The Protein Moiety of Brucella abortus Outer Membrane Protein 16 Is a New Bacterial Pathogen-Associated Molecular Pattern That Activates Dendritic Cells In Vivo, Induces a Th1 Immune Response, and Is a Promising Self-Adjuvanting Vaccine against Systemic and Oral Acquired Brucellosis

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J Immunol published online 29 March 2010
http://www.jimmunol.org/content/early/2010/04/02/jimmunol.0902209

Supplementary Material http://www.jimmunol.org/content/suppl/2010/03/26/jimmunol.0902209.DC1

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Knowing the inherent stimulatory properties of the lipid moiety of bacterial lipoproteins, we first hypothesized that *Brucella abortus* outer membrane protein (Omp)16 lipoprotein would be able to elicit a protective immune response without the need of external adjuvants. In this study, we demonstrate that Omp16 administered by the i.p. route confers significant protection against *B. abortus* infection and that the protective response evoked is independent of the protein lipidation. To date, Omp16 is the first *Brucella* protein that without the requirement of external adjuvants is able to induce similar protection levels to the control live vaccine S19. Moreover, the protein portion of Omp16 (unlipidated Omp16 [U-Omp16]) elicits a protective response when administered by the oral route. Either systemic or oral immunization with U-Omp16 elicits a Th1-specific response. These abilities of U-Omp16 indicate that it is endowed with self-adjuvancing properties. The adjuvanticity of U-Omp16 could be explained, at least in part, by its capacity to activate dendritic cells in vivo. U-Omp16 is also able to stimulate dendritic cells and macrophages in vitro. The latter property and its ability to induce a protective Th1 immune response against *B. abortus* infection have been found to be TLR4 dependent. The facts that U-Omp16 is an oral protective Ag and possesses a mucosal self-adjuvancing property led us to develop a plant-made vaccine expressing U-Omp16. Our results indicate that plant-expressed recombinant U-Omp16 is able to confer protective immunity, when given orally, indicating that a plant-based oral vaccine expressing U-Omp16 could be a valuable approach to controlling this disease. The *Journal of Immunology*, 2010, 184: 000–000.

*B. abortus* is a zoonotic Gram-negative pathogen that causes abortion and infertility in ruminants. In humans, it causes undulant fever, characterized by malaise, aches, and fevers. Human brucellosis can be contracted by accidental contamination from infected animals, handling infected tissues, or consuming undercooked meat or unpasteurized dairy products from infected animals (1). *Brucella* is a facultative intracellular bacterial parasite; the pathogenesis of brucellosis and the nature of the protective immune response are closely related to this property (2). Although both Ab- and cell-mediated immune responses can influence the course of infection with *Brucella*, the latter are essential for clearance of intracellular bacteria. In this respect, IFN-γ plays a central role in acquired resistance against *Brucella*, upregulating macrophage microbial killing (3, 4).

Because brucellosis has serious medical and economic consequences, prevention of animal infection by vaccination is key.
Thus, efforts have been made to prevent the infection through the use of vaccines (3, 5). All commercially available brucellosis vaccines are based on live, attenuated strains of Brucella. Although effective, these vaccines have disadvantages: they can be infectious for humans; they can interfere with diagnosis; they may result in abortions when administered to pregnant animals; and the vaccine strain can spread in the region (3, 6). Currently, no vaccine against human brucellosis is available (7). Therefore, improved vaccines that combine safety and efficacy to all species at risk need to be designed. Within the past few years, we and others have made efforts to develop a vaccine without these drawbacks, a vaccine that would be more effective and safer than those used at present (8–11).

Subunit vaccines, like recombinant proteins, are promising vaccine candidates, because they can be produced at high yield and purity and can be manipulated to maximize desirable activities and minimize undesirable ones. Moreover, they are safer for manipulators, well defined, not infectious, and cannot revert to a virulent strain. However, despite these advantages, they tend to be poorly immunogenic in vivo, and require the coadministration of adjuvants that indirectly enhance the immune response against recombinant proteins. Therefore, recombinant vaccine success is usually dependent on the use of substances endowed with immunomodulatory properties that instruct and control the selective induction of the appropriate type of Ag-specific immune response (12–14).

A promising immune stimulator is the lipid moiety N-palmitoyl-S-(2RS)-2,3-bis-(palmitoyloxy)propyl-cysteine (Pam3Cys). This moiety is found at the N terminus of bacterial lipoproteins and is ubiquitous in and unique to bacteria. Synthetic peptides that are not immunogenic themselves induce a strong Ab response when covalently coupled to Pam3Cys (15, 16). In vitro and in vivo evidence indicates that bacterial lipoproteins and synthetic lipoprotein analogs (lipopeptides) are potent activators of innate immune cells and that these pathogen-associated molecular patterns (PAMPs) trigger cellular activation by binding to pattern recognition receptors on the surfaces of monocytes/macrophages and dendritic cells (DCs) (17, 18). Numerous studies also report different proteins with immunoadjuvant properties, such as outer membrane protein (Omp) A from Klebsiella pneumoniae, Neisseria meningitidis, and the Tat (transcriptional transactivator) protein of HIV-1. These proteins are able to induce strong immune responses in the absence of external adjuvants (19–22).

If we take into account that in brucellosis the infection route usually involves the entry of the pathogen through the mucosal surfaces (23), another key point is the induction of immune responses on the mucosal surfaces. Therefore, an oral vaccine could be a promising candidate to control the disease or to enhance the immune protection provided by currently available vaccines. The protective capacity of several vaccine preparations has already been evaluated by the oral route, including live attenuated strains (24, 25), live recombinant vectors (26, 27), and a few recombinant proteins with mucosal adjuvants (8, 28).

Omp16 of Brucella spp. is a lipoprotein and is expressed in all biovars of B. abortus, B. melitensis, B. suis, B. canis, B. ovis, and B. neotomae (29–31). We have previously reported that B. abortus Omp16 confers protection against a challenge with virulent B. abortus when administered i.p. with systemic adjuvants (IFA or aluminum hydroxide) or orally with a mucosal adjuvant (cholera toxin) (8). Omp16 is also able to activate monocytes, inducing the production of proinflammatory cytokines, a phenomenon mediated by its lipid moiety (32).

We speculate that the N-terminal lipid moiety of Omp16 could confer enough adjuvanticity to evoke a protective immune response against Brucella, abolishing the need of external adjuvants. In the present work, we evaluate this hypothesis.

Materials and Methods

Mice

Female 6- to 8-wk-old specific-pathogen-free BALB/c mice were purchased from the Universidad Nacional de La Plata (La Plata, Argentina). The wild-type (wt) strain C57BL/6 mice were provided by Federal University of Minas Gerais (Belo Horizonte, Brazil), and genetically deficient TLR2−/−, TLR4−/−, and TLR6−/− C57BL/6 mice were provided by S. Akira (Osaka University, Osaka, Japan). B. abortus strain 19 and B. abortus RB51 were obtained from our own laboratory collection (8, 33). Bacterial growth and inocula preparation were performed as previously described (33, 34). All live Brucella manipulations were conducted in biosafety level 3 facilities.

