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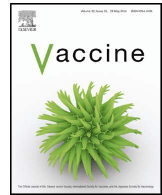

Brucella abortus Omp19 recombinant protein subcutaneously co-delivered with an antigen enhances antigen-specific T helper 1 memory responses and induces protection against parasite challenge

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ABSTRACT

The discovery of effective adjuvants for many vaccines especially those with limited commercial appeal, such as vaccines to poverty-related diseases, is required. In this work, we demonstrated that subcutaneous co-administration of mice with the outer membrane protein U-Omp19 from *Brucella* spp. plus OVA as antigen (Ag) increases Ag-specific T cell proliferation and T helper (Th) 1 immune responses *in vitro* and *in vivo*. U-Omp19 treated dendritic cells promote IFN-γ production by specific $CD4^+$ T cells and increases T cell proliferation. U-Omp19 co-administration induces the production of Ag specific effector memory T cell populations ($CD4^+$ $CD44^{high}$ $CD62L^{low}$ T cells). Finally, subcutaneous co-administration of U-Omp19 with *Trypanosoma cruzi* Ags confers protection against virulent parasite challenge, reducing parasitemia and weight loss while increasing mice survival. These results indicate that the bacterial protein U-Omp19 when delivered subcutaneously could be a suitable component of vaccine formulations against infectious diseases requiring Th1 immune responses.

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1. Introduction

Changes in the nature of vaccine components and routes of administration are imposing the need to develop new adjuvants. Modern adjuvant development, which in spite of many hurdles is progressing, is based on enhancing and shaping vaccine-induced responses without compromising safety by selectively adding well-defined molecules, formulations or both. Adjuvants currently employed in human vaccines licensed for use in the US and/or Europe include aluminum salts, oil-in-water emulsions (MF59, AS03 and AF03), virosomes and AS04 (monophosphoryl lipid A preparation (MPL) with aluminum salt) [1,2].

Worth mentioning, safe and effective adjuvants are required for many vaccines with limited commercial appeal, such as vaccines to infrequent (orphan) diseases or to neglected and poverty-related diseases [3,4]. So, there is also now a major interest in designing vaccines capable of eliciting strong cellular immune responses of the T helper 1 (Th1) type [5]. The induction of such Th1 responses is for example highly desirable for vaccines targeting either chronic parasite and viral diseases, infections linked to intracellular pathogens or cancer (therapeutic vaccines) [6].

Given their potent immunostimulatory capacity, bacteria-derived molecules constitute a major potential source of adjuvants. Unfortunately, whole live or killed microorganisms are too toxic to be used as human adjuvants [7,8]. We have previously reported that intraperitoneal immunization with purified recombinant Omp19 from *Brucella abortus* lipidated (L) or unlipidated (U) version without any adjuvant induced a Th1 response and also conferred protection against *Brucella* infection. Thus, our results indicate that the protein region of the Omp19 lipoprotein (U-Omp19) has self-adjuvant activity on cellular immune responses. Moreover, we demonstrated that U-Omp19 is able to induce the maturation of murine dendritic cells (DCs) *in vivo*. Immune protective responses were independent of TLR4. Also, U-Omp19 co-delivered orally induces specific mucosal immune responses [9–11]. All together these results prompted us to test the hypothesis that U-Omp19

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is an adjuvant for co-administered Ags when delivered subcutaneously. In this work we studied the systemic adjuvant capacity of the protein U-Omp19 when is co-administered with a model Ag (OVA) subcutaneously and also assessed its capacity to increase protection in a vaccine formulation against *T. cruzi* infection in mice.

2. Material and methods

2.1. Ethics statement

All experimental protocols with animals were conducted in strict accordance with international ethical standards for animal experimentation (Helsinki Declaration and its amendments, Amsterdam Protocol of welfare and animal protection and National Institutes of Health, USA NIH, guidelines). The protocols of this work were also approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) from the University of Buenos Aires and CICUAE from University of San Martin.

2.2. Animals

Eight week old female BALB/c or C57BL/6 mice were purchased from University of La Plata or from University of San Martin (UNSAM) and housed in the animal resources facility of University of Buenos Aires (UBA) or UNSAM. OT-II RAG1 (OT-II) and D011.10 mice were obtained from The Jackson Laboratory and were bred in the animal facility of UNSAM.

2.3. Ags and adjuvants

Chicken egg OVA grade V (Sigma-Aldrich) was used as model Ag. Recombinant unlipidated (U)-Omp19 was obtained as previously described [9]. LPS contamination from U-Omp19 was adsorbed with Sepharose-polymyxin B (Sigma). Endotoxin determination was performed with Limulus amoebocyte chromogenic assay (LONZA). In some experiments U-Omp19 fully digested with proteinase K was used as control. U-Omp19 was treated with proteinase K-agarose from *Tricirachium album* (Sigma) as described [10]. All U-Omp19 preparations used contained <0.1 endotoxin units per mg of protein. Incomplete Freund Adjuvant (IFA) and Complete Freund Adjuvant (CFA) were purchased from Sigma.

2.4. Immunizations

BALB/c mice were s.c. immunized once a week during 3 weeks with (i) OVA (50 μg), (ii) OVA (50 μg) plus U-Omp19 (100 μg), (iii) OVA (50 μg) plus CFA (100 μl) or (iv) saline. Three weeks after the last immunization mice were sacrificed to perform cellular experiments.

