Brucella Cyclic β-1,2-Glucan Plays a Critical Role in the Induction of Splenomegaly in Mice

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Abstract

Brucella, the etiological agent of animal and human brucellosis, is a bacterium with the capacity to modulate the inflammatory response. Cyclic β-1,2-glucan (CβG) is a virulence factor key for the pathogenesis of Brucella as it is involved in the intracellular life cycle of the bacteria. Using comparative studies with different CβG mutants of Brucella, cgs (CβG synthase), cgt (CβG transporter) and cgm (CβG modifier), we have identified different roles for this polysaccharide in Brucella. While anionic CβG is required for bacterial growth in low osmolarity conditions, the sole requirement for a successful Brucella interaction with mammalian host is its transport to periplasmic space. Our results uncover a new role for CβG in promoting splenomegaly in mice. We showed that CβG-dependent spleen inflammation is the consequence of massive cell recruitment (monocytes, dendritic cells and neutrophils) due to the induction of pro-inflammatory cytokines such as IL-12 and TNF-α and also that the reduced splenomegaly response observed with the cgs mutant is not the consequence of changes in expression levels of the characterized Brucella PAMPs LPS, flagellin or OMP16/19. Complementation of cgs mutant with purified CβG increases significantly spleen inflammation response suggesting a direct role for this polysaccharide.

Introduction

Brucella is a bacterial pathogen that infects ruminants as the primary host and can be transmitted to humans by consumption of animal-derived contaminated products (e.g. unpasteurized dairy food) leading to brucellosis, a reticular endothelial disease, typically characterized by undulant fever [1]. Brucella, as the result of a longstanding association with the mammalian host, has evolved modified PAMPs (pathogen-associated molecular patterns molecules) such as LPS and flagellin. These modified PAMPs limit host recognition by innate immune receptors (TLRs and NLRs) during the acute phase of the infectious process, reducing the induction of the inflammatory response known to be necessary for an efficient control of the infection [2–4]. In addition Brucella expresses virulence factors that actively interfere with the innate immune response such as the Brucella proteins Btp-A/TcpB and BtpB that down-regulate TLR2/TLR4 signaling [5–7]. Paradoxically, when the host is persistently infected at the chronic phase of brucellosis, inflammation becomes a dominant clinical sign that has been described to affect several organs producing symptoms such as arthritis, endocarditis, meningitis, epididymitis and splenomegaly [8].

To date few virulence mechanisms have been described in Brucella infection and different genomic studies have confirmed the absence of classical virulence factors like fimbriae, pilli, toxins or plasmids [9,10]. In our laboratory, we have identified and characterized a critical molecule for Brucella virulence: the cyclic β-1,2-glucan (CβG). CβG is composed by a family of cyclic polymers of 17 to 25 D-glucose molecules linked by β-1,2 linkages, synthesized by a membrane bound enzyme named Cgs (for cyclic glucan synthase) that utilizes UDP-glucose as the sugar donor [11]. Cgs initiates and elongates a linear chain of glucose covalently bound to the Cgs which is subsequently cyclated and released to the bacterial cytoplasm [12]. A specific Brucella ABC-transporter Cgt (for Cyclic glucan transporter) translocates the CβG to the periplasmic space where they become chemically modified with the addition of succinyl groups, a reaction catalyzed by the membrane enzyme Cgm (for Cyclic glucan modifier) [13,14]. Although CβG is present within the periplasmic space, being this localization critical for hypo-osmotic adaptation, secretion of large amount of this polysaccharide to the supernatant by a non-characterized mechanism has been described in Agrobacterium and Sinorhizobium [15]. Interestingly, Brucella cgs mutant strain presents a defect in intracellular trafficking in epithelial cells that can be complemented by the addition of purified CβG (or Cyclodextrins).
to tissue culture medium [16]. This observation suggests that also in *Brucella*, CβG must be secreted within the host cell to exert its role in virulence. The proposed mechanism of action for CβG in *Brucella* infection is the sequestration of cholesterol from intracellular host membranes leading to lipid raft disorganization and modulation of intracellular trafficking [16].

We have previously observed that mice infected with a cgs mutant had a reduced spleen inflammatory response even though they had a high number of replicating bacteria within this organ [17]. Since inflammation is the consequence of the host induction of microbial PAMPs that ultimately lead to the induction of an inflammatory process, we hypothesized that either the periplasmic CβG may be stabilizing the expression of *Brucella* PAMPs such as LPS, flagellin, and OMPs or that CβG could be recognized by the innate immune receptors in the context of *Brucella* infection triggering inflammation.

