has hydroxyproline racemase activity (24), induces T-cell-independent B-cell proliferation, and is homologous to a B-lymphocyte mitogen of *T. cruzi* that is also involved in virulence (8, 24, 25). A hallmark of *T. cruzi* infection is the polyclonal B-cell proliferation observed during the acute phase that is accompanied by a strong hypergammaglobulinemia (26, 27). Here we report that although PrpA induces B-cell proliferation, it does not bind to B-lymphocytes. Instead, we showed that PrpA acts indirectly by binding to CD11b⁺F4/80⁺ macrophages and inducing them to release one or more soluble fac-

tors responsible for B-cell proliferation. We have additionally identified, by co-immunoprecipitation assays, a putative macrophage target of PrpA. We found that surface-exposed NMM-IIA interacts with PrpA on the membrane of macrophages and that neutralization of NMM-IIA with a polyclonal antibody inhibits binding of PrpA and its B-cell lymphoproliferative activity, suggesting that NMM-IIA could be a PrpA receptor or at least an adaptor protein necessary to engage the functional immune receptor. Moreover, we have demonstrated that PrpA is translocated into the host cell during infection and that it interacts with NMM-IIA during the course of the infection. NMM-II functions as a heterohexamer composed by two heavy chains and two pairs of light chains. The heavy chain possesses enzymatic activity and utilizes ATP to drive actin filament movement. Three isoforms, A, B, and C, of mammalian NMMII have been identified to date that are widely expressed in different tissues and have 64–80% amino acid identity (28). Despite these similarities, they are not function-

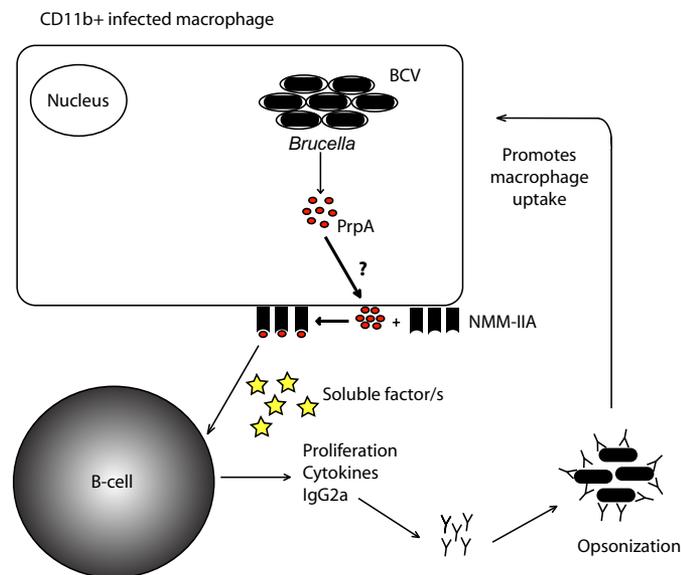


FIGURE 7. Model proposed for the PrpA-dependent B-lymphocyte proliferation. Intracellular *Brucella* secretes and translocates PrpA that traffics to the membrane and engages NMM-IIA. This binding triggers a signal in the macrophages promoting the secretion of soluble factors that induce B-cell proliferation.

ally redundant and appear to have different roles in cell motility, cytokinesis, regulation of cell shape, and intracellular vesicular traffic (29, 30). Although the members of this family are mainly cytoplasmic, there have been many reports indicating that NMM-IIA is also found in the cell membrane, facing the extracellular space (31, 32). NMM-IIA has been recently demonstrated to be a functional receptor for herpesvirus (32), thus opening the possibility of a new function for this protein in the surface of the cell. To our knowledge this is the first report demonstrating the presence of NMM-IIA on the membrane of macrophages and more importantly, its role in triggering polyclonal B-lymphocyte proliferation. Because it is a common strategy of pathogens to hijack physiological signaling pathways of their hosts, it raises interesting questions about the function the macrophage-membrane NMM-IIA may play in the immune system. Are there other components in the membrane necessary for the signal? Which is the signaling cascade triggered in macrophages? Which are the soluble factors secreted by the macrophage that trigger the polyclonal B-lymphocyte proliferation? Preliminary results using neutralizing antibodies indicated that these soluble factors are not IL-10, B-cell-activating factor, or IL-6. Interestingly, many vertebrates, including humans, have genes that code for putative proteins highly similar to PrpA. Maybe these genes play a role in modulating the immune response.

Fig. 7 shows a scheme of the model we propose with the data presented in the present work. In this model *Brucella* secretes and translocates PrpA during its intracellular stage in macrophages and engages NMM-IIA in the surface of the cell, triggering the release of a soluble factor responsible for B-cell proliferation and antibody and cytokine production,⁵ ultimately promoting infection. At this stage we do not know whether the

translocated PrpA traffics to the cell surface and interacts with NMM-IIA or whether this interaction occurs in the cytoplasm and they traffic together as a complex to the cell surface where they trigger the response. If the latter is the case it could be plausible that NMM-IIA functions as a cargo or an adaptor protein for PrpA that is necessary to engage an immune receptor that ultimately triggers the macrophage response. Work is currently in progress to discern between these possibilities.

Finally, to broaden the results of PrpA to its homologue in *T. cruzi* (TcPRAC), we demonstrated that it also binds to macrophages and that this binding, as well as its B-cell proliferative activity, is NMM-IIA-dependent. We have additionally shown that PrpA and TcPRAC compete for the same receptors. The fact that two completely different pathogens (a bacterial and a protozoan pathogen) have evolved a common strategy to modulate the immune response each in its own benefit suggests that the pathway they exploit might be important in the immune regulation in mammals. More studies, including the elucidation of the signaling triggered by macrophages and propagated to B-lymphocytes, must be performed to understand this new pathway.

Acknowledgments—We thank Alberto Carlos Frasch and Jean-Marc Cavaillon for critical reading of the manuscript and useful suggestions.

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A *Brucella* Virulence Factor Targets Macrophages to Trigger B-cell Proliferation

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J. Biol. Chem. 2013, 288:20208-20216.

doi: 10.1074/jbc.M113.453282 originally published online May 29, 2013

Access the most updated version of this article at doi: [10.1074/jbc.M113.453282](https://doi.org/10.1074/jbc.M113.453282)

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