Journal of Controlled Release 220 (2015) 18–28

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

A bacterial protease inhibitor protects antigens delivered in oral vaccines from digestion while triggering specific mucosal immune responses

Andrés Esteban Ibañez [a], Lorena Mirta Coria [a], Marianela Verónica Carabajal [a], María Victoria Delpino [b], Gabriela Sofía Risso [a], Paula Gonzalez Cobiello [c,d], Jimena Rinaldi [e], Paula Barrionuevo [f], Laura Bruno [a], Fernanda Frank [c,d], Sebastián Klinke [e], Fernando Alberto Goldbaum [e], Gabriel Briones [a], Guillermo Hernán Giambartolomei [b], Karina Alejandra Pasquevich [a], Juliana Cassataro [a,*]

[a] Instituto de Investigaciones Biotecnológicas—"Dr. Rodolfo A. Ugalde" Instituto Tecnológico de Chascomús (IIB-INTECH), Universidad Nacional de San Martín (UNSAM) CONICET, San Martín, Buenos Aires, Argentina
[b] Instituto de Inmunología, Genética y Metabolismo (INIGEM), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad de Buenos Aires (UBA) Laboratorio de Inmunogenética, Hospital de Clínicas "José de San Martín", Facultad de Medicina, UBA, Buenos Aires, Argentina
[c] Instituto de Estudios de la Inmunidad Humoral (IDEHU), CONICET-UBA, Facultad de Farmacia y Bioquímica, UBA, Buenos Aires, Argentina
[d] Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, UBA, Buenos Aires, Argentina
[e] Fundación Instituto Leloir, IIBBA-CONICET, Buenos Aires, Argentina
[f] Instituto de Medicina Experimental (CONICET-Academia Nacional de Medicina), Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 11 August 2015
Accepted 6 October 2015
Available online 9 October 2015

Keywords:
Protease inhibitor
Antigen delivery
Oral vaccination
Adjuvant
Brucella spp.

ABSTRACT

We report here that a bacterial protease inhibitor from *Brucella* spp. called U-Omp19 behaves as an ideal constituent for a vaccine formulation against infectious diseases. When co-administered orally with an antigen (Ag), U-Omp19: i) can bypass the harsh environment of the gastrointestinal tract by inhibiting stomach and intestine proteases and consequently increases the half-life of the co-administered Ag at immune inductive sites: Peyer's patches and mesenteric lymph nodes while ii) it induces the recruitment and activation of antigen presenting cells (APCs) and increases the amount of intracellular Ag inside APCs. Therefore, mucosal as well as systemic Ag-specific immune responses, antibodies, Th1, Th17 and $CD8^+$ T cells are enhanced when U-Omp19 is co-administered with the Ag orally. Finally, this bacterial protease inhibitor in an oral vaccine formulation confers mucosal protection and reduces parasite loads after oral challenge with virulent *Toxoplasma gondii*.

1. Introduction

Among different mucosal routes, oral delivery is the natural choice not only for drugs, but also for vaccines, by virtue of its ease of administration and cost. Although oral delivery of vaccines has been the Holy Grail for generations of vaccinologists [1], oral vaccination has been historically thought to be largely ineffective at providing effective mucosal and/or systemic immunity. This is mainly due to the fact that antigens (Ags) undergo proteolytic degradation in the stomach and intestine. Another barrier that would be bypassed by inducing an appropriate inflammatory response is the immune tolerance resulting from Ag feeding [2]. Consequently, to reliably immunize orally with peptide- or protein-based vaccines, Ags must be protected, uptake enhanced and the immune tolerance properly controlled.