It has been shown recently, using in vitro studies and purified CβG, that this molecule acts directly as an agonist of the innate immune system mediated by TLR4 in a MyD88/TRIF dependent fashion (and in a CD14 independent manner) [18]. Here we describe the role of *Brucella* CβG in splenomegaly and inflammation.

**Materials and Methods**

**Ethics statement**

The protocol of this study (reference number 10/2011) was approved by the Committee on the Ethics of Animal Experiments of the Universidad Nacional de San Martin, which also approved protocol development under the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

**Growth of *B. abortus***

Bacterial strains used in this study were: *Brucella abortus* strain 2308, *Brucella abortus* strain S19, *Brucella abortus* cgs08 mutant, *Brucella abortus* cgs19 mutant [17]; *Brucella abortus* cgt19 mutant [13] and *Brucella abortus* cgt19 mutant [14]. All experiments involving live *B. abortus* were conducted in a biosafety level 3 (BSL3) facilities at the University of San Martin, Buenos Aires, Argentina. *B. abortus* strains were grown at 37°C on tryptic soy agar (TSA) and in tryptic soy broth (TSB) on a rotary shaker (250 rpm). If necessary, culture media were supplemented with the appropriate antibiotics at the following concentrations: kanamycin (Km), 30 μg/ml; ampicillin (Amp), 100 μg/ml; and nalidixic acid (NaI), 5 μg/ml.

**CβG purification from *B. abortus***

For preparative purposes, 6-liter of stationary-phase cultures of *B. abortus* S19 grown for 48 h at 37°C (250 rpm) were harvested by centrifugation at 10,000 x g for 10 min. The pellets were extracted with ethanol (70% ethanol; 1 h at 37°C). The ethanolic extracts were centrifuged and the supernatants were dried in a speed-vac centrifugation at 10,000 x g for 1 h at 37°C. The supernatants were removed, weighed, homogenized in PBS, serially diluted, and plated onto TSA with the appropriate antibiotics to determine the number of CFU per spleen.

**Histological analysis of spleens infected with *B. abortus***

Group of mice were i.p inoculated with 1 x 10^6 CFU of *B. abortus* S19 or its isogenic *B. abortus* cgs mutant strain. At two weeks postinfection, spleens were removed, weighed, homogenized in PBS, serially diluted, and plated onto TSA with the appropriate antibiotics to determine the number of CFU per spleen.

**Preparation of single-cell suspensions of spleens infected with *B. abortus***

Spleens were aseptically removed and single cell suspensions were prepared by gently teasing through a sterile stainless steel screen. Erythrocytes were lysed in red blood cell lysis buffer and cells were washed twice in PBS solution.
Determination of inflammatory cell recruitment in spleens infected with B. abortus

BALB/c mice were infected with \(1 \times 10^6\) CFU of B. abortus S19 or its isogenic B. abortus cgs mutant strain. Spleens were obtained at two weeks postinfection and single cell suspensions were prepared. To assess recruitment of different cell subtypes after infection, splenocytes \((2 \times 10^6)\) were stained with anti-CD4 (FITC), -CD8 (PE-Cy5), -CD11c (PE), -CD11b (PE-Cy7), -Ly6G (PE), -Ly6C (PerCP-Cy5.5) and -B220 (PE) and analyzed by flow cytometry using FACSAriaII (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR). Monoclonal antibodies were purchased from eBioscience (San Diego, CA) and BD Biosciences (San Jose, CA).
Determination of cytokines in bone-marrow derived macrophages (BMDM) infected with B. abortus strains