Ags

The recombinant lipopolysaccharide (L- and unlipidated (U-) Omp16 proteins were manufactured as previously described (8, 32). Briefly, recombinant L-Omp16 was isolated from bacterial membranes, and U-Omp16 was isolated from bacterial cytoplasm and then purified by affinity chromatography with an Ni-NTA resin (Qiagen, Hilden, Germany). Expression and purification of the recombinant proteins were checked by SDS-PAGE, followed by silver staining. The identity of the Omp16 Western blot was performed and developed with anti-Omp16-specific mAb (data not shown). Protein concentration was determined by the bicinchoninic acid assay with BSA as a standard (Pierce, Rockford, IL). LPS contamination was adsorbed with Sepharose-polymyxin B (Sigma-Aldrich, St. Louis, MO). Endotoxin determination was performed with Limulus amoeocyte assay (Associates of Cape Cod, Woods Hole, MA). All protein preparations contained <0.25 endotoxin U/mg protein.

HS was obtained as previously described (34). In some experiments, as control, a U-Omp16 enzymatically digested preparation was used. U-Omp16 was treated with proteinase K-agarose from Trichirachium album (Sigma-Aldrich) for 2 h at 37°C, following manufacturer indications. The enzyme immobilized in agarose was then centrifuged out (2000 g, 5 min), and the supernatants were incubated for 1 h at 60°C to inactivate any fraction of soluble enzyme. The complete digestion of the proteins was checked by SDS-PAGE, followed by Coomassie blue staining.

Immunizations and experimental design

Groups of 5–8 mice were anesthetized with methoxyflurane (Mallinckrodt, Phillipsburg, NJ) and then immunized. Some groups of mice were immunized on days 0 and 15 by the i.p. route with 30 μg of 1) L-Omp16, 2) U-Omp16 in PBS, 3) U-Omp16 emulsified in IFA, or 4) PBS-immunized mice as control. As positive control in the protection experiments, a group of mice received a single dose i.p. of 1 × 10^5 CFU live B. abortus strain 19.

Other groups of mice were intragastrically (i.g.) immunized with three consecutive weekly doses of U-Omp16 (100 μg) or PBS in 200 μl 0.1 M bicarbonate buffer (pH 8), as previously described (8, 28). As positive control, a group of mice received a single dose of live B. abortus RB51 (0.5 × 10^7 UFC) by the i.g. route.

At 30 d after the last immunization, mice were challenged with virulent B. abortus for protection experiments or were sacrificed to perform cellular in vitro experiments; other groups of immunized mice were injected in their footpads to perform the delayed-type hypersensitivity (DTH) test. Sera were obtained from blood samples on days 0, 15, 30, and 45 after the first immunization.

Brucella challenge and protective response evaluation

At 1 mo after the last immunization, mice were challenged i.p. with 4 × 10^4 CFU live B. abortus 544 or 2308 or i.g. with 3 × 10^6 CFU B. abortus 2308. At 1 mo postchallenge, their spleens were aseptically removed, homogenized in sterile PBS, diluted, plated, and incubated as described (8, 33), and the number of B. abortus 544 or 2308 CFU was counted. Results were represented as the mean log_{10} CFU ± SD per group. Units of protection were calculated as the difference between the mean log_{10} CFU from the PBS group and the mean log_{10} CFU from the experimental group.
In vitro cellular responses

Single spleen cell suspensions from immunized and control mice were cultured in duplicate at 4 × 10⁶ cells/ml in RPMI 1640 (Life Technologies BRL, Grand Island, NY) supplemented with 10% FCS (Life Technologies), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U penicillin/ml, and 100 µg/ml streptomycin. To stimulate further stimuli were HS (20 µg/ml), U-Omp16 (50 µg/ml), or complete medium alone. When HS was used as the stimulus, the complete medium also contained PB (15 µg/ml; Sigma-Aldrich). After 72 h incubation at 37°C in a humidified atmosphere (5% CO₂ and 95% air), cell culture supernatants were collected. IFN-γ, IL-2, IL-4, IL-5, and IL-10 production was analyzed by sandwich ELISA, using paired cytokine-specific mAbs according to the manufacturer’s instructions (Pharmingen, San Diego, CA). In some experiments, splenocytes were depleted of CD4+ or CD8+ T cells, using mouse CD4 (L3T4) or mouse CD8 (Ly2) Dynabeads according to the manufacturer’s instructions (Dynal Biotech, Oslo, Norway). Depletion was performed before antigenic stimulation. Each T cell population was depleted with an efficacy greater than 99%, as determined by flow cytometry analysis (not shown). After being depleted, cells were suspended in the original volume of complete medium and used in the in vitro stimulation assay.

DTH test

At 4 wk after the last i.g. immunization, mice were injected intradermally in one footpad with 30 µg U-Omp16 and in the contralateral footpad with an equal volume of saline, as negative control. The footpad thickness was measured 24, 48, and 72 h later by using a digital caliper with a precision of 0.01 mm, and the mean increase in footpad thickness (expressed in mm) was calculated according to the following formula: (footpad thickness)U-Omp16 – (footpad thickness)Saline.

In vivo analysis of DC activation

In vivo induction of DC activation was evaluated by measuring the expression of various surface markers by flow cytometry. BALB/c mice were injected i.v. with 100 µg U-Omp16, either untreated or digested with proteinase K, with 50 µg Escherichia coli LPS (Sigma-Aldrich); or with PBS alone. At 20 h postinjection, mice were sacrificed, and spleens from mice were removed and treated for 45 min at 37°C with 400 U/ml collagenase type IV and 50 µg/ml DNase I (Boehringer Mannheim, Indianapolis, IN) in RPMI 1640. After inhibition of collagenase with 6 mM EDTA and 0.5% FCS (Invitrogen Life Technologies, Carlsbad, CA), a single spleen cell suspension was prepared. Spleen cells were incubated for 20–45 min at 4°C in 5% mouse serum in the presence of primary Abs: FITC-, PE-, CyChrome- or PE-Cy5–conjugated Abs specific for CD8α, CD11c, CD40, CD80, CD86, or isotype control. mAbs were purchased from eBioscience (San Diego, CA)and BD Biosciences (Franklin Lakes, NJ). After staining, cells were fixed and analyzed by flow cytometry, using a FACSARia II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

In vitro activation of bone marrow-derived DCs and macrophages

DCs and macrophages were generated from bone marrow (BM) mononuclear cells from wt, TLR2−/−, TLR4−/−, or TLR6−/− C57BL/6 mice, in medium containing rGM-CSF, as previously described (35, 36). Briefly, femora and tibiae were collected from 4- to 12-wk-old mice. After removal of adjacent muscles, bones were flushed throughout their interior with 5 ml HBSS to extract marrow cells. The marrow suspension was filtered through 0.01 mm, and the mean increase in footpad thickness (expressed in mm) was calculated according to the following formula: (footpad thickness)U-Omp16 – (footpad thickness)Saline.