Induction of immune responses following mucosal immunization with non-live vaccines is usually dependent upon the co-administration of appropriate adjuvants that can initiate and support the transition from innate to adaptive immunity [3]. Currently, to induce mucosal adaptive immune responses — in mice — two powerful adjuvants are used: cholera toxin (CT) from *Vibrio cholera* and labile enterotoxin (LT) from *Escherichia coli* [4]. In humans, however, they are responsible for the cholera and "Travelers diarrhea" and therefore cannot be used. In attempt to avoid the enterotoxic effects caused mainly by the A subunit of the cholera toxin, subunit B (CTB) was expressed

Abbreviations: Ab, antibody; Ag, antigen; ALT, alanine aminotransferase; APCs, antigen presenting cells; AST, aspartate aminotransferase; a.u, arbitrary units; *B. abortus*, *Brucella abortus*; BSA, bovine serum albumin; CD, circular dichroism; CFSE, Carboxyfluorescein succinimidyl ester; CT, cholera toxin; CTB, cholera toxin subunit B; DCs, dendritic cells; dmLT, double mutant labile enterotoxin; DTH, delayed type hypersensitivity response; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FI, fluorescence intensity; FITC, fluorescein isothiocyanate; HKS, heat killed extract from *Salmonella typhimurium*; IFN-γ, gamma interferon; Ig, immunoglobulin; IL-, interleukin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; LT, labile enterotoxin; MHC, major histocompatibility complex; MLNs, mesenteric lymph nodes; OD, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline; PPs, Peyer patches; SEM, standard error of the mean; ST, Shiga toxin; Th, T helper; TLR4, Toll Like Receptor 4; U-Omp19, Unlipidated Outer membrane protein 19; U-Omp16, Unlipidated Outer membrane protein 16.

* Corresponding author.
E-mail address: jucassataro@iibintech.com.ar (J. Cassataro).

http://dx.doi.org/10.1016/j.jconrel.2015.10.011

and is now being used in a human vaccine (Dukoral®) [5]. Double-mutant LT (dmLT), with reduced enterotoxicity was developed and shown to increase immune responses [6]. Different strategies to increase Ag delivery like antigen targeting to M cells, nanoparticle-releasing vaccines at the colon, *etc*. for enhancing the efficacy of mucosal vaccines have been and are currently being investigated [7–10]. Meanwhile different new approaches need to be explored to develop novel oral adjuvants and delivery systems [11], especially there is a need of those that can induce T helper (Th)1 and $CD8^+$ T cell responses that can prevent infectious diseases related to intracellular pathogens.

In our laboratory we have been working on the use of a *Brucella* spp. protein devoid of its lipid moiety called U-Omp19 (Unlipidated Outer membrane protein 19) as an antigen for a vaccine against brucellosis. This protein is soluble and easy to express and purify at high scales [12,13]. Oral immunization with U-Omp19 produced either in *E. coli* or in plants without adjuvants conferred significant protection against oral *B. abortus* infection and induced a mixed Th1-17 immune response independent of TLR4. U-Omp19 also induced the maturation of murine dendritic cells (DCs) *in vivo* [14].

Of note, BLAST, Pfam and MEROPS sequence analysis report that U-Omp19 has sequence identity with other bacterial protease inhibitors, particularly with those of the protease inhibitor family inh from *Erwinia chrysanthemi* (family I38) [15,16]. This family of proteins would interact with specific proteases released by plant, insect and animal pathogens [15,17]. Thus, we hypothesize that U-Omp19 would inhibit protease activity at mucosal tissues and would increase the half-life of the co-administered Ag thus increasing its immunogenicity.

In this work we demonstrate that this bacterial protease inhibitor protects antigens delivered in oral vaccines from digestion while triggers specific mucosal immune responses. To our knowledge there are no reports in the literature describing the use of bacterial protease inhibitors in oral vaccine formulations. Therefore, this is an unconventional bacterial molecular pattern never explored before.

2. Materials and methods

2.1. Ethics statement

All experimental protocols of this study 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: Guide for the Care and Use of Laboratory Animals). The protocols of this study were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) from the University of Buenos Aires (Permit Number: 2079) and CICUAE from University of San Martin (Permit Number: 052,014), Buenos Aires, Argentina.

2.2. Animals

Eight week old female BALB/c, C57BL/6 or C3H/HeN mice were purchased from University of La Plata (La Plata, Argentina) or from University of San Martín (UNSAM) and housed in the animal resources facility of University of Buenos Aires (UBA) or UNSAM (Buenos Aires, Argentina). Animals were devoid of food but with free access to water 2 h previous and 2 h after any administration/immunization.