Macrophages were derived from bone marrow of C57BL/6, Mal/tirap, TLR4, TLR6 and TLR9 KO mice as follows. Each femur and tibia was flushed with 5 ml of Hank’s balanced salt solution (HBSS). The resulting cell suspension was centrifuged, and the cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine Serum (FBS; Gibco), 1% penicillin/streptomycin (100 μg/mL) and 10% L929 cell-conditioned medium (LCCM) as a source of macrophage colony-stimulating factor (M-CSF). The cells were distributed in 24-well plates and incubated at 37°C in a 5% CO₂ atmosphere. Three days after seeding, another 0.1 ml of LCCM was added. On the seventh day, the medium was renewed. On the 10th day of culture, the cells were completely differentiated into macrophages [21]. The BMDM were infected with B. abortus S19 or its isogenic B. abortus cgs mutant strain, corresponding to a multiplicity of infection (MOI) of 100:1. After 60 min incubation with the bacteria, wells were washed three times with phosphate-buffered saline (PBS) and incubated with fresh medium containing 50 μg/mL Gm and 100 μg/mL streptomycin to kill non-internalized bacteria. After 24 h postinfection, the level of IL-12 (p40) and TNFα in the supernatants of BMDM were measured by ELISA Duoset kit (R&D, Minneapolis, MN). At 4, 24 and 48 hours postinfection, infected C57BL/6 BMDM were washed three times with PBS and lysed with 500 μl 0.1% Triton X-100 (Sigma-Aldrich Co.). The intracellular CFU was determined by plating serial dilutions on TSA with the appropriated antibiotic.

Results and Discussion

Transport of Brucella CβG to periplasm is required for bacterial-host interaction

As shown in Fig. 1A-a, Cgs, a 320 kDa membrane bound enzyme (the second largest protein in Brucella), is the enzyme responsible for the synthesis of cytoplasmic CβG, which is afterwards translocated to the periplasmic space by Cgt, a CβG-specific ABC transporter, and modified with succynil groups by the activity of Cgm (Fig. 1A-a). In order to determine if the different biosynthetic intermediate states of CβG play differential

Figure 2. B. abortus cgs and cgt mutants elicit a reduced splenomegaly in mice. BALB/c mice were intraperitoneally infected (1×10⁶ CFU) with (A) B. abortus 2308 or (B) B. abortus S19 and their isogenic CβG mutant strains. At two weeks postinfection, spleens were removed, weighed (right panel) and the numbers of CFU recovered were determined by serial dilutions and plating onto TSA (left panel). Five animals were used for each determination. *, P<0.05; **, P<0.01, Mann-Whitney test.

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Figure 3. Splenomegaly induced by B. abortus is dependent on the initial dose of infection. BALB/c mice were intraperitoneally infected with different doses of B. abortus S19 or B. abortus cgs19 mutant. At two weeks postinfection, spleens were removed, weighed and the numbers of CFU recovered from spleens determined by serial dilutions and plating onto TSA. (A) Recovery of viable bacteria from spleens of mice. (B) Spleens weight of infected mice. Bacterial doses were calculated retrospectively by colony counting. *, P<0.05; **, P<0.01, Mann-Whitney test.

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Figure 4. Reduced splenomegaly elicited by cgs B. abortus infection is a consequence of a lesser degree of cell recruitment. (A) BALB/c mice were intraperitoneally infected (1 x 10^6 CFU) with B. abortus S19, B. abortus cgs19 mutant or PBS as a control. Mice were euthanized at two weeks postinfection and spleens were removed and examined by histological analysis. WP: white pulp; RP: Red pulp; CA: central spleen artery; L: L-G-Dependent Splenomegaly.
roles in the bacteria the phenotype of three different \( B. \) \textit{abortus} \textit{CjG} mutants were compared: \textit{cgs} [17], \textit{cgt} [13] and \textit{cgm} mutant [14]. As shown in Fig. 1A–h, deletion of \textit{cgs} gene abolished the presence of \textit{CjG} but mutations in \textit{cgt} or \textit{cgm} lead to the production of neutral \textit{CjG} (Fig. 1A–e and d) with a different cellular localization being cytoplasmic in \textit{cgt} mutant and periplasmic in the case of \textit{cgm}. From the comparison between \textit{cgt} and \textit{cgm} it became evident that \textit{CjG} plays at least two roles: adaptation to hypo-osmotic conditions (Fig. 1B) and \textit{Brucella}-host interaction (Fig. 1C). While the adaptation to hypo-osmotic conditions requires the presence of anionic periplasmic \textit{CjG} (Fig. 1B), the sole requirement for host-pathogen interaction is the transport of \textit{CjG} to the periplasmic space regardless of its anionic charge, as demonstrated by the \textit{cgm} phenotype in intracellular replication and chronic infection in mice (Fig. 1C).

