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The Effector Protein BPE005 from *Brucella abortus* Induces Collagen Deposition and Matrix Metalloproteinase 9 Downmodulation via Transforming Growth Factor β1 in Hepatic Stellate Cells

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The liver is frequently affected in patients with active brucellosis. In the present study, we identified a virulence factor involved in the modulation of hepatic stellate cell function and consequent fibrosis during *Brucella abortus* infection. This study assessed the role of BPE005 protein from *B. abortus* in the fibrotic phenotype induced on hepatic stellate cells during *B. abortus* infection *in vitro* and *in vivo*. We demonstrated that the fibrotic phenotype induced by *B. abortus* on hepatic stellate (LX-2) cells was dependent on BPE005, a protein associated with the type IV secretion system (T4SS) VirB from *B. abortus*. Our results indicated that *B. abortus* inhibits matrix metalloproteinase 9 (MMP-9) secretion through the activity of the BPE005-secreted protein and induces concomitant collagen deposition by LX-2 cells. BPE005 is a small protein containing a cyclic nucleotide monophosphate binding domain (cNMP) that modulates the LX-2 cell phenotype through a mechanism that is dependent on the cyclic AMP (cAMP)/protein kinase A (PKA) signaling pathway. Altogether, these results indicate that *B. abortus* tilts LX-2 cells to a profibrogenic phenotype employing a functional T4SS and the secreted BPE005 protein through a mechanism that involves the cAMP and PKA signaling pathway.

Brucellosis is a worldwide zoonosis characterized by hepatomegaly, splenomegaly, and peripheral lymphadenopathy. It is a chronic and debilitating infection caused by Gram-negative facultative intracellular bacteria that infect domestic and wild animals and that can be transmitted to humans (1, 2). The frequency of liver involvement in active brucellosis ranges from 5% to 52% or more (1). However, although numerous studies have focused on brucellar liver histopathology (1), the pathogenic mechanisms of liver disease caused by *Brucella* have not been completely investigated at the molecular and cellular levels.

Liver fibrosis is a wound-healing response to chronic hepatic injury, which may be caused by alcohol abuse, hepatitis virus infection, or nonalcoholic steatohepatitis, and it is characterized by an excessive accumulation of extracellular matrix proteins in the liver (3, 4). An early event in the development of liver fibrosis is the activation of hepatic stellate cells (HSCs), the major cell type responsible for increased synthesis of extracellular matrix proteins (5). An elevated level of transforming growth factor β1 (TGF-β1) is also observed in the damaged liver, and it has a close correlation with fibrogenic changes in HSCs and liver tissue (6–8). In addition, decreased matrix metalloproteinase 9 (MMP-9) expression was observed in alcoholic liver fibrosis (9). This fibrogenic phenotype involves alterations in the balance of MMPs and their natural inhibitors—tissue inhibitors of metalloproteinases (TIMPs). In particular, MMP-2 and MMP-9 (gelatinase A and B, respectively) are important in regulating fibrogenesis and scar degradation. They can degrade a variety of collagens, including basement membrane (type IV collagen), denatured fibrillar type I collagen (gelatin), and type V collagen (10). Collagen type I is the prototype constituent of the fibril-forming matrix in fibrotic liver (11–13), and TGF-β1, derived from paracrine and autocrine sources, remains the classic fibrogenic cytokine (14, 15).

Previously, we demonstrated that infection of HSCs with *Brucella abortus* induces a series of events characterized by inhibition of MMP-9 secretion, induction of collagen deposition, and increased secretion of TIMP-1. These phenomena were dependent on TGF-β1 induction (16). However, the molecular mechanisms exerted by *Brucella* to activate this fibrogenic phenotype of HSCs have not yet been identified.

Type IV secretion systems (T4SS) are multiprotein complexes that translocate nucleoproteins and/or protein substrates across the bacterial cell envelope to the host cell, generally by a contact-dependent mechanism (17). T4SS protein substrates have been shown to modulate various cellular processes in the host cell, including apoptosis, vesicular traffic, and ubiquitination (18, 19). As the *Brucella* T4SS encoded by the *virB* gene has been shown to be involved in the modulation of the immune response during infection (20–22), we decided to investigate whether the effect of *Brucella* infection on the activation of HSCs is dependent on the presence of a functional T4SS and/or its secreted proteins. To this end, LX-2 cells were infected with *B. abortus* or its isogenic mutants to

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determine the levels of production of MMPs, collagen deposition, and TGF-β1 secretion. The results of the study are presented here.

MATERIALS AND METHODS

Bacterial culture. *Brucella abortus* S2308 or the isogenic *B. abortus virB10* polar, *B. abortus bpe005*, *B. abortus bpe275*, and *B. abortus bpe123* strains and the corresponding complemented mutants (23, 24) were grown overnight in 10 ml of tryptic soy broth (Merck, Buenos Aires, Argentina) with constant agitation at 37°C. Bacteria were harvested and the inocula were prepared as described previously (25). All live *Brucella* manipulations were performed in biosafety level 3 (BSL-3) facilities located at the Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS).