2.5. Determination of T helper immune responses

Splenocytes from immunized mice were cultured and stimulated with OVA, or complete medium alone and production of IFN-γ, IL-4, IL-10 and IL-17 were determined by ELISA as described [12].

2.6. DTH test

Three weeks after the last boost the delayed-type hypersensitivity (DTH) response was measured by determining footpad swelling 48 h after subcutaneous injection of Ag into hind footpad as described [12].

2.7. Proliferation assay

Spleen cells from immunized mice were tested in a standard [^{3}H] thymidine incorporation assay. Effector cells were suspended in complete medium and cultured with soluble OVA (100 or 1000 μg/ml) or Concanavalin A (5 μg/ml) as control. Cultures were incubated for 5 days at 37 °C and pulsed for 18 h with 1 μCi/well of [^{3}H] thymidine. Cells were harvested and radioactivity was measured with a liquid scintillation counter. The mean number of counts per minute (cpm) from triplicate wells was determined. Data are presented as mean ± SD from five animals per group.

2.8. Flow cytometry analysis of $CD4^+$ T cells

Splenocytes from immunized mice were incubated with OVA (10 μg/ml) plus OVA323–339 peptide (KISQAVHAA-HAEINEAGOVA) restricted to MHCII (1 μg/ml) and mitomicyn-treated APCs (A20 J cells stimulated with OVA for 24 h) in complete medium supplemented with IL-2 (10 U/ml) for 5 days at 37 °C and 5% CO2. After stimulation, cells were stained with anti- CD4, CD44 and CD62L. Flow cytometry analysis was performed using FACSAriaII flow cytometer (BD Biosciences) and further analyzed using FlowJo 7.5 software (TreeStar Inc).

2.9. Determination of Ab responses

Serum was obtained 3 weeks after last immunization to study OVA-specific humoral response (total IgG, IgG1 and IgG2a isotypes) by ELISA as described previously [10]. Cut-off values for the ELISA assays were calculated as the mean specific OD plus 3 SD from 20 sera from non-immunized mice. Serum titers were established as the reciprocal of the last dilution yielding an OD higher than the cut-off.

2.10. Bone marrow derived DCs (BMDCs)

DCs were generated from bone marrow (BM) mononuclear cells from wild-type BALB/c or C57BL/6 mice as described [12].

2.11. In vitro Ag-presentation assay

BMDCs ($CD11c^+$ $MHCII^{low}$) from BALB/c mice (1×10^6) were pulsed with complete medium, OVA (50 μg/ml), OVA plus U-Omp19 (100 μg/ml) or OVA plus LPS (1 ng/ml) for 24 h. Then, DCs were washed and co-cultured with transgenic DO11.10 spleen cells (4×10^6 cells) at 37 °C for 72 h. During the last 5 h cells were treated with brefeldin A. Afterwards, cells were washed and stained with anti-CD4 and then fixed, permeabilized with saponin and stained with anti-IFN-γ. Production of intracellular IFN-γ by $CD4^+$ T cells was assessed by flow cytometry. Similar experiments were performed using the chimera protein (GFP-OTII) as Ag [13]. In these experiments the IFN-γ production was measured in $CD4^+$ T cells from OTII mice.

2.12. Adoptive transfer of OT-II cells and in vivo $CD4^+$ T-cell proliferation

Splenocytes from OT-II mice were labeled with 5 μM CFSE (Molecular Probes) prior to i.v. injection. One day before immunization, 10×10^6 OT-II cells were injected i.v in C57BL/6 sex-matched recipients. Transferred mice received a single s.c. dose of OVA (30 μg) plus U-Omp19 (100 μg), proteinase K digested U-Omp19 (100 μg), LPS (1 μg) or saline. Three days after immunization, mice were sacrificed and spleen cells suspensions were obtained to study the proliferation of $CD4^+$ T cells by flow cytometry.

2.13. *Trypanosoma cruzi* protection assay

T. cruzi antigens present in a whole homogenate (WH) of parasites were prepared from epimastigote forms as described [14]. BALB/c mice were immunized s.c with 50 μg of WH alone or plus U-Omp19 (100 μg) or CFA (100 μl, first dose and IFA the second and third doses) at day 0, 7 and 14. Three weeks after last immunization mice were injected i.d with 1×10^3 bloodstream trypomastigotes of RA strain. Parasitemia was monitored by daily counting of the number of trypomastigotes per ml of fresh blood, and mortality was recorded.

2.14. Statistical analysis

Statistical analysis and plotting were performed using GraphPad Prism 5 software (GraphPad, San Diego, CA). In experiments with more than 2 groups, data were analyzed using one-way ANOVA with Bonferroni's post-test. If necessary, a logarithmic transformation was applied to obtain data with a normal distribution. When bars were plotted results were expressed as mean ± SEM for each group. In survival experiment Kaplan–Meier curve was generated using GraphPad Prism 5 software and analyzed with log-rank test.