\textit{B. abortus} mutants unable to produce \textit{CjG} or to transport it to the periplasm elicit a reduced splenomegaly in mice

As we reported, deletion of \textit{cgs} gene either in the wild type strain \textit{B. abortus} \textit{2308} or in the vaccine strain \textit{B. abortus} \textit{S19} (which is virulent for humans) reduced their ability to infect mice and hampered their efficiency to reach the intracellular replication niche in epithelial cells [16,17]. Although attenuation of virulence occurs in both \textit{Brucella} backgrounds, in the \textit{S19} strain the phenotype is already evident after 4 weeks postinfection while in the \textit{2308} background, attenuation is observed after 12 weeks postinfection [17]. Interestingly, at two weeks postinfection when the infection is still not resolved and the number of bacteria in the spleens are equivalent (in the case of \textit{B. abortus cg08} mutant, Fig. 2A left panel) or slightly reduced (in the case of \textit{B. abortus cg19} mutant, Fig. 2B left panel) compared to their respective wild type parental strains, the splenomegaly was significantly reduced in mice infected with both \textit{cgs} isogenic mutants (Fig. 2A and 2B, right panel). In an earlier report, Crawford et al. observed that splenomegaly elicited by \textit{Brucella melitensis} infection (which peaks from two to three weeks postinfection) is dependent on the initial dose of infection rather than on the bacterial burden, concluding that very early events in the \textit{Brucella} infection are the driving force controlling the severity of the inflammatory response in the spleen [22].

Since \textit{cgs} mutants induced a reduced splenomegaly phenotype in both \textit{B. abortus} \textit{2308} and \textit{B. abortus S19} backgrounds, we decided to use the vaccine strain of \textit{B. abortus} \textit{S19} for the next set of experiments as the wild type control because it elicited a significantly increased splenomegaly compared to the one induced by the \textit{B. abortus} \textit{2308} strain [23] allowing us to develop a more sensitive assay. Additionally, since \textit{B. abortus S19} presents an intracellular trafficking defect in epithelial cells [24], similar to the defect described for \textit{B. abortus cg19} mutant strains, we reasoned that the use of \textit{S19} as our wild type control would reduce also the experimental variability due to differences in intracellular localization. Fig 2B (right panel) shows that the \textit{B. abortus S19} and \textit{B. abortus cgm19} mutant strains evolved a significantly increased inflammatory response in the spleens in comparison with the mutants \textit{B. abortus cg19} and \textit{B. abortus cg19}, suggesting that the splenomegaly correlated with the presence of \textit{CjG} within the periplasmic space. As mentioned above, \textit{CjG} is likely secreted within the host cell and therefore periplasmic localization requirement might be potentially a prerequisite for its delivery outside the bacteria.

Splenomegaly is dependent on the initial dose of \textit{B. abortus} infection

To study the impact of the initial dose of infection on the intensity of the splenomegaly, BALB/c mice were intraperitoneally infected with different doses of \textit{B. abortus S19} \textdagger{10\textsuperscript{5}}, \textdagger{10\textsuperscript{6}} and \textdagger{10\textsuperscript{9}} CFU (Fig. 3) and after two weeks postinfection spleens were removed, weighed, homogenized and the number of bacteria determined by serial dilution and plating to determine CFUs. Fig. 3 shows that, at two weeks postinfection although the numbers of replicating \textit{B. abortus S19} recovered from spleens were similar (about \textdagger{10\textsuperscript{5}–\textdagger{10\textsuperscript{9}} CFU per spleen) (Fig. 3A), the splenomegaly varied from negligible (about 0.1 grams) to a massive one (1 gram) (Fig. 3B) depending on the initial dose of infection, confirming previous observations in \textit{Brucella melitensis} [22]. To estimate the impact of \textit{cgs} phenotype on spleen inflammation, we infected mice with different doses of \textit{B. abortus cgs19} mutant strain and at two weeks postinfection, splenomegaly was determined. The results showed that, to achieve an equivalent degree of splenomegaly elicited by \textdagger{10\textsuperscript{6}} \textit{B. abortus S19}, it was necessary to increase a thousand times the initial dose of the \textit{B. abortus cgs19} \textdagger{10\textsuperscript{5}} (Fig. 3B).

Figure 5. \textit{B. abortus CjG} partially complement splenomegaly in mice. BALB/c mice were intraperitoneally infected with \textdagger{10\textsuperscript{6}} CFU of \textit{B. abortus S19} or \textdagger{10\textsuperscript{6}} CFU of \textit{B. abortus cgs19} mutant. Sets of five mice inoculated with \textit{B. abortus cgs} mutant were injected intraperitoneally with 15 µg of purified \textit{CjG} during the first five days of infection. At two weeks postinfection, mice were euthanized, and spleens were removed, weighed (A) and the number of CFU recovered determined by serial dilutions and plating onto TSA (B). *, \( P < 0.05, \) Mann-Whitney test.