Construction of the *Brucella abortus* BPE005, BPE123, and BPE275 mutants. Unmarked deletion mutants with mutations of the selected candidate genes (*bpe005* and *bpe275*) were generated as follows. DNA fragments (~500 bp) containing the flanking regions of BAB1_2005 and BAB1_1275 were amplified from *B. abortus* 2308 genomic DNA with modified primers carrying BamHI and EcoRI restriction sites at the 5′ and 3′ ends, respectively. The PCR amplicons were ligated to the BamHI and EcoRI sites of mobilizable suicide vector pK18mobsacB, and the resulting plasmid was transformed in *Escherichia coli* S17 λ-*pir* and subsequently conjugated to *B. abortus* 2308. Single recombinants were selected with kanamycin and replica plated in Trypticase soy agar (TSA) supplemented with 10% sucrose to counterselect the double recombinants. Deletion of the selected gene was confirmed by colony PCR and sequence analysis. Using these procedures, the *B. abortus* Δ*bpe005* and *B. abortus* Δ*bpe275* mutant strains were generated. To obtain the *bpe123* deletion mutant, a DNA fragment of 500 bp coding for BPE123 was amplified by PCR using primers 20123 BamHI and 20123 SpeI. The PCR product was ligated to pGem-T-Easy (Promega), and the resulting plasmid was linearized with HindIII and blunt ended with T4 DNA polymerase (New England BioLabs). Linearized pGem-T-*bpe123* was ligated to a HincII DNA fragment coding for a nonpolar kanamycin resistance cassette to generate pGem-T*bpe123*::*Kan*. This plasmid was electroporated into *B. abortus* 2308, where it is incapable of autonomous replication. Homologous recombination events were selected using kanamycin resistance and ampicillin sensitivity. Deletion of the selected gene was confirmed by colony PCR and sequence analysis. To generate the complemented strain of the *B. abortus bpe005* mutant, the construct pLF-*bpe005* (containing BAB1_2005 fused to a 3× FLAG tag) was transferred by biparental conjugation to the *B. abortus* deletion mutant, and the resulting complemented strain was selected with ampicillin.

Cell culture. The LX-2 cell line, a spontaneously immortalized human hepatic stellate cell line, was kindly provided by Scott L. Friedman (Mount Sinai School of Medicine, New York, NY). LX-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies/Invitrogen, Carlsbad, CA, USA) and supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% (vol/vol) fetal bovine serum (FBS; Gibco/Invitrogen, Carlsbad, CA, USA). All cultures were grown at 37°C and 5% CO_2.

Cellular infection. LX-2 cells were infected with *B. abortus* S2308 or its isogenic mutants at different multiplicities of infection (MOI; 100 to 1,000). After the bacterial suspension was dispensed, the plates were centrifuged for 10 min at 2,000 rpm and then incubated for 2 h at 37°C under a 5% CO_2 atmosphere. Cells were extensively washed with DMEM to remove extracellular bacteria and incubated in medium supplemented with 100 μg/ml gentamicin and 50 μg/ml streptomycin to kill extracellular bacteria. LX-2 cells were harvested at different times to determine cytokine production, MMP secretion, and collagen deposition. To determine the role of cyclic AMP (cAMP) in the inhibition of MMP secretion and collagen deposition, some infection experiments were performed in the presence of 0.001, 0.01, 0.1, and 1 μM dibutyryl cAMP (B2cAMP) (Sigma-Aldrich de Argentina SA, Buenos Aires, Argentina).

Zymography. Gelatinase activity was assayed by the method of Hibbs et al. with modifications as described previously (25–27).

Measurement of cytokine concentrations. Secretion of TGF-β1, interleukin-6 (IL-6), IL-8, and monocyte chemotactic protein 1 (MCP-1) in the supernatants was quantified by enzyme-linked immunosorbent assay (ELISA) (BD Biosciences). TIMP-1 was quantified in culture supernatants by ELISA (R&D Systems).

Assessment of collagen deposition using Sirius red staining. Collagen deposition was quantified using Sirius red (Sigma-Aldrich, Argentina), a strong anionic dye that binds strongly to collagen molecules (28). Sirius red staining was performed as previously described (16).

Transfection experiments. LX-2 cells were transfected with BPE005 plasmid DNA or pRSV-PKI plasmid (kind gift of J. Silvio Gutkind), and pCDNA3-c-myc (2 mg/ml of plasmid DNA in each well transfected for 5 h) was used as a control for analysis of transfection efficiency. Transfection was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. MMP secretion and collagen deposition were determined at the end of cultures by zymography and Sirius red staining, respectively. Transfection efficiency was determined with mouse anti-c-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in phosphate-buffered saline (PBS)–0.1% Tween for 30 min at room temperature and then with fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear staining for 30 min at room temperature. After they were washed in PBS, cells were mounted and then were analyzed by fluorescence microscopy. These assays determined the presence of more than 70% transfected cells.

Evaluation of hepatic fibrosis in a mouse model of infection. Six- to 8-week-old female BALB/c mice were infected through the intraperitoneal route with 5×10^5 CFU of *B. abortus* S2308, *B. abortus bpe005* mutant, or vehicle (PBS). Mice were sacrificed at 4 and 12 weeks postinfection. To determine the levels of CFU, TGF-β1, and collagen production in mice livers, a liver lobe from each mouse was excised and placed immediately into 1 ml of cold PBS. Liver extractions were performed by using a tissue homogenizer. Aliquots of homogenates were serially diluted in sterile PBS and plated for CFU determinations, the remained homogenates were centrifuged at 2,000 × *g* for 20 min at 4°C, and the supernatants were stored at −70°C until TGF-β1 and collagen measurements were performed. In another group of mice, histological examination of liver was carried out at week 4 postinfection after routine fixation and paraffin embedding. Five-micrometer-thick sections were cut and stained with hematoxylin and eosin and with Masson's trichrome stain. Masson's trichrome staining was conducted according to the manufacturer's instructions (Sigma-Aldrich). Collagen-positive areas were visualized by light microscopy and quantified using Image Pro-Plus 6.0 software (Media Cubernetics, Inc.). The animal housing and all animal manipulations were conducted in the animal BSL-3 facility at Instituto de Investigaciones Biotecnológicas, Universidad Nacional de San Martín (IIB-UNSAM), under the guidance of and using protocols approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL-UNSAM).

Statistical analysis. Statistical analysis was performed with one-way analysis of variance (ANOVA), followed by a *post hoc* Tukey test using GraphPad Prism 4.0 software. Data were represented as means ± standard errors of the means (SEM).