3. Results

3.1. Subcutaneous U-Omp19 co-administration with OVA induces OVA-specific Th1 cellular immune responses

To study if U-Omp19 has adjuvant properties when co-delivered subcutaneously (s.c) with an Ag, OVA was used as model Ag. Thus, mice were s.c. immunized with saline, OVA, OVA + U-Omp19 or OVA + CFA and three weeks later OVA-specific cellular immune responses were evaluated. U-Omp19 co-administration with OVA induced higher levels of IFN-γ production upon OVA stimulation compared with the immunization with OVA alone ($P < 0.01$ *vs* OVA immunized mice, Fig. 1A). Immunization with CFA also induced production of IFN-γ by splenocytes ($P < 0.001$ *vs* OVA). In contrast, there were no differences in the levels of IL-4 or IL-10 produced by spleen cells from the different groups (Fig. 1B and C). Furthermore, there was a slight increment in IL-17 production by OVA-stimulated cells from U-Omp19 + OVA immunized mice but this increment was not statistically significant compared with the production by cells from OVA-immunized mice (Fig. 1D). Immunization of mice with U-Omp19 alone did not induce statistically significant production of cytokines after OVA stimulation (data not shown). Con A induced the production of the corresponding cytokines in all groups (data not shown). These results indicate that subcutaneous U-Omp19 co-administration with OVA enhances OVA-specific Th1 cytokine responses.

Spleen cells from OVA + U-Omp19 or OVA + CFA immunized mice induced a more potent T cell proliferation than the response elicited by splenocytes from OVA administered mice ($P < 0.05$, $P < 0.001$ *vs* OVA, Fig. 1E). Moreover, U-Omp19 + OVA immunized mice showed an increment in the footpads thickness (DTH) compared with mice immunized with OVA alone ($P < 0.05$ *vs* OVA, Fig. 1F). Immunization with CFA also induced a significant DTH response ($P < 0.01$ *vs* OVA). Of note, U-Omp19 did not induce any visible adverse effects on inoculated tissues as elicited by CFA inoculation (Fig. 6). All together these results indicate that U-Omp19 s.c co-delivered with an Ag induces Ag-specific Th 1 cellular immune responses *in vitro* and *in vivo*.

3.2. U-Omp19 co-admnistration promotes Ag-experienced effector CD4⁺ T cells

To determine whether T cells from immunized mice develop into memory T cells or maintain an activated phenotype, the Ag-experienced T cells were identified by the expression of CD44 [15,16] in splenocytes from immunized mice after *in vitro* restimulation. Memory T cells can be further divided based on CD62L (L-selectin) expression into lymph node-homing T central memory cells (TCM, $CD62L^{high}$) and tissue homing T effector memory cells (TEM, $CD62L^{low}$) [17]. Then, the percentage of cells expressing CD44 and the activation marker CD62L was analyzed in $CD4^+$ T cell subset.

Phenotypic analysis of memory $CD4^+$ T cells [15–17] showed that T cells from mice s.c. administered with OVA + U-Omp19 exhibited a high proportion of the $CD44^{high}$ and $CD62^{low}$ phenotype in $CD4^+$ T cells (33.29%) indicating that a large population of these cells are Ag-experienced cells compared with cells from mice immunized with OVA alone (20.83%) and further indicates that Ag-experienced $CD4^+$ T cells are maintained as effector, rather than central, memory cells in OVA plus U-Omp19 immunized mice (Fig. 2). OVA + CFA immunization induced a similar profile of effectors T cells to OVA + U-Omp19 immunized mice (31.43% $CD44^{high}$ $CD62^{low}$ $CD4^+$ T cells, Fig. 2).

These data suggest that the vast majority of $CD4^+$ T cells induced after Ag plus U-Omp19 s.c. co-administration have a phenotype of activated, not resting, memory cells.

3.3. U-Omp19 subcutaneously co-administered with Ag alters Ag-specific IgG isotype profile but does not enchance the magnitude of Ag-specific IgG humoral immune response

No differences in the serum titer of OVA-specific IgG antibodies were found after immunization with OVA alone or in combination with U-Omp19 (Fig. 3A). As expected, OVA + CFA vaccination increased significantly OVA-specific IgG titer in sera ($P < 0.001$ *vs* OVA, Fig. 3A). Of note, OVA + U-Omp19 modified the ratio between anti-OVA IgG1 and IgG2a anti-OVA titers ($P < 0.05$ *vs* OVA, Fig. 3B). While IgG1 titres predominated over IgG2a in sera from mice injected with OVA or OVA + CFA, the opposite was found in mice injected with OVA + U-Omp19. These results indicate that U-Omp19 as adjuvant, delivered by s.c route, is not able to increase Ag-specific IgG responses while it can change the elicited antibody subtype profile.

3.4. U-Omp19 treated DCs promote IFN-γ production by specific CD4⁺ T cells and increases T cell proliferation

To study if U-Omp19 has the capacity to directly activate DCs and promote effector specific $CD4^+$ T cells responses, D011.10 mice were used as a source of OVA-specific $CD4^+$ T cells against OVA. Bone marrow derived dendritic cells (BMDCs) from BALB/c mice were treated with OVA alone, OVA plus U-Omp19 or OVA plus LPS for 24 h, washed and then co-cultivated with $CD4^+$ T cells from spleen DO11.10 mice for 3 days. DCs incubated with OVA + U-Omp19 were able to induce a higher level of IFN-γ production in DO11.10 $CD4^+$ T cells (11.1%) compared to T cells co-cultivated with DCs pulsed with OVA alone (6.35%, Fig. 4A). LPS treated BMDCs also induced a strong IFN-γ production by D011.10 cells (48.6%).