2.3. Ags and adjuvants

Chicken egg OVA grade V (Sigma) was used as a model Ag. Recombinant unlipidated (U)-Omp19 was obtained as previously described [13]. LPS contamination from U-Omp19 was adsorbed with Sepharose-polymyxin B (Sigma). Endotoxin determination was performed with Limulus amoebocyte chromogenic assay (LONZA). All U-Omp19 preparations used contained <0.1 endotoxin units per mg of protein. CT and aprotinin were purchased from Sigma.

2.4. Determination of protease inhibitor activity in vitro and in vivo

Protease activity was determined using a casein fluorimetric kit (EnzChek, Invitrogen). This kit has casein-BODIPY-FL, whose fluorescence is quenched. Protease-catalyzed hydrolysis relieves this quenching, yielding bright green fluorescent peptides. The increase in fluorescence emission is proportional to casein digestion and protease activity. Pepsin (0.483 μM, Sigma), pancreatic Elastase (0.965 μM, Sigma), Trypsin (0.965 μM, Sigma), α-Chymotrypsin (0.965 μM, Sigma), Carboxypeptidase A (0.160 μM, Sigma) and B (0.065 μM, Sigma) were incubated with U-Omp19 at different molar ratios protease:U-Omp19 (1:1, 1:5, 1:10 and 1:50 — only for pepsin). As positive control a mammalian protease inhibitor cocktail was used. Bovine serum albumin (BSA) and U-Omp16 – a *Brucella* spp. protein with a similar molecular weight to U-Omp19 and expressed and purified in the same way – were used as negative controls. Each reaction mix was incubated at room temperature (RT) for 1 h and then substrate (casein-BODIPY-FL, 1 μg/ml) was added. Fluorescence was measured with a fluorescence plate reader (FilterMaxF5 Molecular devices).

To study pH stability of the inhibitory activity of U-Omp19, U-Omp19 was pre-incubated at RT for 1 h at different pH (2, 5, 7.5 and 8) buffers containing 10 mM sodium phosphate and 50 mM NaCl and then adjusted to the optimum pH for α-Chymotrypsin (pH = 7.8). Microcon 3 (Biopore, Germany) was then used to exchange buffers. Residual inhibitor activity was assessed using casein-BODIPY (1 μg/ml) and α-chymotrypsin (0.965 μM) as model protease in a molar ratio protease: U-Omp19 of 1:10. Thermal stability of the inhibitory activity of U-Omp19 was assessed by incubating different tubes containing U-Omp19 at different temperatures (25–100 °C) for 1 h and subsequently adjusting them at RT. Residual inhibitor activity was assessed as above described for pH stability experiments using casein-BODIPY-FL and α-chymotrypsin or elastase (0.965 μM) as model proteases. Far-UV circular dichroism (CD) spectra of U-Omp19 under different pHs and temperatures were conducted (see supp. Materials and methods).

To further study the protease inhibitory mechanism of U-Omp19, the kinetic of trypsin, α-chymotrypsin and pancreatic elastase inhibition was performed (see supp. Materials and methods).

To evaluate if U-Omp19 inhibits proteolytic activity of stomach and intestine extracts, stomach or intestine extracts from mice were pre-incubated with buffer, different amounts of U-Omp19, Inhibitor cocktail or BSA as negative control. Then, the mixtures were incubated with casein BODIPY-FL for 1 h or with OVADQ (quenched protein that releases fluorescence upon digestion) for 4 h and the fluorescence increment was determined.

To study protease inhibitor activity *in vivo* BALB/c mice were orally — intragastrically (i.g.) — administered with casein-BODIPY alone (100 μg), plus U-Omp19 (150 μg) or aprotinin (1 μg). Stomachs were removed 15 min and 1 h after oral delivery and extracts of the organs were obtained. Afterwards Ag digestion was determined by fluorescence emission. The dose of aprotinin used in this work was chosen because it did not produce adverse effects on treated animals and also induced proteolysis inhibition of the antigen similar to U-Omp19 (data presented in this manuscript and not shown).

To study Ag fate after delivery BALB/c mice were i.g. administered with PBS, OVA-FITC (100 μg) alone or OVAFITC (100 μg) plus U-Omp19 (150 μg). Stomachs and small intestines were removed 90 min after oral delivery and extracts of the organs were obtained. Afterwards Ag presence was determined by fluorescence emission.