RESULTS

***B. abortus* induces a profibrogenic phenotype in LX-2 cells in a VirB-dependent manner.** The type IV secretion system (T4SS) VirB has been shown to be involved in the capacity of different *Brucella* species to establish an intracellular replication niche (24). In addition, this system has been involved in the induction of inflammatory response during *B. abortus* infection (21, 22). Taking into account that the major mediators of liver fibrosis are liver injury and inflammation (29), we decided to test whether VirB is involved in the ability of *B. abortus* to replicate within LX-2 cells. To this end, LX-2 cells were infected with *B. abortus* and its iso-

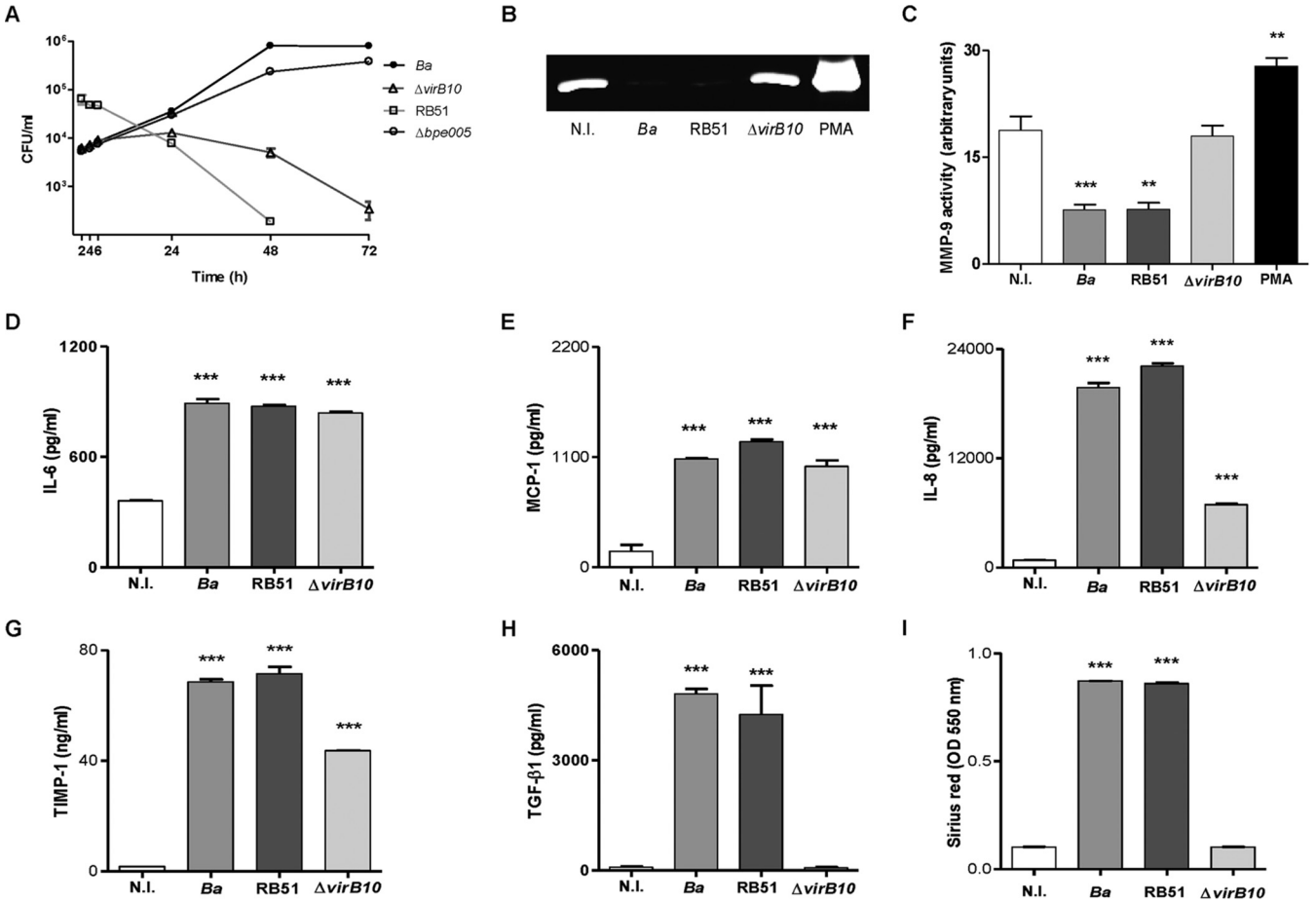

FIG 1 The *B. abortus virB10* mutant (Δ*virB10*) does not induce a profibrogenic phenotype in LX-2 cells. (A) LX-2 cells were infected with *B. abortus* (*Ba*), *B. abortus* RB51 (RB51), or the *B. abortus bpe005* mutant (Δ*bpe005*) or with the Δ*virB10* mutant at an MOI of 1,000, and CFU levels were determined 2, 4, 6, 24, 48, and 72 h postinfection. (B) MMP-9 production assessed by zymography at 24 h postinfection, (C) Densitometric analysis of results from three independent experiments performed as described for panel B. (D to H) ELISA determinations of levels of IL-6 (D), MCP-1 (E), IL-8 (F), TIMP-1 (G), and TGF-β1 (H) were performed in culture supernatants from LX-2 cells infected for 24 h. (I) Collagen deposition was assessed by quantification of Sirius red at 7 days postinfection. MMP-9 activity, cytokine secretion, and collagen deposition analyses were performed using LX-2 cells infected at an MOI of 1,000. PMA, phorbol myristate acetate. Data are given as the means ± SEM of results from experiments performed in duplicate. Data shown are from a representative experiment of three performed. **, $P < 0.01$; ***, $P < 0.001$ (versus noninfected [N.I.]).

genic *B. abortus virB10* mutant, which has been shown to be incapable of replicating within several cell types (24, 30, 31). *B. abortus* intracellular replication in LX-2 cells was dependent on the presence of a functional T4SS. In addition, and as occurs in other cell types (32), the *B. abortus* RB51 rough mutant was also unable to replicate in LX-2 cells (Fig. 1A).