The priming capacity of U-Omp19 pulsed DCs on $CD4^+$ T cells was also assayed using a different Ag (chimeric GFP). Soluble chimeric GFP contains the MHC-II restricted OVA peptide epitope (323-339, ISQAVHAAHAEINEAGR) recognized by $CD4^+$ OT-II cells (GFP-OTII). Of note, this chimeric protein is not internalized by lectin-like receptors as OVA [13]. After co-incubation with GFP-OTII plus U-Omp19, DCs were able to increase their Ag presentation to $CD4^+$ T cells as revealed by a greater percentage of IFN-γ producing OTII cells (1.96%) compared with DCs incubated with GFP-OTII alone (0.49%, Fig. 4B). In conclusion, U-Omp19 s.c. co-delivered with an Ag promotes Ag presentation by DCs and the induction of IFN-γ producing $CD4^+$ T cells *in vitro*.

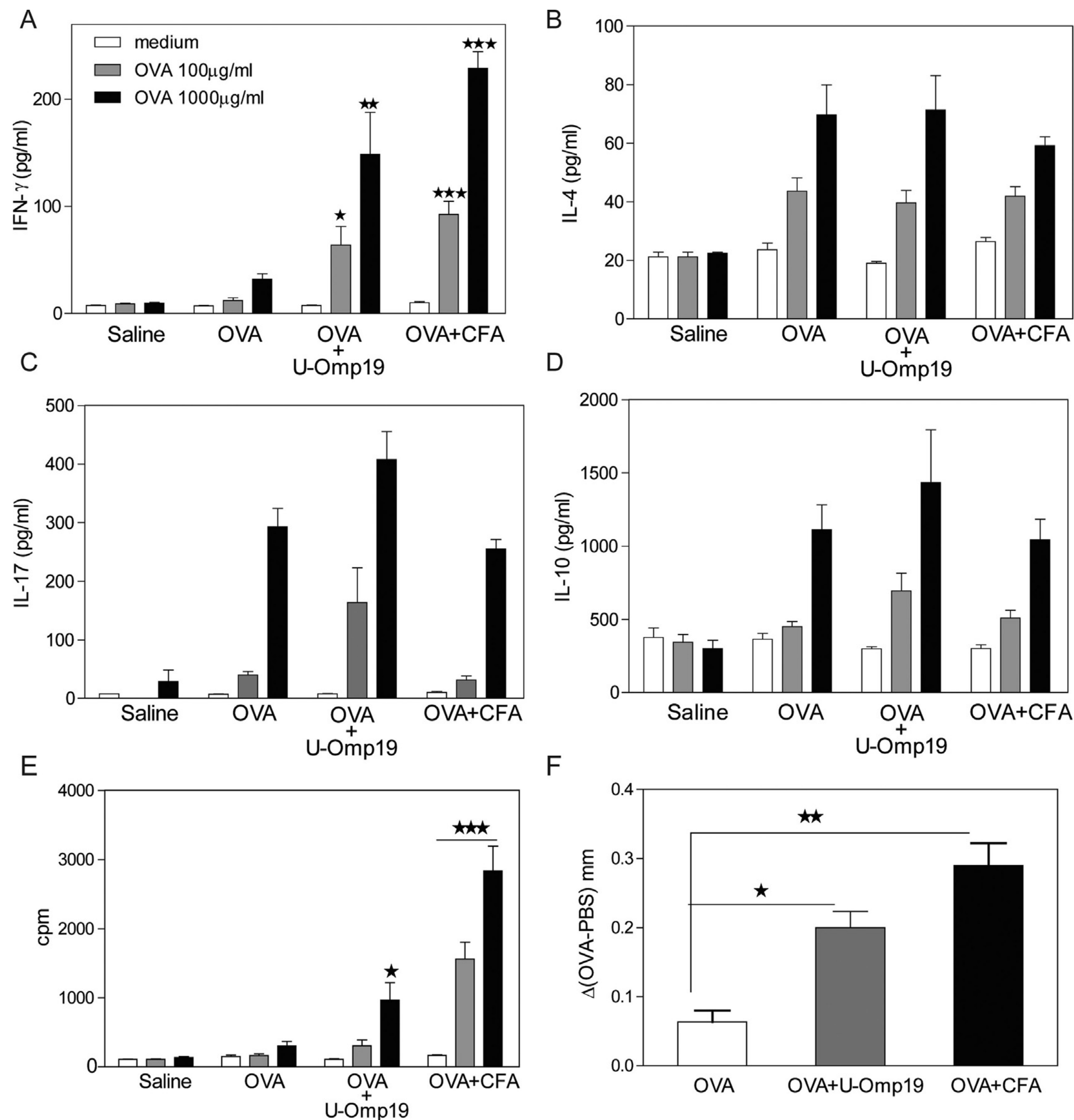

Fig. 1. U-Omp19 s.c. co-administration with an Ag increases Ag-specific Th1 immune responses in BALB/c mice. Mice (n = 5/group) were subcutaneously administered with OVA, OVA + U-Omp19, OVA + CFA or saline. Spleens from mice were obtained 3 weeks after the last immunization. Cytokine production was determined by ELISA on supernatants harvested 5 days after stimulation of spleen cells with OVA or complete medium. Production of IFN-γ (A), IL-4 (B), IL-17 (C) and IL-10 (D) is shown. Results are presented as mean ± SEM for each group and are representative of 3 independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001 *vs* OVA group. Delayed-type hypersensitivity (DTH) response to OVA (E) was assayed 3 weeks after the last boost to evaluate the cellular immune response *in vivo*. Twenty microgram of OVA were injected into one footpad, and saline was injected into the contra lateral footpad, as a negative control. The thickness of both footpads was measured 48 h later and the increment between OVA and saline injected was plotted. (* P < 0.05, ** P < 0.01 *vs* OVA group). T-cell proliferation from immunized mice (F). Splenocytes were stimulated *in vitro* with OVA (100 or 1000 μg/ml) for 5 days and proliferation was assessed by thymidine labeling. The number of counts per minute (cpm) was determined. Data are presented as mean ± SD. (* P < 0.05, *** P < 0.001 *vs* OVA group).