Plant-expressed protein extraction, purification, and immuno blot analysis

For rapid protein detection, 100–200 µg frozen plant material was ground and mixed with 200 µl extraction buffer (25 mM Na₂HPO₄, 17 mM NaH₂PO₄, and 100 mM NaCl, pH 7.4), and incubated on ice for 20 min. After centrifugation at 4°C and maximum speed for 20 min, the supernatant was collected and centrifuged for a second time under the same conditions.

Recombinant protein was purified from plant material, using the Protino Protein Purification System from Ni-NTA (Qiagen, Hilden, Germany) according to the supplier’s protocol. Total soluble protein and purified protein concentration were determined with the BCA Protein Assay Kit (Pierce).

Defined amounts of total plant protein (0.1 µg, 0.5 µg, 1 µg, 5 µg, and 15 µg) and of purified Omp16 (0.05 µg, 0.1 µg, and 0.25 µg) in a volume of 20 µl were mixed with 5 µl SDS sample buffer (250 mM Tris-Cl, pH 6.8; 10% [v/v] SDS; and 0.02% [w/v] bromophenol blue) and incubated at 95°C for 10 min and subjected to SDS-PAGE (15%; see below). Electrophoresis was performed in 1 × SDS running buffer (25 mM Tris, pH 8.3; 192 mM glycine; and 0.1% [v/v] SDS) with 40 V for 1 h and 100 V for the remaining run time. After electrophoresis, the gels were equilibrated in transfer buffer (25 mM Tris; 192 mM glycine; 20% [v/v] methanol) for 10 min, and proteins were transferred to a polyvinylidenefluoride membrane (Hybond-P; Amersham Biosciences, Freiburg, Germany), using Pequlab tank-blot equipment (Pequlab, Erlangen, Germany) at 100 V for 1 h. The membrane was blocked in 5% (w/v) dried milk powder in 1 × PBS-T (5.6 mM Na₂HPO₄, 12 mM H₂O₂; 2.9 mM K₂HPO₄; 2.9 mM NaCl; 0.05% [v/v] Tween 20; and pH 7.2–7.4). Subsequently, the membrane was incubated with the primary Ab (murine anti-Omp16 mAb) at a dilution 1:2000 in 1 × PBS-T at room temperature. After washing three times in 1 × PBS-T for 10 min, the membrane was incubated with a HRP-conjugated secondary Ab (goat anti-mouse IgG-HRP; Santa Cruz Biotechnology, Heidelberg, Germany; dilution 1:10000 in 1 × PBS-T) for 1 h at room temperature. Washing was repeated as described, and immunoblots were developed using the Chemiluminescence Detection Kit (AppliChem, Darmstadt, Germany) according to the supplier’s instructions.

Sequence analysis

The amino acid sequence of U-Omp16 was used to perform searches of nonredundant protein databases. The BLAST program available at www.ncbi.nlm.nih.gov/BLAST/ and also the Phylogeny.fr BLAST platform at www.phylogeny.fr/version2_cgi/index.cgi were run (using MUSCLE for multiple alignment and PhyML for phylogeny) (40, 41).

Differentiated BM-derived DCs or macrophages were stimulated for 24 h with complete medium alone or complete medium with: U-Omp16 (1, 5, 10, or 50 µg/ml), E. coli LPS (1 µg/ml), or Pam3CSK4 (1 µg/ml; InvivoGen, San Diego, CA). A plant-made U-Omp16 was used as the stimulant protein in some assays. For control groups, in some experiments, cells were stimulated either with proteinase K-digested U-Omp16 (50 µg/ml) or with U-Omp16 (50 µg/ml) plus PB (15 µg/ml). After stimulation, cell-free supernatants were collected and assayed by sandwich ELISA for TNF-α and IL-12 (p40) production, using paired cytokine-specific mAbs according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

Transient expression of U-Omp16 in Nicotiana benthamiana plants

The sequence of omp16 lacking the segment coding for the N-terminal signal peptide was amplified by PCR, using primers PO16-102 (5′-TCTTGCTTCAAGGATGAACTTCTTGAAAATGCGGCTGAC- TCGG-3′) and PO16-201 (5′-GCTCTAGATCTGTTGTTGTTGTTGTTGTTGTC-3′). Primer PO16-201 also included the nucleotides encoding for a 6× histidine extension that was already present in the template vector [pET-U-Omp16 (32)]. The PCR product was cloned into the vector pCRBhunt (Invitrogen, Karlsruhe, Germany), and its identity was confirmed by sequencing. For subcloning, a Bsd-XhoI fragment from this vector was inserted into the vector pCH10990 (38); the resulting vector was termed PO16-6121 and mobilized into agrobacteria by chemical transformation (39). Infiltration of N. benthamiana plants was carried out as described by Marillonnet et al. (36).

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Results

Omp16 confers protection against B. abortus challenge without the need of external adjuvants or its lipid moiety

As stated before, it was already described that the lipid moeity of lipoproteins has adjuvant activity per se (42). To address whether this was the case for the B. abortus Omp16 lipoprotein, a group of mice was immunized i.p. with the lipoprotein alone, whereas other groups of animals were immunized with PBS (negative control) or the vaccine strain B. abortus S19 (positive control). Immunized mice were challenged with virulent B. abortus 544, and the potential of the L-Omp16 per se to impart protection without adjuvants was evaluated on the basis of its ability to eliminate the bacterial burden (B. abortus 544) in spleens of immunized mice at day 30 postchallenge. Immunization with L-Omp16 without adjuvants induced a significant level of protection (1.49 U of protection, p < 0.01) when compared with the control PBS-immunized mice (Table I). This result indicates that L-Omp16 is endowed with an intrinsic adjuvant activity that allows it to confer protection against a challenge with virulent B. abortus, without the need for external adjuvants.

To corroborate the finding that the intrinsic adjuvanticity of L-Omp16 was due to its lipid moiety, another experiment has been conducted in which mice were immunized with L-Omp16 or with U-Omp16, in both cases without adjuvants. As before, L-Omp16 conferred protection (1.59 U of protection, p < 0.01 versus PBS), but unexpectedly U-Omp16 also induced significant protection levels against an experimental B. abortus infection (1.68 U of protection, p < 0.01 versus PBS). Notably, the protection levels elicited by U-Omp16 were similar to those elicited by its respective lipidated version (p > 0.05), indicating that the molecular region responsible for the observed self-adjuvanticity is the protein portion of this lipoprotein. It is worth mentioning that the protection levels elicited by U-Omp16 were similar to those elicited by the attenuated vaccine strain B. abortus S19 (p > 0.05) (Table II).