2.5. Determination of Ag fate and proteolysis in vivo

To study Ag proteolysis *in vivo* Casein-BODIPY-FL or OVADQ were used. To evaluate Ag fate and internalization *in vivo* OVA-AlexaFluor647 or OVA-FITC were used. If Ags (OVA-FITC or casein-BODIPY-FL) were used alone, mice were fed Ag (100 μg) plus i) buffer,

ii) U-Omp19 (150 μg), iii) CT (10 μg), iv) aprotinin (1 μg) or buffer as control. To examine simultaneously Ag fate and degradation, mice were fed both Ags simultaneously (100 μg OVA-AlexaFluor647 and 100 μg OVADQ) plus i) buffer, ii) U-Omp19 (300 μg), iii) CT (20 μg), iv) aprotinin (2 μg) or buffer as control. At 2, 6, 12 and 18 h post administration mice were sacrificed and Peyer's Patches (PPs) and Mesenteric Lymph Nodes (MLNs) were aseptically removed. Single cell suspensions were prepared [18], and washed twice in PBS solution to eliminate any extracellular Ag, thus the fluorescence determination was proportional to Ag presence or proteolysis inside the cells. Total viable cells were counted using Trypan Blue. Fluorescence intensity (FI) in arbitrary units was determined in 1×10^6 cells. % of max. internalization of OVA or % of OVA degradation was calculated as described in supplemental material and methods.

To study cell subtypes involved in Ag internalization and processing, cells were stained with fluorochrome conjugated antibodies: anti-CD11c, anti-CD8α, anti-CD11b and anti-MHC-II or isotype controls and analyzed by flow cytometry using FACSAriaII and FlowJo software (Tree Star, OR).

2.6. Immunizations

BALB/c mice were orally immunized as described for other adjuvants [19,20] on days 1, 2, 3, 8, 9 and 10 with OVA (100 μg), OVA + U-Omp19 (150 μg), OVA + CT (10 μg) or OVA + aprotinin (1 μg). C57BL/6 mice were immunized on days 0, 7 and 14. One month after the last immunization mice were sacrificed to study immune responses.

2.7. Determination of T helper immune responses

Spleen, PPs and MLNs single cell suspensions from immunized mice were cultured in duplicates at 4×10^6 cells/ml in RPMI 1640 supplemented with 10% FCS (Life Technologies), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin 100 U/ml, and streptomycin (100 mg/ml) (complete medium) and stimulated with OVA, or complete medium alone. After 72 h or 5 days of incubation at 37 °C in a humidified atmosphere (5% CO_2) cell culture supernatants were collected. IFN-γ, IL-4, IL-10 and IL-17 were determined by ELISA (Pharmingen and R&D systems).

2.8. Intracellular IFN-γ determination

Splenocytes from immunized mice were cultured (4×10^6 cells/well) in presence of complete medium (supplemented with IL-2) or Ag stimuli (IL-2 plus OVA — 500 μg/ml, A20JOVA mitomicyn-treated cells as a source of APCs (25:1) and OVA_{323} peptide) for 18 h. Next, brefeldin A was added for 5 h more to the samples. After that, cells were treated and analyzed as described previously [13].

2.9. Determination of Ab responses

BALB/c mice were orally immunized as described on days 1, 2, 3, 8, 9 and 10 with CT (10 μg) or CT + U-Omp19 (10 + 150 μg). Same immunization schedule was used when Shiga toxin (ST) was selected as Ag. Other groups of mice were orally immunized with heat killed extract from *Salmonella*-HKS (60 μg) or HKS + U-Omp19 (60 + 150 μg) on days 0, 7 and 14. Feces and sera were obtained as described previously [21] 2 weeks after last immunization to study Ag-specific- or U-Omp19-specific IgA or IgG responses. Cut-off values for the ELISA assays were calculated as the mean specific OD plus 3 SD from 20 sera or feces from non-immunized mice. Sera or feces titers were established as the reciprocal of the last dilution yielding an OD higher than the cut-off.