To determine *in vivo* the primary Ag-specific clonal expansion of T cells following U-Omp19 s.c. administration, adoptive transfer assays using TCR transgenic mice OTII were performed. Thus, C57BL/6 mice were adoptively transferred with CFSE- labeled OT-II T cells. One day later, mice were s.c. immunized with OVA co-administered with: U-Omp19, U-Omp19PK (protein digested with Proteinase K used as control, Fig. 4D), LPS or saline. Immunization with OVA plus U-Omp19 induced greater OT-II T cell proliferation (61.7%) than immunization with OVA alone (31.7%, Fig. 4C). When U-Omp19 digested with proteinase K was co-delivered with OVA its activity was lost since it induced a degree of proliferation (38.7%) similar to OVA delivered alone. These results indicate that the measured activity resides in the protein rather than in another non protein contaminant. As expected, a large percentage of proliferating cells was obtained when LPS was co-administered with OVA (86.5%, Fig. 4C).

3.5. U-Omp19 subcutaneously co-administered with parasite derived antigens extends the survival of mice after virulent parasite challenge

Finally U-Omp19's adjuvant capacity using microbial derived Ags was evaluated. A whole homogenate (WH) from *T. cruzi* parasites was used as Ag. Mice were s.c inoculated with WH alone, WH plus U-Omp19 or WH emulsified in Incomplete Freund Adjuvant (IFA) and 3 weeks later challenged with a lethal dose of bloodstream trypomastigotes of the virulent RA strain. Mice s.c immunized with WH plus U-Omp19 presented a significant reduction in weight

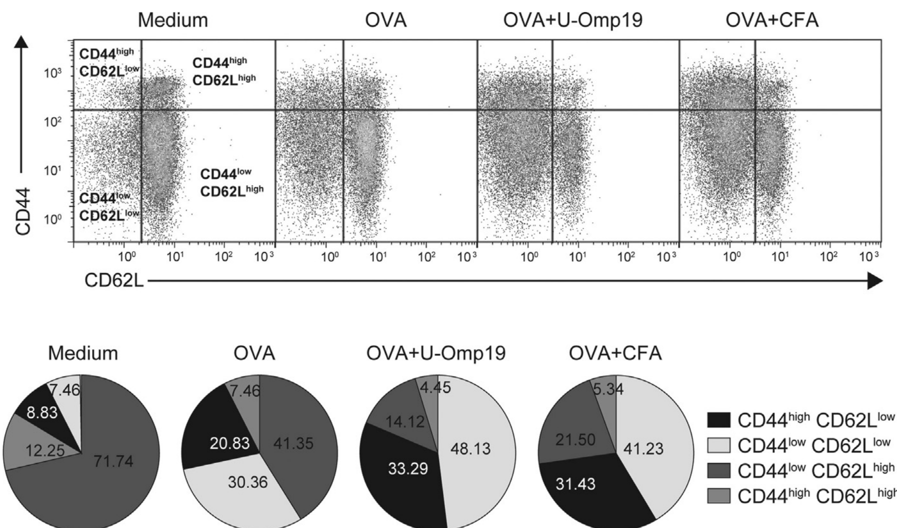

Fig. 2. U-Omp19 as adjuvant by s.c route promotes memory Th1 cellular immune responses. Phenotypic analysis of memory T cells by flow cytometry. Animals were immunized as described in Fig. 1. Splenocytes from immunized mice were stimulated with the Ag *in vitro* for 5 days. Percentage of CD62L and CD44 markers expression in CD4T cells was determined by flow cytometry. Subpopulations are shown in dot plots and proportions are shown in pie chart below.

loss and peak of parasitemia when compared with animals that received the antigen alone after challenge (Fig. 5A and B, $P < 0.05$). Most importantly, WH plus U-Omp19 immunization significantly reduced mouse mortality when compared with immunization with WH alone (Fig. 5C, $P < 0.05$). Immunization with WH plus IFA did not elicit a significant protective immune response against parasite challenge (Fig. 5A–C).

Taken together these results indicate that U-Omp19 could be a suitable adjuvant in vaccine formulations against pathogens in which protection is reached by promoting Th1 immune responses.