The persistence of an infectious stimulus might drive liver fibrosis because its presence could induce marked alterations in a variety of immune and structural cells. In this context, hepatic stellate cells have a fundamental role in tissue homeostasis and injury repair through the production of extracellular matrix proteins (33). We have demonstrated that infection of LX-2 cells with *Brucella* inhibits the spontaneous secretion of MMP-9 (as determined by ELISA and by measurement of its activity by zymography) and induces collagen deposition (16). To determine whether intracellular replication is critical for inhibition of MMP-9 secretion and for induction of collagen deposition, experiments were performed using the *B. abortus virB10* mutant and the RB51 rough mutant. Inhibition of MMP-9 secretion induced by *B. abortus* was dependent on the expression of a functional T4SS, since the levels of MMP-9 activity did not differ significantly between LX-2 cells infected with a *B. abortus virB10* mutant and uninfected controls. However, this phenomenon was not dependent on intracellular replication, since RB51 was able to inhibit MMP-9 secretion by LX-2 cells (Fig. 1B and C), indicating that truncated bacterial intracellular replication is not a mandatory step in the inhibition of MMP-9 secretion in LX-2 cells. In addition, collagen deposition and TGF-β1 secretion were also dependent on the expression of a functional T4SS and are not related to bacterial replication, since the levels of collagen deposition and TGF-β1 secretion did not differ significantly between LX-2 cells infected with a *B. abortus virB10* mutant and uninfected controls whereas *B. abortus* RB51-infected cells showed increased TGF-β1 secretion and collagen deposition (Fig. 1H and I). In contrast, IL-6, IL-8, MCP-1, and TIMP-1 secretion was induced by wild-type *B. abortus*, *B. abortus* RB51, and *B. abortus virB10* mutant infection (Fig. 1D to G). However, levels of IL-6, IL-8, MCP-1, and TIMP-1 (Fig. 1C to F) were lower for the *B. abortus virB10* mutant than for the other

Brucella strains tested. The reduction of the levels of all these mediators was not due to bacterium-induced cell death. Measured at an MOI of 1,000 by Hoechst staining, by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, or by annexin V-phosphatidylinositol staining, *B. abortus* infection did not induce apoptosis of LX-2 cells (data not shown).

These results indicated that expression of TGF-β1, the main profibrogenic cytokine, is modulated by the T4SS system of *B. abortus*. However, this system is not involved in the modulation of expression of IL-6 and of chemokines MCP-1 and IL-8 (attractants for neutrophils and monocytes, respectively), indicating that T4SS could not modulate the inflammatory infiltrate.

The participation of MMPs and their specific inhibitors, TIMPs, together with collagen deposition, is implicated in the formation and recovery processes of liver fibrosis (33). However, although the *B. abortus* wild type and the *virB10* mutant modulate the expression of MMP-9 in different ways, both strains induce TIMP-1 secretion.

Together, these results indicate that upon infection of LX-2 cells, *B. abortus* inhibits MMP-9 secretion and induces concomitant collagen and TGF-β1 secretion in a VirB-dependent manner.

VirB-dependent effector protein BPE005 is responsible for the profibrogenic phenotype induced by *B. abortus*. Recently, secreted proteins of *B. abortus* have been identified that require a functional VirB system to be translocated into the host cells (23). *B. abortus* mutants of three of them (BPE005, BPE275, and BPE123) were obtained, and their potential profibrogenic effect on LX-2 cells was studied. To this end, the levels of secretion of MMP-9 and TGF-β1 and collagen deposition were determined using LX-2 cells that were infected with *B. abortus bpe005*, *bpe275*, and *bpe123* mutants. *B. abortus bpe005* mutant bacteria infect and replicate in LX-2 cells (Fig. 1A), and the results mimic the LX-2 responses mediated by the *B. abortus virB10* mutant. In contrast, the *B. abortus bpe275* and *bpe123* mutants inhibited MMP-9 activity and increased TGF-β1 secretion and collagen deposition in a manner similar to that seen with *B. abortus* wild-type infection (Fig. 2).

Together, these results indicate that upon infection of LX-2 cells, *B. abortus* inhibits MMP-9 secretion and induces concomitant TGF-β1 secretion and collagen deposition and that these phenomena are dependent on the presence of T4SS effector protein BPE005.

Expression of BPE005 in LX-2 cells mimics the profibrogenic effect caused by *B. abortus* infection. In order to determine whether the observed profibrogenic effect caused by *B. abortus* infection of LX-2 cells is dependent on BPE005 protein expression, cells were transfected with a eukaryotic expression vector harboring the *bpe005* gene and the levels of MMP-9 activity, collagen deposition, and TGF-β1 secretion were determined. Expression of BPE005 protein in LX-2 cells was able to inhibit MMP-9 activity and to induce collagen deposition and TGF-β1 secretion in a manner similar to that seen with *B. abortus* infection (Fig. 3). This result corroborated that this effector protein is the protein responsible for triggering the profibrogenic phenotype observed in LX-2 cells.

Butiril-cAMP restores the ability of BPE005-transfected LX2 cells to secrete MMP-9 and inhibits TGF-β1 secretion. BPE005 is a protein containing a cyclic nucleotide monophosphate binding domain. Although its function is unknown, the predicted structure suggests that it might have an effect on cAMP-dependent signaling pathways in the host cell (23). Previous studies (34, 35) revealed that MMP-9, TGF-β, and collagen secretion could be regulated via activation of the cAMP/protein kinase A (cAMP/PKA) pathway. Our hypothesis is that *B. abortus*, through the activity of BPE005 protein, could alter this pathway, modulating cellular responses in LX-2 cells. Experiments were then conducted to evaluate whether cAMP could restore the effect triggered by BPE005 in LX-2 cells. To this end, LX-2 cells were transfected with the plasmid carrying *bpe005* in the presence or absence of dibutyryl cyclic AMP (B2cAMP). This treatment completely reversed the inhibition of MMP-9 activity and TGF-β1 secretion induced by BPE005 expression in LX-2 cells (Fig. 4A and B). To corroborate this finding during *B. abortus* infection, LX-2 cells were infected with *B. abortus* in the presence of different concentrations of B2cAMP. Treatment with B2cAMP reversed the inhibitory effect induced by *B. abortus* infection on MMP-9 production by LX-2 cells in a dose-dependent manner (Fig. 4C). Our results indicated that cAMP could be involved in the mechanisms by which *B. abortus* could drive LX-2 responses to a profibrotic phenotype.