2.10. Pathogen challenge assays

C3H/HeN mice were orally immunized on days 1, 2, 3, 8, 9 and 10 with PBS, purified recombinant *E. coli* GRA4 (30 μg), GRA4 + U-Omp19 (100 μg) or GRA4 + CT (10 μg). Two weeks after the last immunization, some mice per group were injected intradermally in one footpad with 20 μg of Ag (GRA4) and in the contralateral footpad with an equal volume of saline. Footpad thickness was measured 72 h later using a digital caliper with a precision of 0.01 mm, and the mean increase in footpad thickness (mm) was calculated as: (footpad thickness) Ag — (footpad thickness) saline. Three weeks after last immunization other mice were orally infected with 20 *T. gondii* tissue cysts of the ME49 strain. Serum samples were obtained 8 days post challenge and the activity of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and alanine aminotransferase (ALT) were measured as an indication of liver damage [22] (Wiener Lab., Argentina). One month after challenge, mice were sacrificed and their brains removed and homogenized to determine the mean number of cysts per brain by observation under optical microscope as described [23].

2.11. Statistical analysis

Statistical analysis and plotting were performed using GraphPad Prism 4 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 prior to the analysis to obtain data with a normal distribution. In experiments with 2 groups, Unpaired T Test was used. A P value less than 0.05 was considered significant. When bars are plotted results are expressed as mean ± SEM for each group.