4. Discussion

Adjuvants should be selected on the basis of the type of immune response that is desired for a particular vaccine. In this work we demonstrated that U-Omp19 from *Brucella* increases Th1 immune responses when is co-delivered s.c. with an Ag since it increases *in vitro* Ag-specific T cell responses with IFN-γ production. Moreover, it induces *in vivo* proliferation of CD4$^+$ T cells and DTH responses after i.d challenge with the Ag. Of note and in contrast to other known Th1 adjuvants-like CFA or CpG-U-Omp19 co-administered s.c. does not increase antibody responses while is able to change the isotype profile further indicating its immunomodulatory properties.

Induction of memory T cells is a key event in the generation of protective immunity. *In vitro* stimulation of CD4$^+$ T cells from mice s.c immunized with U-Omp19 exhibits a larger percentage of effector memory T cells (CD4$^+$ CD44^{high} CD62L^{low} T cells). Central memory T cells expressing CD62L^{high} reside in the T-cell zone of lymphoid organs and respond quickly to Ag re-stimulation proliferating and differentiating into effector cells. Moreover, in this memory subpopulation the Ag experienced T cells expressing the CD44 marker were increased when U-Omp19 is co-delivered.

In previous reports we have shown that U-Omp19 can activate DCs *in vivo* by up-regulating CD40, CD80 and CD86 [10]. In this work, *in vitro* assays indicate that U-Omp19 treated DCs promote IFN-γ production by specific CD4$^+$ T cells and increased T cell proliferation indicating a direct effect on DCs Ag presentation. Immunization with OVA plus U-Omp19 induced greater OVA-specific CD4$^+$ T cell proliferation than immunization with OVA alone. This activity is completely dependent on the presence of the intact protein, since it was lost when the digested protein U-Omp19 (proteinase K treated) was used as stimulant.

A common adverse effect using adjuvant compounds is their local reactogenicity at the site of injection. Macroscopic analysis of the site of injection after s.c. immunization with OVA plus U-Omp19 or plus CFA shows that CFA promotes the formation of a pronounced and visible granuloma and inflammation signs while there was not any visible local reaction after U-Omp19

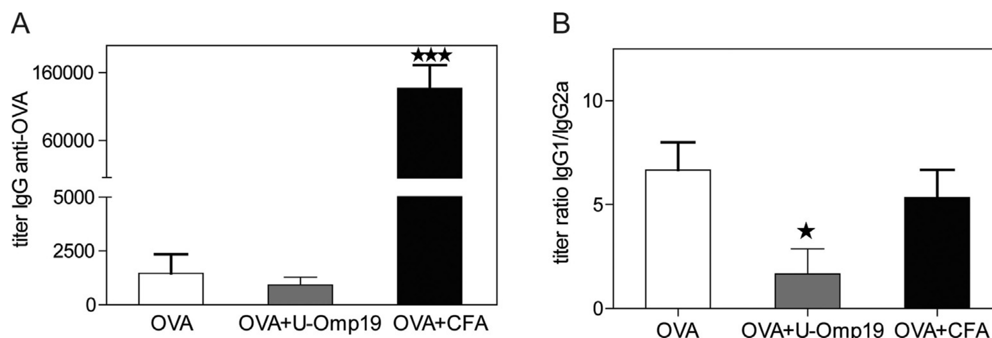

Fig. 3. U-Omp19 delivered s.c. does not increase IgG humoral response against co-administered Ag. Animals were immunized as described in Fig. 1. Specific-OVA IgG, IgG1 and IgG2a titers were determined by ELISA in serum from immunized mice. * $P < 0.05$, *** $P < 0.001$ *vs* OVA group.