It is important to bear in mind that the purification yield of L-Omp16 is much lower than that obtained when producing U-Omp16. Moreover, it is necessary to make extra LPS depletion rounds to get the recombinant lipoprotein LPS-free (8). Those aspects and its self-adjuvanticity led us to focus on the protein region of Omp16. Besides, the addition of an external adjuvant, like IFA, did not improve the U-Omp16–elicited protective responses (U-Omp16 + IFA: 1.46 versus U-Omp16: 1.33 U of protection; p > 0.05) (Table III).

Altogether, these results suggest that the protein portion of the B. abortus Omp16 has an inherent adjuvant activity that allows it to induce a protective immune response against an in vivo Brucella challenge.

Immunization with U-Omp16 induces a specific Th1 cellular immune response

Given the intracellular nature of Brucella spp., it is well known that cellular immune responses (in particular, Th1) are crucial for conferring protection against this pathogen (43). Thus, we decided to test whether immunization with U-Omp16 without adjuvants is able to induce a specific cellular immune response. Splenocytes from U-Omp16–immunized mice were stimulated in vitro with a known Brucella membrane extract that contains native Omp16 (HS). After stimulation, splenocytes from U-Omp16–immunized mice produced large amounts of IFN-γ in comparison with the same splenocytes incubated with complete medium alone (p < 0.01). This effect was specific, because spleen cells from U-Omp16–immunized mice produced higher levels of IFN-γ than did cells from PBS-immunized mice in response to the same stimulus (p < 0.01) (Fig. 1A). The splenocytes from both groups of mice did not produce IL-2, IL-4, IL-5, or IL-10 in response to HS (data not shown). To evaluate which cells were responsible for the specific IFN-γ production, we selectively depleted CD4+ T cells or CD8+ T from whole splenocytes. When splenocytes from U-Omp16–immunized mice were depleted of the CD4+ T cell population, the specific IFN-γ production in response to HS was abrogated (p < 0.01 versus not depleted) (Fig. 1B). On the contrary, CD8+ T cell population depletion did not affect the specific IFN-γ production (Fig. 1B). This result indicates that U-Omp16 immunization

Statistical analysis

Statistical analysis and plotting were performed using GraphPad Prism 4 software (GraphPad, San Diego, CA). Protection results were evaluated using one-way ANOVA and the Dunnett multiple-comparison posttest. The data from cytokine production, DTH response, and flow cytometry were analyzed using one-way ANOVA with Bonferroni’s posttest. When data were not normally distributed, a logarithmic transformation was applied prior to the analysis; then parameters showed normal distribution. A p value < 0.05 was taken as the level of significance.

Table I. Protection against B. abortus 544 in BALB/c mice immunized with L-Omp16 without adjuvant

<table>
<thead>
<tr>
<th>Vaccine (n = 6)</th>
<th>Log_{10} CFU of B. abortus 544 at Spleen (mean ± SD)</th>
<th>Units of Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Omp16</td>
<td>4.25 ± 0.44abc</td>
<td>1.49</td>
</tr>
<tr>
<td>B. abortus strain 19</td>
<td>3.71 ± 0.17bc</td>
<td>2.03</td>
</tr>
<tr>
<td>PBS</td>
<td>5.74 ± 0.15b</td>
<td>0</td>
</tr>
</tbody>
</table>

The content of bacteria in spleens is represented as the mean log_{10} CFU ± SD per group.

Significantly different from PBS-immunized mice; p < 0.01 estimated by Dunnett’s test.

Significantly different from B. abortus strain 19 immunized mice; p < 0.01 estimated by Dunnett’s test.

Table II. Protection against B. abortus 544 in BALB/c mice immunized with L-Omp16 or U-Omp16 without adjuvant

<table>
<thead>
<tr>
<th>Vaccine (n = 5)</th>
<th>Adjuvant</th>
<th>Log_{10} CFU of B. abortus 544 at Spleen (mean ± SD)</th>
<th>Units of Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Omp16</td>
<td>None</td>
<td>4.68 ± 0.55abcd</td>
<td>1.59</td>
</tr>
<tr>
<td>B. abortus strain 19</td>
<td>None</td>
<td>4.59 ± 0.42bc</td>
<td>1.68</td>
</tr>
<tr>
<td>PBS</td>
<td>None</td>
<td>6.27 ± 0.14b</td>
<td>0</td>
</tr>
</tbody>
</table>

The content of bacteria in spleens is represented as the mean log_{10} CFU ± SD per group.

Significantly different from PBS-immunized mice; p < 0.01 estimated by Dunnett’s test.

Significantly different from B. abortus strain 19 immunized mice; p < 0.01 estimated by Dunnett’s test.

Table III. Protection against B. abortus 544 in BALB/c mice immunized with U-Omp16 without adjuvant or in IFA

<table>
<thead>
<tr>
<th>Vaccine (n = 5)</th>
<th>Adjuvant</th>
<th>Log_{10} CFU of B. abortus 544 at Spleen (mean ± SD)</th>
<th>Units of Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-Omp16</td>
<td>None</td>
<td>4.37 ± 0.14b</td>
<td>1.33</td>
</tr>
<tr>
<td>U-Omp16</td>
<td>IFA</td>
<td>4.24 ± 0.18b</td>
<td>1.46</td>
</tr>
<tr>
<td>PBS</td>
<td>None</td>
<td>5.70 ± 0.20b</td>
<td>0</td>
</tr>
</tbody>
</table>

The content of bacteria in spleens is represented as the mean log_{10} CFU ± SD per group.

Significantly different from PBS-immunized mice; p < 0.01 estimated by Dunnett’s test.
FIGURE 1. U-Omp16 induces a specific cellular immune response when administered i.p. without adjuvants. A, Spleen cells of PBS- or U-Omp16-immunized mice (4 × 10^6/ml) were stimulated in vitro with an extract of Omps of Brucella (HS) or complete medium alone (−). Cell-free culture supernatants were collected at 72 h poststimulation for the measurement of IFN-γ (pg/ml) by ELISA. Results are shown as mean ± SEM for each group and are representative of three independent experiments. **Significantly different from the same stimulus in PBS-immunized mice (p < 0.01). B, CD4+ or CD8+ T cell IFN-γ responses in U-Omp16-immunized mice. Splenocytes from PBS- or U-Omp16-immunized mice were depleted of CD4+ cells (−CD4+) or CD8+ T cells (−CD8+), using mouse CD4 (L3T4) Dynabeads or mouse CD8 (Lyt2) Dynabeads or were not depleted (T). These cells were stimulated, and IFN-γ was measured in culture supernatants as described in A. **Significantly different from analogous treated cells from PBS-immunized mice (p < 0.01). ##Significantly different from nondepleted cells (T) from the same group stimulated with the same stimulus (p < 0.01).