The PKA signaling pathway is involved in the profibrogenic response of LX-2 cells upon *B. abortus* infection. The action of cAMP in eukaryotic cells was thought to occur mainly via activation of protein kinase A (PKA) and PKA-mediated changes in protein expression and function (36, 37). Therefore, experiments were conducted to determine whether PKA was involved in the profibrogenic response of the cells during *B. abortus* infection. To this end, LX-2 cells were infected with *B. abortus* or its isogenic *bpe005* mutant in the presence or absence of KT5720 (PKA inhibitor) and MMP-9 and TGF-β1 production were evaluated. KT5720 treatment induced the inhibition of MMP-9 secretion in LX-2 cells infected with *B. abortus bpe005* mutant. KT5720 treatment also increased the inhibition of MMP-9 production in *B. abortus* wild-type-infected LX-2 cells (Fig. 5A).

In addition, KT5720 treatment induced TGF-β1 secretion in LX-2 cells infected with the wild-type *B. abortus* strain or the mutant strain (Fig. 5A and B). To corroborate these results, LX-2 cells were transfected with pRSV-PKI plasmid, an expression vector containing heat-stable inhibitor PKI of cAMP-dependent PKA (38). Transfection with PKI of LX2 cells infected with the *B. abortus bpe005* mutant induced inhibition of MMP-9 secretion; in addition, transfection with PKI of LX-2 cells infected with the *B. abortus* wild type increased the inhibition of MMP-9 secretion even more (Fig. 5C). Taken together, these results indicated that the cAMP/PKA signaling pathway is involved in the modulation of responses induced during *B. abortus* infection.

Infection of mice with the *B. abortus bpe005* mutant induced a diminished fibrotic lesion in livers. Finally, to determine the *in vivo* relevance of our hypothesis, BALB/c mice were infected with *B. abortus* and its isogenic *bpe005* mutant.

As shown in Fig. 6A, CFU levels recovered from livers after 4 weeks of infection in mice infected with the *B. abortus* wild type and in those infected with the *B. abortus bpe005* mutant were similar, indicating that the differences in the fibrotic phenotype were not dependent on bacterial replication.

Masson's trichrome staining revealed that the level of fibrotic patches is lower in mice infected with the *B. abortus bpe005* mutant than in those infected with the *B. abortus* wild type (Fig. 6B and C). Moreover, the levels of collagen, measured by Sirius red staining, were significantly increased in the livers of mice infected with wild-type *B. abortus* compared with the livers of mice in-