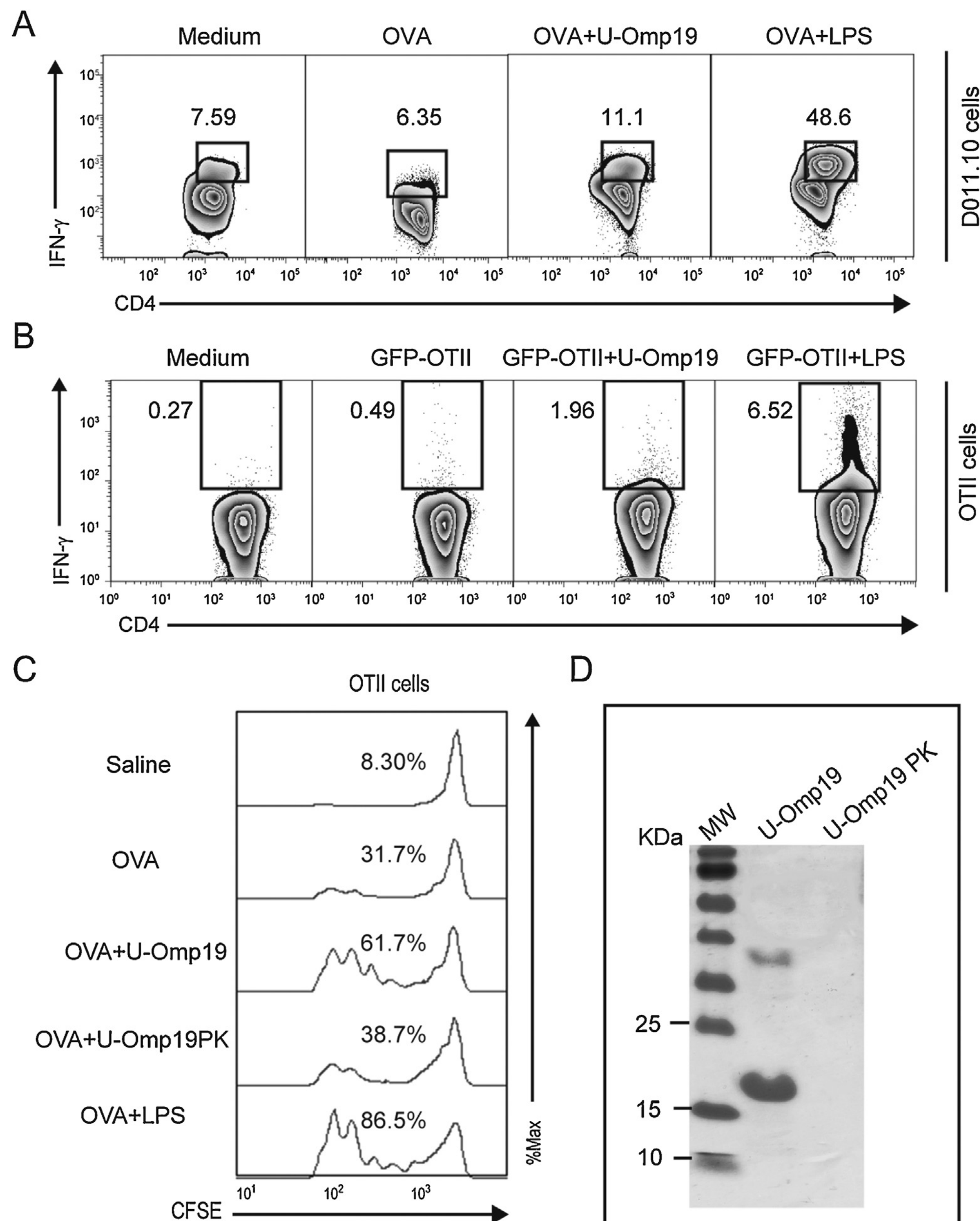

Fig. 4. U-Omp19 promotes Ag presentation by DCs *in vitro*. (A) Production of IFN-γ by D011.10 CD4+ T cells. BMDCs were incubated with OVA alone, plus U-Omp19 or LPS for 20h and then washed and co-incubated with DO11.10T cells for 3 days. Determination of intracellular IFN-γ in CD4+ T cells was evaluated by flow cytometry. IFN-γ producing CD4+ T cells frequencies is shown. (B) Production of IFN-γ by OTII CD4+ T cells. BMDCs were incubated with GFP-OTII chimera protein alone, plus U-Omp19 or LPS for 20h and then washed and co-incubated with OTII T cells. Intracellular IFN-γ staining was performed and the percentage of IFN-γ producing OTII T cells was determined by flow cytometry. (C) Proliferation *in vivo* of OTII CFSE labeled T cells after s.c. administration of (i) saline, (ii) OVA, (iii) OVA + U-Omp19, (iv) OVA + U-Omp19PK or (v) OVA + LPS. Three days later spleen cells were harvested and cell suspensions were analyzed by flow cytometry. Results are shown as representative histograms and data represented as percentage of OTII CD4+ CFSE+ proliferative T cells (n = 3/group). Representative of three different experiments. (D) Degradation of U-Omp19 with Proteinase-K. U-Omp19 was treated with proteinase K-agarose from *Tricirachium album* for 1 h at 37 °C. The enzyme immobilized in agarose was then centrifuged out and the supernatants were incubated for 2 h at 60 °C to inactivate any fraction of soluble enzyme. The complete digestion of U-Omp19 was checked by SDS-PAGE followed by Western Blot analysis. Line 1: Molecular weight marker, line 2: U-Omp19, line 3: U-Omp19 PK.

administration at the injection site (Fig. 6). Besides, U-Omp19 s.c. co-administration does not have an effect in the magnitude of the specific humoral response elicited but it has an effect on the isotypes profile of this response. The generation of a humoral response balanced to the generation of specific-IgG1 antibodies could be interesting to polarize immune responses to a desired profile. For example, in many allergic processes turning the allergen specific Th2 response to a modulatory Th1 response could be beneficial to resolve or prevent allergic responses [18]. Indeed, one of the most accepted mechanisms to explain the occurrence of autoimmunity following infection/vaccination is molecular mimicry [19]. Thus, if using U-Omp19 the mentioned problem would not be expected.

The development of effective vaccine formulations against life-threatening diseases especially those associated with poverty as well as neglected infectious diseases is an actual scientific priority. In this work we have shown that U-Omp19 is a suitable adjuvant to be included in future vaccine formulations against *T. cruzi* infection since U-Omp19 s.c. co-delivered with whole homogenate (WH) from *T. cruzi* parasites induced protection and significantly reduced mouse mortality. Of note, immunization with WH plus IFA did not