Oral delivery of U-Omp16 confers protection against B. abortus challenge and elicits a Th1 immune response without the need of external adjuvants

Oral infection is one of the principal ways in which the disease is acquired, in both humans and animals (23, 28). For that reason, we sought to evaluate the protection afforded when mice were immunized i.g. with U-Omp16 without adjuvants. As negative control, a group was immunized similarly with PBS, and as positive control another group received the already reported oral protective vaccine strain B. abortus RB51 (24, 25). Oral immunization with U-Omp16 without adjuvants induced a significant protective response against an oral challenge with B. abortus 2308 (Table IV). This immunization elicited a specific DTH response at 48 and 72 h post–U-Omp16 intradermal injection (Fig. 2A). Furthermore, splenocytes from mice orally immunized with U-Omp16 secreted IFN-γ after an in vitro stimulation with HS (p < 0.05 versus PBS-immunized mice) (Fig. 2B). In contrast, splenocytes from all immunized mice did not produce IL-2, IL-4, IL-5, or IL-10 in response to HS (data not shown).

All in all, these results indicate that the protein region of the Omp16 lipoprotein, when delivered by the oral route without adjuvants, has the ability to induce a Th1 immune response in vitro as well as in vivo while mediating immunoprotection against an oral Brucella challenge.

<table>
<thead>
<tr>
<th>Vaccine (n=5)</th>
<th>Expressed in</th>
<th>Adjuvant</th>
<th>Log_{10} CFU of B. abortus 2308 at Spleen (mean ± SD)</th>
<th>Units of Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-Omp16 E. coli</td>
<td>None</td>
<td></td>
<td>4.61 ± 0.17</td>
<td>0.94</td>
</tr>
<tr>
<td>U-Omp16 Tobacco</td>
<td>None</td>
<td></td>
<td>4.29 ± 0.08</td>
<td>1.26</td>
</tr>
<tr>
<td>B. abortus –</td>
<td>None</td>
<td></td>
<td>3.74 ± 0.44</td>
<td>1.81</td>
</tr>
<tr>
<td>RB51 –</td>
<td>None</td>
<td></td>
<td>5.55 ± 0.05</td>
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</table>

The content of bacteria in spleens is represented as the mean log_{10} CFU ± SD per group.

*Significantly different from PBS-immunized mice; p < 0.01 estimated by Dunnett’s test.

Significantly different from B. abortus RB51 immunized mice; p < 0.01 estimated by Dunnett’s test.

**Significantly different from B. abortus RB51 immunized mice; p < 0.05 estimated by Dunnett’s test.
U-Omp16 activates DCs in vitro and in vivo

DCs are initiators and modulators of immune responses with the ability to activate naïve T cells. Therefore, we evaluated the ability of U-Omp16 to induce the activation of DCs in vitro. BM-derived DCs of C57BL/6 mice were incubated in vitro with U-Omp16, and the TNF-α production was measured. As controls, BM-derived DCs were incubated with U-Omp16 plus PB or with U-Omp16 completely digested with proteinase K (Fig. 3A); at the same time, complete medium and E. coli LPS were used as negative and positive controls, respectively. After being stimulated with U-Omp16, DCs produced significant amounts of TNF-α (p < 0.001 versus complete medium-stimulated cells). The ability of U-Omp16 to activate DCs was not modified by the addition of PB in the medium (Fig. 3B), indicating that the result is not an outcome of LPS contamination. In the same way, when U-Omp16 digested with proteinase K was used to stimulate DCs, its activating ability was lost (p > 0.05 versus complete medium-stimulated cells) (Fig. 3B), indicating that the measured activity resides in the protein. This result was also specific for U-Omp16 because in the same stimulation conditions, another B. abortus recombinant protein (U-Omp19) was unable to stimulate DCs to produce cytokines in vitro (data not shown).

The latter results indicate that U-Omp16 is able to activate DCs in vitro, but did not ensure that the same would occur in vivo; therefore, we decided to evaluate the U-Omp16 ability to activate DCs in vivo. To achieve this, BALB/c mice were injected i.v. with U-Omp16 (Fig. 3A). As controls, other groups of mice were injected with either U-Omp16 completely digested with proteinase K (Fig. 3A), E. coli LPS (positive control), or PBS alone (negative control). At 20 h postinjection, splenic CD11c+ DCs were analyzed directly ex vivo by flow cytometry for the expression of the surface markers CD40, CD80, and CD86. DCs from U-Omp16-inoculated mice showed an increased expression of the costimulatory molecules CD40, CD80, and CD86, compared with the expression on DCs from control mice receiving PBS (Fig. 3C). Similarly, E. coli LPS induced the upregulation of the three costimulatory molecules on splenic DCs from injected mice (Fig. 3C). The ability of U-Omp16 to induce DC maturation in vivo was totally abrogated by protein digestion with proteinase K, whereas the same treatment did not influence the E. coli LPS effect on these maturation DC markers (Fig. 3C; data not shown).

**FIGURE 3.** U-Omp16 induces in vitro and in vivo activation of DCs. A, Coomassie blue stained 12.5% SDS PAGE of U-Omp16 untreated or digested with proteinase K (U-Omp16 + PK) that was used for the in vivo DC activation assay. B, In vitro stimulation of BM-derived DCs. C57BL/6 BM-derived DCs were incubated in vitro with complete medium (–), U-Omp16 (50 μg/ml) (NT U-Omp16), U-Omp16 (50 μg/ml) with PB (15 μg/ml) (Pol B U-Omp16), proteinase K digested U-Omp16 (50 μg/ml) (PK U-Omp16), or E. coli LPS (1 μg/ml) (LPS). At 24 h later, the TNF-α (pg/ml) concentration was measured by ELISA in supernatants. Results are shown as mean ± SEM for each stimulation condition. **p**Significantly different from the TNF-α amounts in complete medium stimulated cells (p < 0.001). C, In vivo stimulation of splenic DCs. BALB/c mice were injected i.v. with 100 μg of U-Omp16 either untreated or digested with proteinase K or with E. coli LPS or PBS alone as controls. At 24 h postinjection, splenic CD11c+ DCs were analyzed for their activation status by assessing the surface expression of CD40, CD80, and CD86 molecules by flow cytometry. This experiment was conducted three times with similar results. Histograms display results from one representative experiment. D, CD40 upregulation on the different DC subpopulations. BALB/c mice were injected i.v. as in C, and the surface expression of CD40 was analyzed on total splenic CD11c+ DCs, myeloid CD11c+CD8α− DCs, or lymphoid CD11c+CD8α+ DCs. Histograms display results from one representative experiment out of three. E, Mean fluorescence intensities for the CD40 expression on the indicated subpopulations from three different mice per group are shown in the bar graph. Significant differences between U-Omp16– or LPS-injected mice and PBS-injected mice are denoted on the graph: *p < 0.05, **p < 0.01. Results are representative of two independent experiments.
Spleenic conventional CD11c+ DCs can be subdivided phenotypically into three different subsets based on surface marker expression: CD8α+CD4− DCs (myeloid DCs), CD8α−CD4+ DCs, and CD8α−CD4− DCs; the last two subsets are often referred to as CD8α− DCs (lymphoid DCs) (44, 45). The effect of U-Omp16 on CD8α+ DC and CD8α− DC subsets in vivo is shown in Fig. 3D. Total splenic DCs of mice treated with U-Omp16 upregulated the expression of the costimulatory molecule CD40 (Fig. 3C, 3D), this upregulation involved both splenic DC subpopulations: myeloid DCs (CD11c+CD8α+, Fig. 3D) and lymphoid DCs (CD11c+CD8α−, Fig. 3D), the effect being more evident in the myeloid subpopulation (Fig. 3E).