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Reduced splenomegaly elicited by the cgs mutant is the consequence of a lesser degree of cell recruitment

At two weeks postinfection spleens from *Brucella* infected mice were processed for histological analysis and the results are shown in Fig 4A. While spleens from wild type strain infected mice showed an increase in the global cellularity with a massive infiltration of the red and white pulp (Fig. 4A–c), spleens from *cgs* infected mice showed a reduced degree of cellular infiltration (Fig. 4A–b) similar to what was observed in spleens of non-infected animals (Fig. 4A–a). Remarkably, at 400× magnification, the presence of neutrophils and macrophages within the red pulp of *B. abortus* S19 infected mice was observed (Fig. 4A–i) indicating massive cell recruitment to the spleen. In addition, spleen white pulp of *B. abortus* S19 and *B. abortus* cgs19 infected mice presented reactive lymphocytes (larger and medium lymphocytes) likely due to antigen stimulation (Fig. 4A–e and f).

To further characterize the spleen cell population, antibodies against specific markers were used to identify and quantify neutrophils, monocytes, B cells, T cells and dendritic cells by flow cytometry. As shown in Fig 4B spleens from *B. abortus* S19 infected mice presented eight times more neutrophils, ten times more monocytes, three times more dendritic cells and two times more B cells than spleens infected with *B. abortus* cgs19 mutant strain. No difference was observed in T-cell recruitment (not shown).

One possible explanation for the reduced splenomegaly and cell recruitment associated with the lack of CβG biosynthesis or CβG transport to the periplasm could be that *cgs/cgt* strains have a differential expression of PAMPs that might lead to a less efficient engagement of the host innate immune receptors and consequently to a diminished inflammatory response.

*B. abortus cgs* mutant has normal expression of flagellin and Omps, displaying normal amounts of smooth LPS (S-LPS) on the membrane

It has been demonstrated that *Agrobacterium* and *Sinorhizobium cgs* mutants have a reduced expression of flagellin and a defect in flagella assembly that consequently leads to a non-motile phenotype [25] [26]. Since bacterial flagellins are powerful agonists of innate immune receptors, being recognized extracellularly by the surface receptor TLR-5 and intracellularly by Nod-like receptors (such Ipaf or Naip5) [2], we explored if the *Brucella cgs* mutant has an altered expression of flagellin that might explain the diminished inflammatory response. In *Alphaproteobacteria* (including the *Brucella* genus) flagellin has a modification in the protein domain recognized by the TLR-5 innate immune receptor supporting the idea that *Brucella* flagellin has evolved to escape the host innate immune recognition [2]. In addition, in *Brucella*, flagellin expression is tightly controlled and only expressed under very strict culture conditions [19] and, because it has never been identified in any intracellular *Brucella* proteomic studies, this suggests that it is expressed poorly within the host cell [27,28]. In order to determine if the absence of periplasmic CβG affects flagellin expression, *B. abortus* S19 and its isogenic cgs, cgt and cgm mutants were grown in the conditional media to allow flagella assembly as described in Materials and Methods. Expression of *Brucella* flagellin was monitored by Western blot analysis, and the results, shown in Fig. S1A, indicates no changes in flagellin expression. Afterwards, we studied the expression of other critical *Brucella* PAMPs such as OMP16, OMP19 [29] or LPS [3,4][31] and no differences in expression were observed associated to any *B. abortus* CβG mutant strains (Fig. S1A and S1B).

Purified *Brucella* CβG partially complemented the splenomegaly defect in cgs-infected mice

As was mentioned above, purified CβG is capable to restore the intracellular replication deficiency of cgs-mutant strain in HeLa cells [16] and this observation suggests that CβG must be secreted within the host cell to exert its role in virulence. To evaluate the direct role of *Brucella* CβG in the inflammatory response we designed a trans-complementation experiment using purified CβG. For this, we added the purified carbohydrate to the bacterial initial inoculum and during the following five days postinfection by i.p injection. Splenomegaly determined at two weeks postinfection showed an increase in spleen weight in the CβG complemented mice compared to the cgs mutant although...
not to the degree of the Brucella wild type strain (Fig. 5A). Although significant, trans-complementation with purified CjG elicits a moderate increase on spleen enlargement compared with cgs mutant strain, an effect that can be explained as the result of the intrinsic limitations of this experimental approach. For instance, since injected CjG is diluted within the peritoneal cavity it is not possible to know the effective CjG concentration at the Brucella intracellular replicative niche. However, these results suggest that Brucella CjG plays a direct role in the induction of the inflammatory response in the spleen.