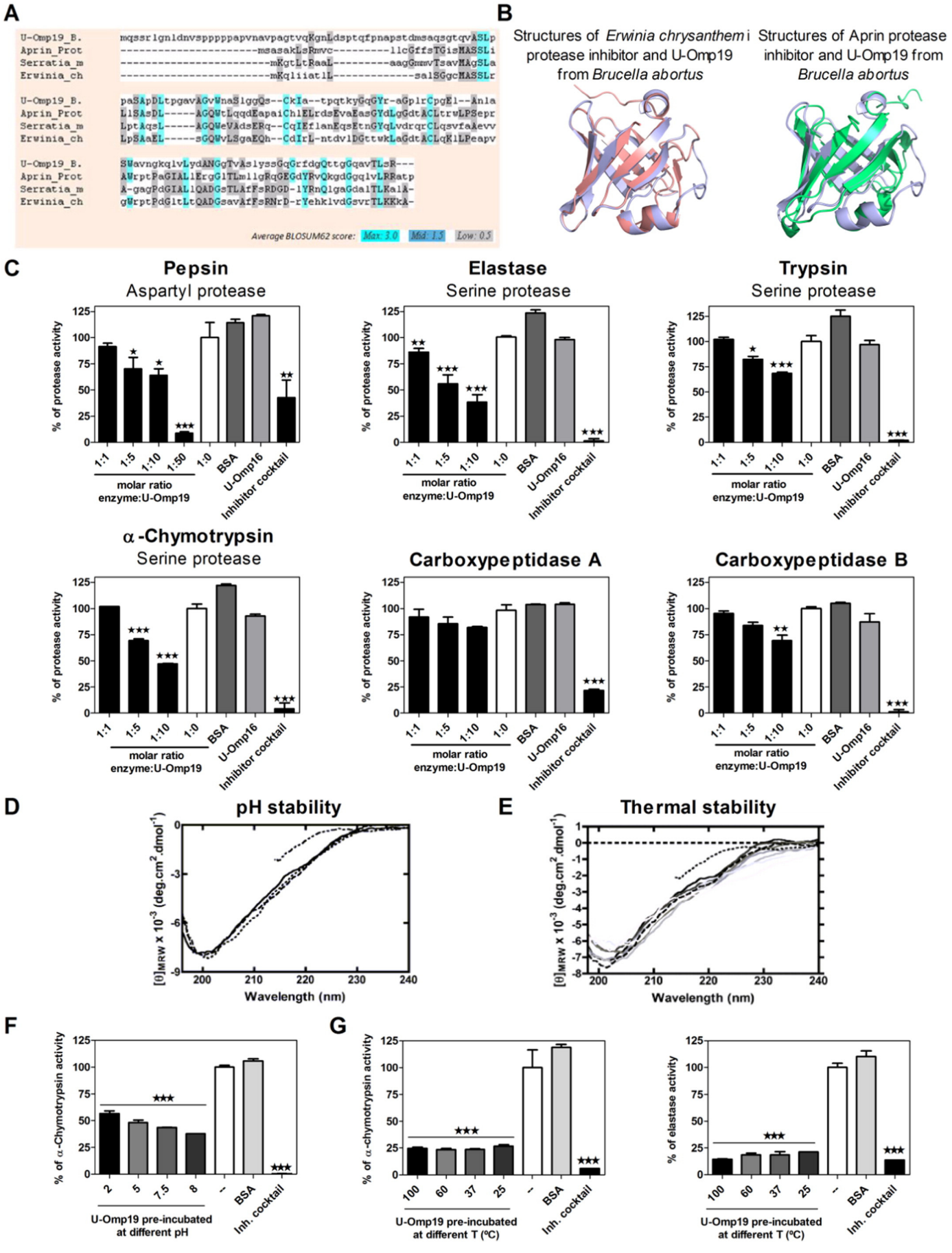
3. Results

3.1. The protein moiety of B. abortus Omp19 (U-Omp19) is a protease inhibitor

BLAST, Pfam and MEROPS sequence analysis report that U-Omp19 has significant sequence identity with other bacterial protease inhibitors, particularly with those representatives of inh protease inhibitor family (family I38, clan IK): *Pseudomonas aeruginosa* protease inhibitor (APRin), *Serratia marcescens* protease inhibitor and *E. chrysanthemi* protease inhibitor (Inh) [15,16] (Fig. 1A and supp. Materials and methods). Superposition of the known three-dimensional structures of Inh or APRin with the homology model of *Brucella* U-Omp19 (obtained with the SWISS MODEL server [24]) were performed. U-Omp19's beta barrel core is predicted to be structurally similar to Inh and APRin (Fig. 1B).

Then, the ability of U-Omp19 to inhibit gastrointestinal proteases was studied for the first time. U-Omp19 is a protease inhibitor since it was able to inhibit *in vitro* the activity of proteases present in the stomach (pepsin, $P < 0.001$) and proteases secreted from the pancreas to the intestine (elastase, trypsin and α-chymotrypsin, $P < 0.001$). Also it partially inhibited the activity of carboxypeptidase B ($P < 0.01$) but could not inhibit carboxypeptidase A (Fig. 1C). Bovine Serum Albumin (BSA) and U-Omp16 from *Brucella* (a *Brucella* Omp with a molecular weight very similar to U-Omp19) did not inhibit the enzymatic activity of any of the proteases tested when used in a similar molar ratio to U-Omp19, further indicating the specificity of the reaction assayed (Fig. 1C).

These assays were performed under the optimal pHs of each protease. Of note, U-Omp19 inhibited *in vitro* pepsin activity at pH 2 suggesting that its structure is stable at this low pH present at the stomach. To confirm this, Far-UV circular dichroism (CD) spectra of U-Omp19 under different pHs and temperatures were conducted, which indicated that U-Omp19 did not suffer any evident structural change under pH 2, 5 or 7.5. A control performed with U-Omp19 in a buffer containing 6 M guanidinium chloride showed the loss of CD signal between 210 and


A
B
Structures of Erwinia chrysanthemi protease inhibitor and U-Omp19 from Brucella abortus
Structures of Aprin protease inhibitor and U-Omp19 from Brucella abortus
C
Pepsin
Aspartyl protease
Elastase
Serine protease
Trypsin
Serine protease
α-Chymotrypsin
Serine protease
Carboxypeptidase A
Carboxypeptidase B
% of protease activity
molar ratio enzyme:U-Omp19
BSA
U-Omp16
Inhibitor cocktail
D
pH stability
E
Thermal stability
Wavelength (nm)
F
% of α-Chymotrypsin activity
U-Omp19 pre-incubated at different pH
G
% of α-chymotrypsin activity
% of elastase activity
U-Omp19 pre-incubated at different T (ºC)
Inh. cocktail

240 nm expected for a denatured U-Omp19. Moreover, the thermal denaturation experiment showed no significant denaturation as CD signal between 210 and 235 nm was still present after heating U-Omp19 to 90 °C (Fig. 1D–E). Consequently U-Omp19 retained its full protease inhibitor activity when previously exposed to a broad pH (2–8) or temperature (25–100 °C) range ($P < 0.001$) (Fig. 1F–G).