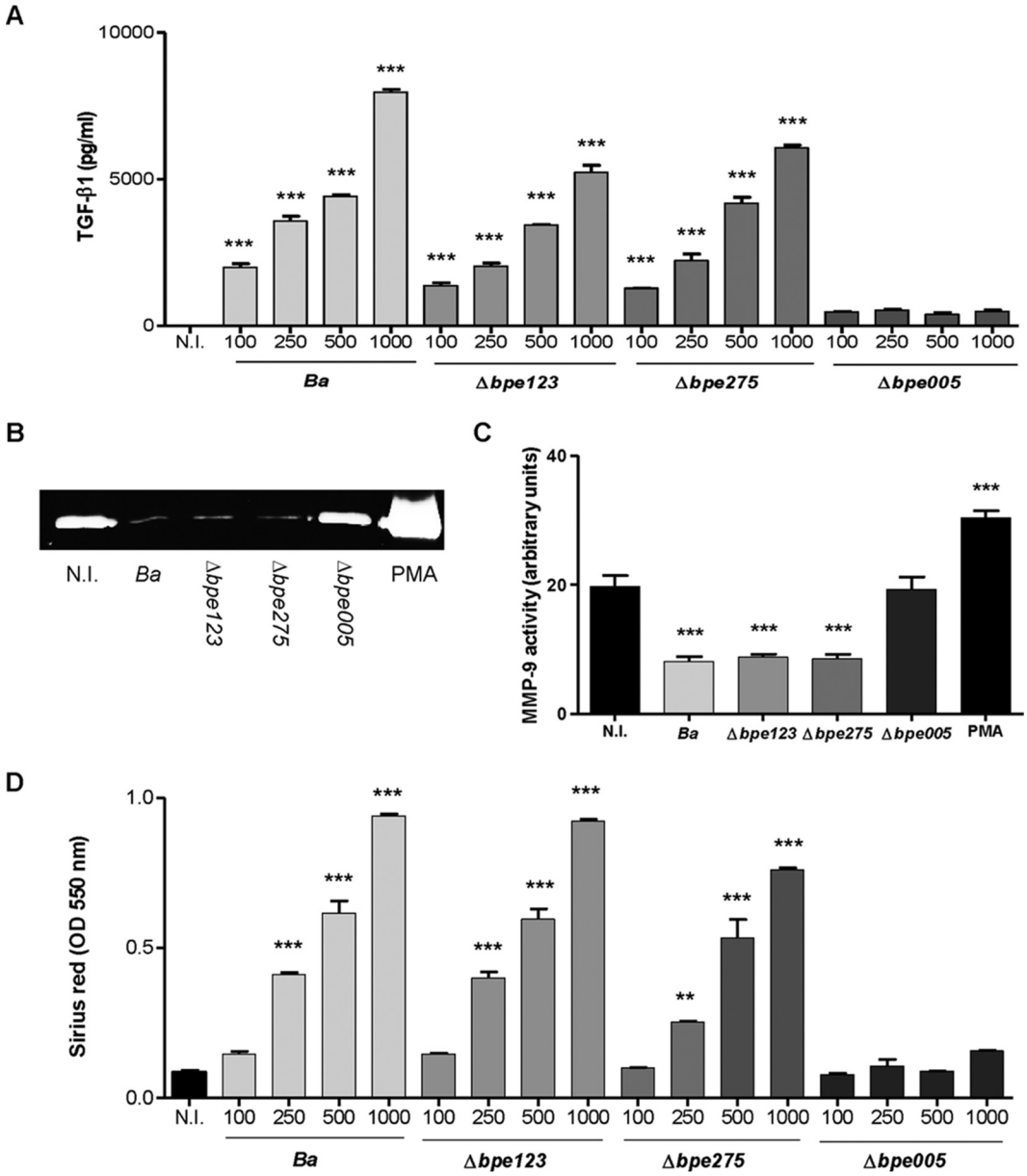

FIG 2 BPE005 inhibits MMP-9 secretion and induces collagen deposition and TGF-β1 secretion in LX-2 cells. (A and B) LX-2 hepatic stellate cells were infected with *B. abortus* (*Ba*) or its isogenic *bpe123* (Δ*bpe123*), *bpe275* (Δ*bpe275*), and *bpe005* (Δ*bpe005*) mutants, and at 24 h postinfection, supernatants were harvested to analyze TGF-β1 secretion by ELISA (A) and MMP-9 production by zymography (B) in culture supernatants from LX-2 cells infected at an MOI of 1,000. (C) Densitometric analysis of results from three independent experiments performed as described for panel B. (D) Collagen deposition was assessed by quantification of Sirius red at 7 days postinfection. PMA, phorbol myristate acetate. Data are given as the means ± SEM of results from experiments performed in duplicate. Data shown are from a representative experiment of three performed. **, $P < 0.01$; ***, $P < 0.001$ (versus noninfected [N.I.]).

fected with the *B. abortus bpe005* mutant (Fig. 6D). In addition, these parameters correlated with the lower increase in TGF-β1 secretion in livers from *B. abortus bpe005* mutant-infected mice than in those infected with the *B. abortus* wild type (Fig. 6E). These results indicated that BPE005 plays a key role in modulation of fibrosis during *B. abortus* infection.

DISCUSSION

The liver is frequently affected in patients with active brucellosis. Different histology patterns can be observed in liver involvement in brucellosis, the most common being (i) granuloma formation with inflammatory infiltrations and (ii) parenchymal necrosis (39). In any event, he persistence of an infectious stimulus might drive liver fibrosis because its presence could induce marked alterations in a variety of immune and structural cells, leading to a healing phenotype which is characterized by the deposition of extracellular matrix (40).

Hepatic stellate cells are recognized as the main source of liver fibrosis (5). Liver fibrosis is characterized by the deposition of extracellular matrix. An infectious stimulus, such as *B. abortus* infection, and the sustained injury might drive fibrosis (40).

We have previously demonstrated that *B. abortus* bacteria in-

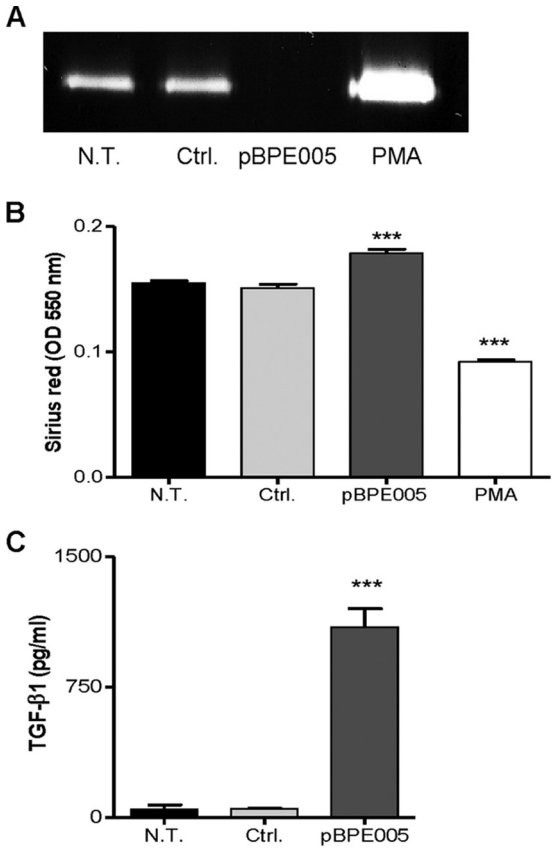

FIG 3 BPE005 protein mimics the *B. abortus* effect on MMP-9, collagen deposition, and TGF-β1 secretion by LX-2 hepatic stellate cells. LX-2 cells were transfected with BPE005 plasmid DNA (pBPE005) or with pcDNA3-c-myc as a control (Ctrl.). (A) MMP-9 production was assessed by zymography in culture supernatants harvested 48 h after transfection. (B) At 5 days posttransfection, collagen deposition was analyzed by Sirius red quantification. (C) ELISA determination of TGF-β1 levels in supernatants of LX-2 after 24 h of transfection. PMA, phorbol myristate acetate. Data are given as the means ± SEM of results from experiments performed in duplicate. Data shown are from a representative experiment of three performed. ***, $P < 0.001$ (versus nontransfected [N.T.]).