B. abortus cgs mutant strain elicits a reduced inflammatory response in BMDM

In previous studies, Zhan et al demonstrated that the macrophage-synthesized cytokines IL-12 and TNF-α are required for an efficient control of Brucella infection. In addition, it was shown that depletion of both pro-inflammatory cytokines by antibody treatment abolished the development of splenomegaly in animals infected with B. abortus S19 at two weeks postinfection [34]. To understand if the reduced spleen inflammation observed in mice infected with B. abortus cgs19 was due to a lower induction of IL-12 or TNF-α, we performed an in vitro infection experiment with naive BMDM. Differently to the defective intracellular replication phenotype reported for B. abortus cgs mutant strains in HeLa cells [17], in BMDM B. abortus cgs19 mutant strain showed no defect in intracellular replication in comparison with its parental wild type strain (Fig. 6A). The same phenotype was also reported for cgs mutant strain for intracellular replication in dendritic cells [5]. As shown in Fig. 6B and 6C, wild type BMDM infected with B. abortus S19, secreted higher levels of IL-12 (Fig. 6B) and TNF-α (Fig. 6C) to the supernatant than cells infected with B. abortus cgs19 mutant strain, suggesting that CjG promotes the induction of pro-inflammatory cytokines from BMDM. To understand if this CjG-dependent IL-12/TNF-α induction is dependent on Toll-like receptor (TLR) recognition, an in vitro experiment with BMDM from Mal/Tirap (the TLR2/TLR4 adapter protein), TLR4, TLR6 and TLR9 KO mice was performed (Fig. 6B and C). As shown in Fig. 6B, CjG-dependent IL-12 induction was independent on the presence of TLR2, TLR4, TLR6 or TLR9 (Fig 6B) while CjG-dependent TNF-α induction was independent on the presence of TLR4 or TLR9 (Fig. 6C). In absence of TLR2 or TLR6, B. abortus S19 and its isogenic cgs mutant strain elicited similar levels of TNF-α (Fig. 6C). These results suggest that TLR2 and TLR6 are potentially involved in the TNF-α induction elicited by CjG. It is interesting to notice that TLR2 and TLR6 are able to interact to form a heterodimer which is responsible for bacterial deacylated lipoproteins recognition [35].

Taken together all these results suggest that the reduced splenomegaly observed in cgs mutant strain infected mice is a consequence of a lower induction of proinflammatory cytokines that lead to a lesser cell recruitment to this organ.

Concluding Remarks

In the present study we describe the role of the Brucella cyclic β-1,2-glucan in promoting spleen enlargement during bacterial infection. Splenomegaly was the result of massive cell recruitment, mediated by the induction of pro-inflammatory cytokines. Since mutants deficient in CjG biosynthesis in the soil bacteria Sinorhizobium and Agrobacterium have shown to have membrane alterations that lead to non-motile phenotypes and an increased sensitivity to dyes and detergents, we evaluated if the low-inflammation phenotype observed with the cgs/cgt mutants was due to changes in expression of membrane bound complexes with inflammatory activity. No differences in flagellin, OMPs or LPS expression were evident and results suggested that CjG per se is responsible for the splenomegaly observed. The molecular mechanism underlying CjG induced splenomegaly remains to be identified and further studies will be performed to characterize this process.

Supporting Information

Figure S1 Western blot analysis of flagellin, outer membrane proteins (Omps) (A) and LPS (B) in B. abortus CjG mutant strains. Immunoblotting was performed using: rabbit polyclonal antibodies against Brucella flagellin, monoclonal antibodies against Omp16 and Omp19, and O-antigen specific monoclonal antibody (M84). SDS-PAGE and Western blot were carried out as described in Materials and Methods. The same amount of total protein extracts were loaded into the gels. The estimated molecular weight of each protein is shown.

(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: MSR GHG SCO JC GB. Performed the experiments: MSR AEI JASF JMS LM GB. Analyzed the data: MSR GB. Contributed reagents/materials/analysis tools: LM. Wrote the paper: MSR GB.

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