Thus, U-Omp16 self-adjuvanticity could be mediated, at least in part, by the stimulation of DC maturation in vivo, in particular, activation of myeloid DCs.

**TLR-4 is involved in U-Omp16 self-adjuvanticity**

Signaling through TLRs has been shown to be immunostimulatory, inducing host cells to proliferate, secrete cytokines and chemokines, and upregulate costimulatory molecules. The latter characteristic renders TLR ligands a potent addition to the vaccine adjuvant repertoire by possessing the capability to link the innate and adaptive immune systems, thus inducing not only an inflammatory response but also activation of the adaptive arm of the immune system (46). Among the TLRs, TLR2 and TLR4 mediate the response to the most diverse set of molecular structures, including some bacterial proteins (47), and it has been reported that some Omps interact with heterodimers of TLR2 and TLR6 (48). To determine if TLR2, TLR4, or TLR6 is involved in the immunostimulant activity of U-Omp16, BM-derived DCs or macrophages from TLR2−/−, TLR4−/−, TLR6−/−, or wt C57BL/6 mice were stimulated in vitro with different doses of U-Omp16 (1, 5, 10, and 50 μg/ml), complete medium alone (unstimulated control), E. coli LPS, or Pam3Cys. After 24 h of stimulation, the TNF-α and IL-12 p40 concentrations in supernatants were measured. Both BM-derived DCs and macrophages from C57BL/6 wt mice produced significant levels of both cytokines after stimulation with U-Omp16 in a dose-dependent fashion (Figs. 4, 5, respectively) when compared with the same cells treated with culture media alone (p < 0.01), indicating that both cell types were stimulated by U-Omp16 in vitro. The same pattern of response to U-Omp16 stimuli in vitro was elicited in BM-derived DCs and macrophages from TLR2−/− or TLR6−/− C57BL/6 mice, indicating that neither TLR2 nor TLR6 is involved in the activation of both cell types by U-Omp16 in vitro. In contrast, U-Omp16 was unable to stimulate BM-derived DCs or macrophages from TLR4−/− C57BL/6 mice to secrete these cytokines, suggesting that the stimulating activity of U-Omp16 in vitro would be mediated by this receptor. E. coli LPS and Pam3Cys stimuli were included as controls, and, as expected, they induced the activation of all cell types excluding those derived from TLR4−/− mice stimulated with E. coli LPS or TLR2−/− mice stimulated with Pam3Cys.

Then we sought to determine if in vivo TLR4 plays a role in the generation of the adaptive response elicited by U-Omp16. For that purpose, C57BL/6 wt or TLR4−/− mice were vaccinated with U-Omp16 without adjuvants, and the elicited cellular and protective responses were evaluated. Spleen cells of immunized mice were cultured in vitro in the presence of U-Omp16 or culture medium alone. The antigenic stimulus produced significantly large levels of IFN-γ from the splenocytes of U-Omp16–vaccinated mice, compared with the production in spleen cells of the control mice (p < 0.05) (Fig. 6). In agreement with our previous in vitro results, splenocytes from mice lacking TLR4 did not respond to this stimulus in vitro, indicating that U-Omp16 requires TLR4 in vivo for eliciting a cellular Th1 immune response. Similarly, immunization with U-Omp16 did not elicit significant protection against B. abortus infection in TLR4−/− mice (0.51 U of protection, p > 0.05 versus PBS). As expected, U-Omp16 delivery in the absence of external adjuvants elicited 1.15 U of protection (p < 0.01 versus PBS) in C57BL/6 wt mice (Table V).

Overall, these results indicate that U-Omp16 requires the presence of TLR4 for displaying its self-adjuvanting properties, activating macrophages and DCs while eliciting a protective Th1 immune response against *Brucella*.

**U-Omp16 is a new bacterial PAMP**

The ability of U-Omp16 to interact with the innate immune system through TLR4 suggested that it would be a PAMP. As PAMPs are generally constituted by conserved structures that are widespread on different pathogens (49), we decided to investigate whether...
The elicited U-Omp16–specific cellular Th1 immune response is TLR4 dependent. Spleen cells of PBS- or U-Omp16–immunized C57BL/6 wt or TLR4$^{-/-}$ mice were stimulated in vitro with complete medium alone (–) or complete medium containing either U-Omp16 (1, 5, 10, or 50 μg/ml), E. coli LPS (1 μg/ml), or Pam3Cys (1 μg/ml). Cell-free supernatants were collected, and the concentration of IFN-γ (A) or IL-12 p40 (B) was determined by ELISA. Results are shown as mean ± SEM for each group and are representative of three independent experiments. *p < 0.05, significantly different from PBS-treated control (−). #p < 0.01, significantly different from C57BL/6 wt control (−).

It is noteworthy that BLAST and pfam sequence analyses stated the protein portion of Omp16 (U-Omp16) revealed high homology—near to identity (identities, 100–76%; scores, 297–215 bits)—with other Omps of the α Proteobacteria class, order Rhizobiales, particularly in the Brucellaceae, Rhizobiaceae, Aurantimonadaceae, and Phyllobacteriaceae families (identical proteins are present in 27 different organisms among them) (Supplemental Fig. 1). Moreover, U-Omp16 is highly conserved (identities, 67–49%; scores, 189–142) among the α Proteobacteria class, homologous proteins being present in 99 organisms from Caulobacterales, Rhizobiales, Rhodobacterales, Rhodospirillales, and Sphingomonadales, as well as in the B. abortus species, such as in Helicobacter bizzozeronii (Supplemental Fig. 2).

A plant-based U-Omp16 vaccine confers protection against B. abortus

The notion that U-Omp16 evokes a protective and cellular Th1 immune response when given orally without adjuvants prompted us to develop a plant-made vaccine based on U-Omp16. One major prerequisite for vaccine production in plants is the development of a reliable system that allows the expression of the desired Ag. As a first approach, we studied the transient expression of U-Omp16 in N. benthamiana plants.

The protein portion of Omp16 was generated by PCR and cloned into the 3′-provector module pICH10990 for transient nuclear expression. The resulting vector termed p016 6122 was mobilized into agrobacteria and infiltration performed with the 5′-module pICH15879 and recombinase module pICH10881, as described (38). Expression of the recombinant U-Omp16 was monitored by SDS-PAGE and immunoblot. As illustrated in Fig. 7A, U-Omp16 accumulated to significant amounts in infiltrated N. benthamiana leaves. Determination of Omp16 content by immunoblot revealed that a fraction of 2% of the total soluble protein consists of the C-terminal region of many Gram-negative bacterial Omps—for example, porin-like integral membrane proteins (such as OmpA), small lipid-anchored proteins (such as pal), and MotB proton channels. In terms of the innate immune system, published studies have indicated that K. pneumoniae and E. coli OmpA induce DC activation (19, 50); therefore, we conducted a multiple sequence alignment comparing B. abortus U-Omp16 with E. coli OmpA and K. pneumoniae OmpA. Although amino acids are different, U-Omp16 from Brucella has significant homology with K. pneumoniae OmpA (24% identity; score, 45.4 bits) and from E. coli OmpA (25% identity; score, 48.5) (Supplemental Fig. 3).