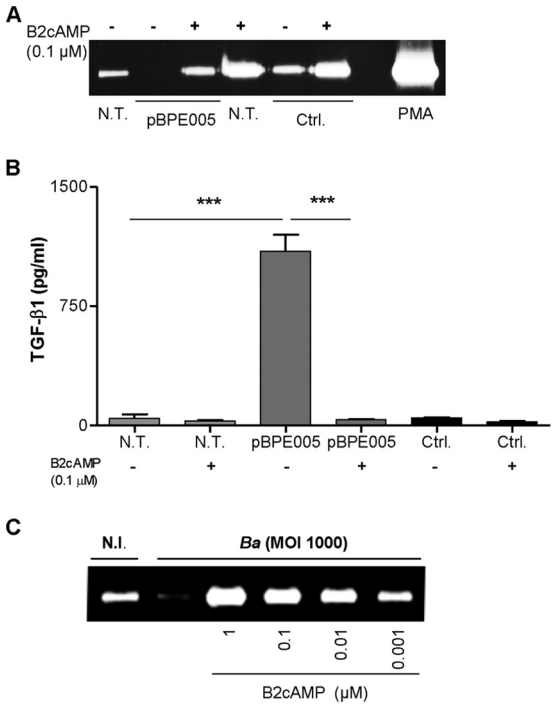

FIG 4 Butiril-cAMP restores the ability of BPE005-transfected LX2 cells to secrete MMP-9 and inhibits TGF-β1 secretion. (A and B) LX-2 cells were transfected with BPE005 plasmid DNA (pBPE005) or with pcDNA3-c-myc (Ctrl.) in the presence or absence of B2cAMP. At 24 h posttransfection, MMP-9 production was assessed by zymography (A) and TGF-β1 secretion by ELISA (B). (C) Cells were infected with *B. abortus* (*Ba*) at an MOI of 1,000 in the presence or absence of B2cAMP. At 24 h after infection, supernatants were harvested and MMP-9 levels were determined by zymography. Data are given as the means ± SEM of results from experiments performed in duplicate. Data shown are from a representative experiment of three performed. ***, $P < 0.001$ (versus nontransfected [N.T.]) or transfected and treated [pBPE005, B2cAMP]).

fect and replicate in hepatic stellate cells *in vitro*; *in vivo*, however, the cells would not represent a replicative niche but could be infected transiently or stimulated by *Brucella* antigens or by cytokines present in the inflammatory milieu generated by the infection. *B. abortus* infection inhibits basal levels of MMP-9 secretions and induces collagen deposition and TIMP-1 secretion by hepatic stellate cells. These phenomena were dependent on TGF-β1 induction (16).

As occurs in other bacterial infections (41, 42), a fibrotic phenotype may contribute to the persistence of infection, leading to poor penetration of antibiotics and immune mediators into the lesion. In addition, a fibrotic response could contribute to granuloma formation. The compact structure of granuloma successfully prevents the dissemination of the microorganisms; however, a negative facet of the granuloma is that the lesion may harbor within its burnt-out or calcified structure residual viable bacteria.

On the other hand, taking into account the role played by MMPs in infectious disease, orchestrating the recruitment of innate inflammatory cells and regulating their effector functions subsequent to cellular activation (43), the inhibition of MMP-9 secretion induced by *B. abortus* infection in hepatic stellate cells could contribute to partially inhibit the immune response, contributing to the chronicity of infection.

In the present study, we demonstrated that the ability of *B. abortus* to activate hepatic stellate cells is dependent on the presence of a functional T4SS and the secreted protein BPE005. Bacteria use T4SS for genetic exchange and to deliver effector molecules to eukaryotic target cells. The T4SS system plays a crucial role in the intracellular replication of *B. abortus* (44). Recently, it has been demonstrated that *Brucella* bacteria are able to modulate the host cell secretory pathway via multiple T4SS effector proteins (45). Yet BPE005 inhibits MMP-9 secretion but does not affect the secretion of other cytokines and collagen deposition. Therefore, our results indicate that BPE005 could have a specific role in the inhibition of MMP-9 secretion; however, we could not rule out other different molecular mechanisms that could be implicated such as the alteration of MMP-9 expression or the inhibition of the host secretory pathway. Also, it has been demonstrated that *Brucella* bacteria could use this secretion system to translocate effector proteins into host cytosol or even nucleic acids to further activate the inflammasome in a way that involved NLRP3 (22). In addition, it has been demonstrated that NLRP3 inflammasome

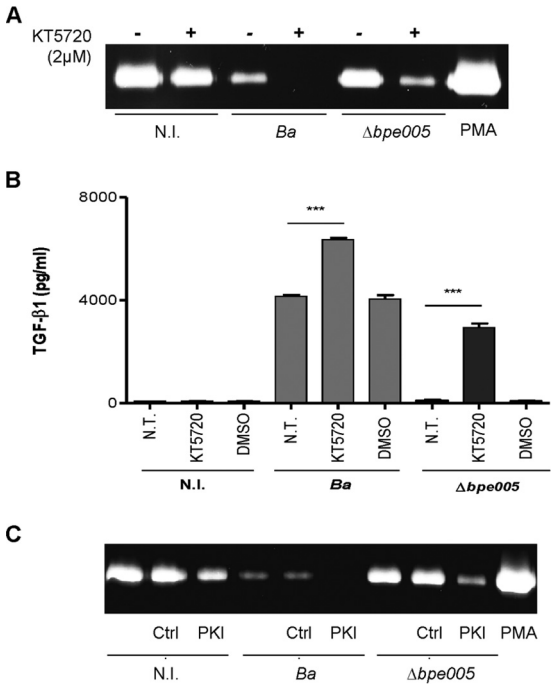

FIG 5 The PKA signaling pathway is involved in the modulation of LX-2 cells in response to *B. abortus* infection. (A and B) LX-2 cells were infected with *B. abortus* S2308 (*Ba*) or its isogenic *bpe005* mutant (Δ*bpe005*) in the presence or absence of KT5720 (PKA inhibitor) (2 μM), and 24 h after infection, MMP-9 levels were determined by zymography (A) and TGF-β1 production by ELISA (B). (C) Noninfected LX-2 cells and *B. abortus- or* Δ*bpe005* mutant-infected LX-2 cells were transfected with pRSV-PKI plasmid (PKI) or with pcDNA3-c-myc (Ctrl.), and MMP-9 production was assessed by zymography. PMA, phorbol myristate acetate. Data are given as the means ± SEM of results from experiments performed in duplicate. Data shown are from a representative experiment of three performed. ***, $P < 0.001$ (versus nontreated [N.T.]).

activation in hepatocytes and nonparenchymal liver cells results in the induction of proinflammatory signaling and hepatocyte pyroptotic cell death (46). Then, the T4SS from *B. abortus* could be involved in the modulation of immune response in liver through activation of inflammasomes with concomitant fibrosis induction to repair inflammatory damage, depending on the secreted effector and the target cell. This response may therefore enable *Brucella* survival by subverting the immune response *in vivo* through an altered acute inflammatory response, resulting in impaired bacterial clearance and the establishment of chronic disease.