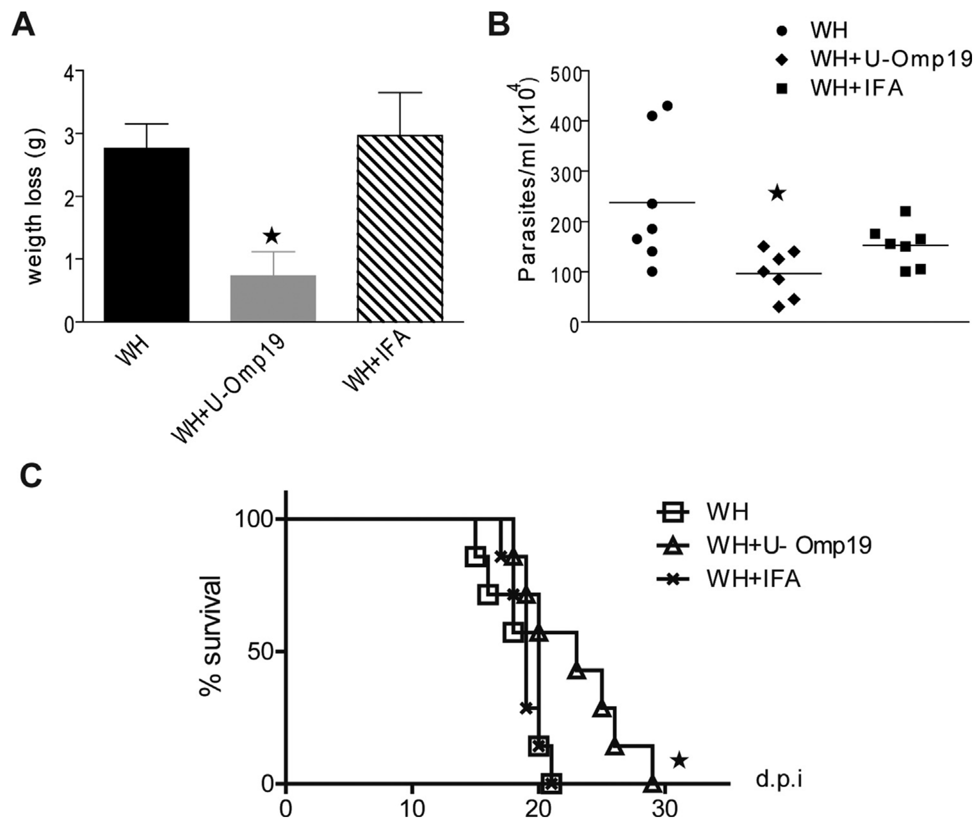

Fig. 5. U-Omp19 s.c. co-administration induces protection against *T. cruzi* challenge. BALB/c mice (n/group = 7) were s.c. immunized at days 0, 7 and 14 with (i) whole homogenate (WH) from *T. cruzi* parasites, (ii) WH + U-Omp19 or (iii) WH + IFA. Three weeks after last boost mice were i.p. challenged with bloodstream trypomastigotes (RA strain). Weight loss, blood parasite levels and survival were monitored until day 30 after challenged. Data are presented as mean of grams of weight loss per group (A), parasites/ml per mouse at the peak of parasitemia (B) and percent of survival of mice (C). * $P < 0.05$ *vs* WH group.

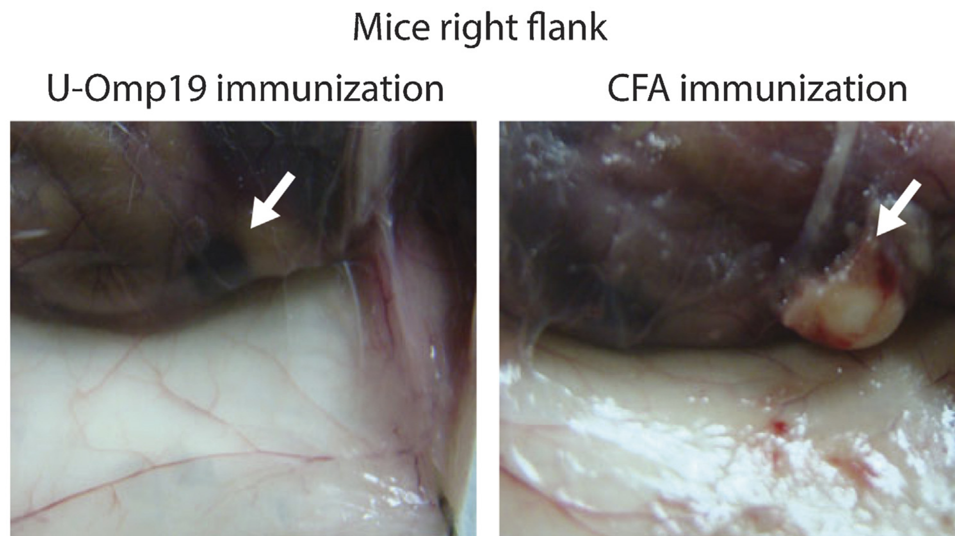

Fig. 6. U-Omp19 does not induce any visible adverse effect at the site of injection. BALB/c mice were s.c. immunized at the right flank with OVA + U-Omp19 or OVA + CFA at day 0, 7 and 14. Three weeks after last immunization pictures were taken to determine any visible inflammation signs in the tissues at the site of infection. Representative pictures are shown.

elicit a significant protective immune response against parasite challenge. Further indicating the importance of evaluating different formulations/adjuvants to the development of vaccines against infectious diseases.

Altogether the results presented in this work indicate that U-Omp19 delivered subcutaneously could be a suitable adjuvant in vaccine formulations against pathogens in which protection is mediated by Th1 cell immune responses.

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