Taken together, these results indicate that Omp16 would constitute a highly conserved protein among α Proteobacteria. By recognizing this protein, the innate immune system could also sense the presence of these particular types of bacteria.
recombinant bacterial protein. Total expression level was rather moderate for this protein; however, sufficient plant material could be provided for purification of U-Omp16 for subsequent immunization studies. Purification of the recombinant protein was achieved owing to the C-terminal poly-histidine extension (6×His tag) by immobilized metal affinity chromatography. Up to 20 μg of U-Omp16 could be purified from 1 g of fresh leaf material. The U-Omp16 preparation from plants showed a high purity, as exemplified by SDS-PAGE and subsequent Coomassie staining (Fig. 7A). All visible protein bands also gave a positive signal on Western blot with the appropriate anti-Omp16 Ab (Fig. 7B); therefore, we conclude that they constitute different manifestations of Omp16.

To evaluate whether the plant-made U-Omp16 contained the immunostimulating properties from its E. coli-made pair, an in vitro BM-derived DC or macrophage stimulation assay was performed. When wt-derived cells were incubated in vitro with plant-made U-Omp16, both cell types produced large amounts of TNF-α and IL-12 p40 in response to the stimulus (p < 0.01 versus nonstimulated cells), whereas TLR4−/− cells did not produce any from the measured cytokines (Fig. 8). This result indicates that, as occurs with E. coli U-Omp16, plant-made U-Omp16 stimulates the innate immune system by a TLR4-mediated pathway.

To assess the protective capacity of plant-made U-Omp16, groups of mice were orally immunized in parallel either with tobacco-expressed purified U-Omp16 or with E. coli-expressed purified U-Omp16, and the elicited protective response was evaluated. Plant-made U-Omp16 was able to elicit a significant level of protection (1.26 U of protection, p < 0.01 versus PBS) against oral B. abortus infection. The elicited protection was similar to that induced by U-Omp16 produced in E. coli (p > 0.05 tobacco versus E. coli) (Table V).

Altogether, these results indicate that plant-made U-Omp16 can be correctly expressed in a plant system and conserve its intrinsic immunogenicity: it activates the innate immune system through its interaction with TLR4, allowing it to induce a protective response against an oral Brucella challenge. Therefore, a plant-made vaccine could be a successful approach to control of brucellosis.

### Discussion

Effective vaccines have three key components: 1) an Ag against which adaptive immune responses are generated, 2) an immune stimulus or adjuvant to signal the innate immune system to potentiate the Ag-specific response, and 3) a delivery system to ensure that the Ag and adjuvant are delivered together at the right time and location (51).

Available brucellosis vaccines are live attenuated strains that have all these key components but also have several disadvantages; thus, new improved vaccines need to be developed. Highly purified Ags offer potential advantages over traditional vaccines, including a high degree of safety and the capacity of eliciting highly specific immune responses, but in general they need to be coadministered with additional immunostimulant substances (adjuvants) because they are poorly immunogenic (12–14).

Knowing the inherent stimulatory properties of the lipid moiety of bacterial lipoproteins (32, 52), we hypothesized that B. abortus Omp16 lipoprotein would be able to elicit a protective immune response without the need of external adjuvants. Our results confirm that the lipoprotein Omp16 of B. abortus as a recombinant Ag is able to induce a protective immune response against a challenge with virulent B. abortus when given to mice without external adjuvants, indicating that Omp16 lipoprotein has an intrinsic adjuvanticity. To our surprise, however, U-Omp16 administered without adjuvants elicited similar levels of protection, suggesting that the protein portion of Omp16 has enough intrinsic adjuvant activity.

This property is not unique to Omp16, because several bacterial proteins have intrinsic adjuvant activity that allows them to induce specific and effective immune responses without the help of external adjuvants, such as the K. pneumoniae OmpA or the Omp complex of N. meningitidis serogroup B. The self-adjuvanting properties of these proteins were attributed to their ability to activate different cellular types, mainly DCs (19–21, 53). Recently, other investigators have identified a Borrelia burgdorferi lipoprotein (BmpA) whose protein portion stimulates the secretion of proinflammatory cytokines in human synovial cells (54).
FIGURE 8. Plant-made U-Omp16 stimulates DCs and macrophages in vitro in a TLR4-dependent fashion. BM-derived DCs (A, B) and macrophages (C, D) from wt and TLR4−/− C57BL/6 mice were treated in vitro for 24 h with complete medium alone (−) or complete medium containing U-Omp16 (50 μg/ml for DCs or 1, 5, and 10 μg/ml for macrophages) or E. coli LPS (1 μg/ml). Cell-free supernatants were collected, and the concentration of TNF-α (A, C) or IL-12p40 (B, D) was determined by ELISA. Results are represented as mean ± SEM of triplicate measurements. *p < 0.001, significant differences between stimulated and complete medium alone treated cells.

The ability of U-Omp16 to induce a protective immune response without the addition of adjuvants is not a frequently occurring characteristic in the brucellosis field. Indeed, other Brucella proteins have been evaluated for their ability to induce protective responses when administered without adjuvants, giving unsatisfactory results. None of the Brucella p39, BLS, or L7/L12 proteins elicited a significant protective response when administered without adjuvants (33, 55, 56), whereas immunization with the DnaK protein or a fusion protein of Omp16 and L7/L12 proteins induced moderate protection levels, but lower than those induced by the control attenuated vaccine strain B. abortus S19 or the same proteins with adjuvants (10, 11). In the case of the fusion protein containing Omp16, the lower protection levels elicited by this preparation compared with those elicited by U-Omp16 in this report could be due to the lower doses used in immunization or to differences between the immunization routes used.

The idea that protection elicited by Omp16 without adjuvants was independent of protein acylation represents an advantage in vaccine development, particularly in the scale-up for bulk manufacture and in the final cost of the product, the production of L-Omp16 being a more time-consuming and expensive process than the production of U-Omp16 (8). Moreover, the protection levels induced by U-Omp16 in the absence of external adjuvants were statistically similar to those elicited by the vaccine strain B. abortus S19 and were not improved by the addition of an adjuvant, such as IFA, indicating that U-Omp16 is a worthy vaccine candidate for brucellosis control.