The modulation of collagen deposition and MMP secretion in HSCs involved the cAMP/PKA signaling pathway, as previously demonstrated in other cell types (47–49). It has been demonstrated that cAMP is a key messenger of many hormones and neuropeptides, some of which modulate the composition of extracellular matrix, and, recently, cAMP/PKA signaling was hypothesized to be involved in the proliferation and activation of rat HSCs (50–53). The action of cAMP in eukaryotic cells was thought to occur mainly via activation of PKA, and this molecule mediates changes in protein expression and function (36, 37). cAMP inhibits TGF-β-induced collagen synthesis in fibroblasts; in fact, several studies have shown that elevation of intracellular cAMP levels with phosphodiesterase inhibitors such as pentoxifylline inhibits fibroblast growth and collagen synthesis induced by serum and/or fibroblast-activating cytokines (54–56). It has been reported that, in this way, cAMP-elevating agents have the potential to act as antifibrotic therapeutics in a mechanism(s) that involves TGF-β (57). The ability of TGF-β to stimulate the proliferation of extracellular matrix production by cultured fibroblasts is well documented (58, 59).

We demonstrated that the capacity of *B. abortus* to induce fibrosis upon infection is dependent on the modulation of the

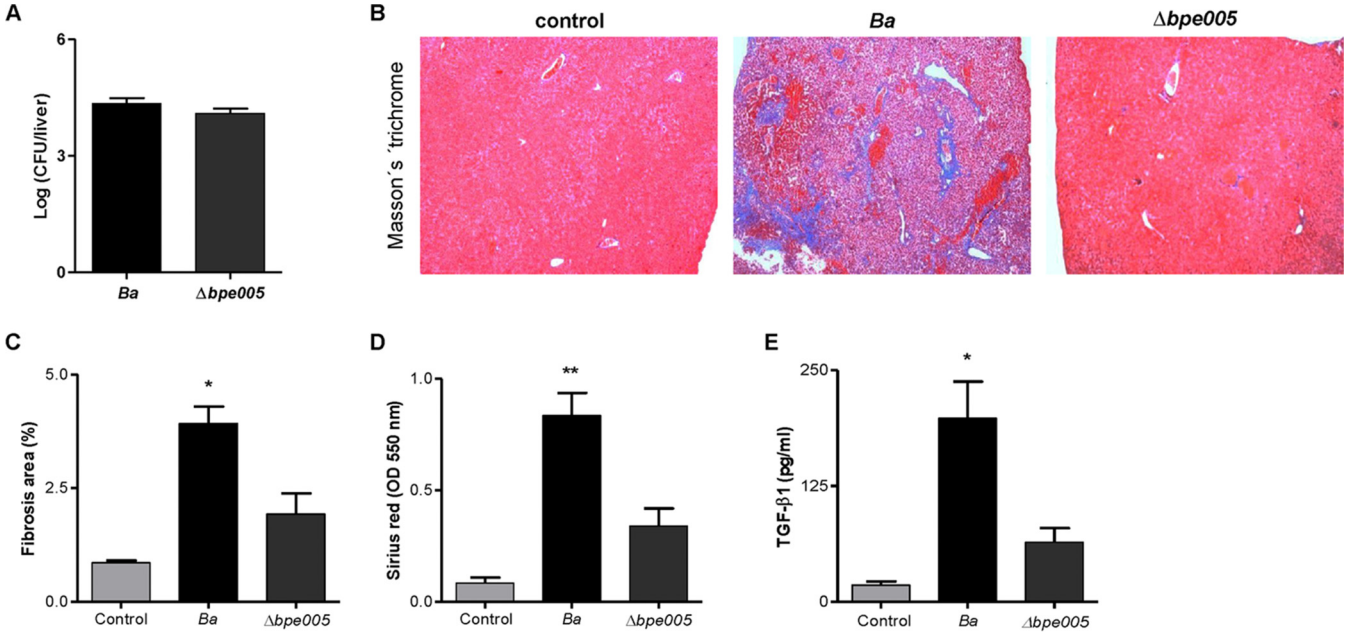

FIG 6 The *B. abortus bpe005* mutant induced a mild fibrotic lesion in livers. (A) Liver colonization by *B. abortus* (Ba) or the *B. abortus bpe005* mutant (Δ*bpe005*) following intraperitoneal infection of mice. (B) Representative photomicrographs of liver sections from control mice, *B. abortus* (*Ba*)-infected mice, and *B. abortus bpe005* isogenic mutant (Δ*bpe005*)-infected mice ($n = 5$) stained with Masson's trichrome staining. (C to E) Collagen-positive areas were quantified using Image Pro-Plus 6.0 software (C), collagen production was determined by quantification of Sirius red (D), and TGF-β1 secretion was measured by ELISA in liver extracts (E) 4 weeks after infection. *, $P < 0.1$; **, $P < 0.01$ (versus control).

cAMP/PKA signaling pathway via the BPE005 effector protein, since the observed effect on the fibrotic phenotype of HSCs was increased by adding an inhibitor of PKA, KT5720, or by transfecting cells with pRSV-PKI plasmid. In addition, the effect was also dependent on the presence of cAMP since the phenomenon was restored when we performed the infection with *B. abortus* in the presence of B2-cAMP. We demonstrated that the T4SS BPE005 effector plays a main role in the modulation of fibrosis as revealed by experiments performed with the *B. abortus bpe005* mutant or with cells transfected with a plasmid encoding BPE005 protein.

BPE005 is a protein containing a cyclic nucleotide monophosphate binding domain. Although its function is unknown, the predicted structure suggests that it might have an effect on cAMP-dependent signaling pathways in the host cell blocking the binding between cAMP and PKA.

Importantly, the histological results corroborated the main role of BPE005 in the modulation of the fibrotic phenotype during *B. abortus* infection, thus confirming the relevance of the *in vitro* findings.

All together, these results indicate that upon infection of LX-2 cells, *B. abortus* triggers a profibrotic response characterized by inhibition of MMP-9 secretion, inducing concomitant collagen deposition and TGF-β1 secretion in a way that involved a functional T4SS and its BPE005 effector protein through a mechanism(s) that involved the cAMP and PKA signaling pathway.

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