To our knowledge, Omp16 is the first Brucella protein able to induce protection levels similar to those induced by the control live vaccine strain without the requirement of external adjuvants. This unique quality represents an exceptional benefit because external adjuvants might sometimes present risk, inducing adverse reactions, such as local inflammation at the injection site with the induction of granuloma or sterile abscess formation (57). Moreover, U-Omp16 lost its DC-activating capacity when completely digested with proteinase K, indicating that the in vivo activation of DCs, leading to the priming of specific naive T cells and evoking a protective Th1 immune response against Brucella infection, because IFN-γ plays a central role in protective responses against Brucella (61).

Induction of adaptive specific immune responses involves the activation of specific T cells by APCs. Among them, DCs are the key mediators of adaptive immunity. The quality of signals received by DCs in response to PAMPs influences the nature of the evoked adaptive response. Following stimulation with PAMPs, DC maturation occurs, a process in which DCs undergo phenotypic changes resulting in an improved ability to promote T cell responses. Many immunopotentiators mediate their effect by activating DCs (62, 63). Our results show that U-Omp16 is able to stimulate DC activation in vitro and in vivo. This stimulation could not be due to LPS contamination in the preparations, because the protein preparations were exhaustively depleted of LPS with Sepharose-PB, as assessed by Limulus amoebocyte assay. Moreover, U-Omp16 lost its DC-activating capacity when completely digested with proteinase K, indicating that the in vivo elicited DC activation was due to the protein rather than to another nonprotein contaminant. The same treatment did not affect the stimulating properties of E. coli LPS.

The DC subpopulation (CD8α+) is able to produce large amounts of IL-12 when activated with several immunostimulants, being involved in the induction of Th1 adaptive immune responses (44, 45, 63–65). In the current study, we have demonstrated that the DC stimulating activity of U-Omp16 was more prominent in the CD8α+ DC subpopulation. Therefore, our results suggest that the intrinsic adjuvant activity of the protein portion of Omp16 could be mediated, at least in part, by the in vivo activation of DCs, leading to the priming of specific naive T cells and evoking a protective Th1 immune response against Brucella infection.
It is now known that signaling of one or more receptors on immune cells (for example, TLRs) results in a rapid inflammatory response, leading to enhanced presentation of Ags to the immune system (47, 66, 67). In the current study, we have demonstrated that the ability of U-Omp16 to stimulate innate immune cells (DCs and macrophages) as well as to induce an adaptive specific immune response in the absence of adjuvants required the presence of TLR4. Although U-Omp16 immunization did not induce a statistically significant protection against Brucella in TLR4-/-mice, the protection was not totally abrogated, suggesting that other mechanisms (additive or redundant) would account for the elicited protection against Brucella. The best characterized TLR4 agonist is *E. coli* LPS. However, there are several agonists for this receptor that are not related to LPS, including different proteins. Among the protein TLR4 agonists are endogen molecules, such as the extradomain A of fibronectin (68, 69) and gp96 (70), and pathogen-derived proteins, such as the peptidyl-propyl cis-trans isomerase from *Helicobacter pylori* (71), the DnAK protein from *Francisella tularensis* (72), Hsp60 from *Chlamydia pneumoniae* (73), and the Brucella lumazine synthase BLS from *B. abortus* (74). Altogether, our results indicate that U-Omp16 could constitute a new PAMP recognized by TLR4.

A previous report by our laboratory indicates that *B. abortus* L-Omp16 possesses proinflammatory activity on human monocytes in a TLR2-dependent fashion. This activity was dependent on protein lipidation (32). In that report, assays were designed to imitate the lipoprotein concentration present on the bacterial surface at the site of infection (0.01–1 μg/ml). The current study is different because the protein concentrations used (5–50 μg/ml) attempted to resemble those present at the site of injection after vaccine administration (150 μg/ml). Therefore, the absence of response by human monocytes following U-Omp16 stimulus reported in our previous paper could be due to the lower concentrations used in such assays.

PAMPs are generally well conserved structures among different pathogens (49). The U-Omp16 sequence is highly conserved among α Proteobacteria, and BLAST and pfam sequence analyses revealed that U-Omp16 belongs to a family of proteins with a peptidoglycan binding domain similar to the C-terminal domain of OmpA. Well-studied members of this family include the *E. coli* OmpA, the *E. coli* lipoprotein PAL, and *N. meningitidis* RmpM, which interact with the outer membrane, as well as the *E. coli* motor protein MotB and the *Vibrio* flagellar motor proteins PomB and MotY, which interact with the inner membrane. From the standpoint of the innate immune system, it has been stated that *K. pneumoniae* OmpA induces DC maturation by binding TLR2 (19). In addition, *E. coli* OmpA induces DC maturation independently of TLR4 (50). Although U-Omp16 from *Brucella* has significant homology with *K. pneumoniae* OmpA and *E. coli* OmpA, there are some differences in their amino acid sequences that may explain the differences in TLR binding. A similar situation has already been described in the case of flagellin from *H. pylori*, which no longer recognizes TLR5 as the flagellin from *E. coli* because of changes in its amino acid sequence (75). At present, it is not known which domain of *K. pneumoniae* OmpA binds TLR2, but it is important to have in mind that the homology with *B. abortus* U-Omp16 is predominantly at the C-terminal portion.

Vaccine production in plants is attractive in terms of safety and cost-effectiveness. From a safety perspective, the plant expression system would produce a vaccine free of animal pathogens and animal proteins. The production of plant-derived vaccines is, in principle, almost limitless and may require little or no downstream processing. An edible vaccine could be very practical to administer in cattle, but in this situation Ag and adjuvant must be expressed together. The latter notion, together with our findings showing that oral U-Omp16 delivery without adjuvants induced protection against *Brucella*, led us to develop a plant-made vaccine expressing U-Omp16. As a first approach, we studied the transient expression of U-Omp16 in *N. benthamiana* plants. The fact that U-Omp16 expressed in *N. benthamiana* leaves demonstrates a TLR4-mediated capacity to activate in vitro DCs and macrophages indicates that it retains its immunostimulating activity. The latter result, in conjunction with the demonstration that *E. coli*-made U-Omp16 retains its stimulating activity when it is co-incubated with PB and loses its activity when completely digested with proteinase K, contributes to the interpretation that the measured immunostimulating activity is not an outcome of contaminating LPS but rather a specific effect of the U-Omp16 protein. Moreover, tobacco-made U-Omp16 induced the same protective efficacy as *E. coli*-expressed U-Omp16, indicating that the plant-made U-Omp16 retains its self-adjuvanticity and immunogenicity. These findings suggest that future attempts to obtain an edible vaccine for cattle in another plant system (for example, alfalfa or barley) hold much promise.

Finally, this report presents U-Omp16 as a new bacterial PAMP that signals through TLR4, is able to activate in vivo DCs, induces a Th1 immune response, and is a very promising self-adjuvanting vaccine against systemic as well as orally acquired brucellosis. This study also set the precedent for development of a plant-made vaccine against brucellosis.

**Acknowledgments**

We thank Icon Genetics (Halle, Germany) for providing vectors for transient plant expression.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