To further study the protease inhibitory mechanism of U-Omp19, the kinetic of trypsin (Fig. 2A), α-chymotrypsin (Fig. 2B) and pancreatic elastase (Fig. 2C) inhibition was studied (see Supp. Materials and methods). Lineweaver–Burk and Dixon plots indicated that U-Omp19 inhibited the three serine proteases in a mixed noncompetitive manner [25] (Fig. 2 left and middle panels), indicating that U-Omp19 binds both, the free proteases and the protease-substrate binary complexes. As U-Omp19 does not compete with the substrates for binding to the free proteases; it binds these serine proteases at a site distinct from the active site. Global nonlinear fit of data was used to estimate K_i and alpha values. Determined K_i values (Mean; Std Error) were: (77.39 μM; 21.23) for Trypsin, (55.75 μM; 10.40) for α-Chymotrypsin and (128.0 μM; 41.6) for pancreatic Elastase. For the three proteases the estimated values of alpha were between 1.8 and 3.4, indicating that U-Omp19 bound both, the free proteases and the protease-substrate binary complexes, but with different affinities (Fig. 2, right panels). Together these results indicate that U-Omp19 exhibits a mixed noncompetitive type inhibitory activity against main gut proteases (trypsin, α-chymotrypsin and elastase) with K_i values in the μM range.

As the *in vivo* content and proportion of proteases in the gastrointestinal tract is diverse in the different organs, the ability of U-Omp19 to inhibit proteases activity in stomach or intestine extracts was studied. U-Omp19 was able to partially (40–60%) inhibit *in vitro* the protease activity of stomach ($P < 0.001$) or intestine ($P < 0.001$) extracts from mice using two different substrates: casein and ovalbumin (OVA) (Fig. 3A–B). As expected, BSA did not inhibit the proteolytic activity of stomach or intestine extracts *in vitro* whereas a protease inhibitor cocktail did (Fig. 3A–B).

To study *in vivo* the biological activity of U-Omp19 animals were i.g. administered, on one occasion, with casein-BODIPY (model Ag which exposes its dyes and fluoresces when digested) alone, plus U-Omp19 or plus aprotinin. Stomachs were removed 15 min and 1 h (h) later and casein digestion was determined. Oral co-administration of U-Omp19 prevented Ag degradation *in vivo* (~50%) in the stomach ($P < 0.001$) of mice at 15 min (Fig. 3C). The proteolysis inhibition was a slight lesser than that obtained when aprotinin was used. At 60 min there is still significant reduction ($P < 0.05$) in Ag digestion (~25%) at the stomach (Fig. 3C). Moreover, using OVA-FITC as a model Ag *in vivo* there was an increase, at 90 min post-delivery, in the amount of Ag at the stomach (slight but not significant) and intestine ($P < 0.05$) when it was co-administered orally with U-Omp19 (Fig. 3D).

Taken together these and above results indicate that U-Omp19 is a pH- and temperature-resistant, protease inhibitor of stomach and gut proteases.

3.2. Oral U-Omp19 co-administration limits proteolysis of the Ag within cells from Peyer's Patches and Mesenteric Lymph Nodes, increasing the amount of Ag at these sites

To induce an adaptive immune response Ags must reach inductive sites (PPs and MLNs) of the gastrointestinal immune system. Oral delivery of U-Omp19 increased the amount of the co-administered model Ag OVA-AlexaFluor647 (Figs. 4A and S1A fluorescence arbitrary units shown) or OVA-FITC (Fig. S2A) inside cells from PPs at 2, 6 and 12 h ($P < 0.001$, $P < 0.001$, $P < 0.01$ *vs* OVA delivered alone) and in cells from MLNs at 6, 12 and at 18 h post administration ($P < 0.05$, $P < 0.01$, $P < 0.001$). Neither CT nor aprotinin -a known eukaryotic serine protease inhibitor- co-administration was as efficient as U-Omp19 at increasing the amount of intracellular Ag at the inductive sites.

Digestive enzymes break down large polypeptides into non-immunogenic di- and tri-peptides, too small to bind to major histocompatibility complex (MHC) molecules [26]. Accordingly with the inhibition of the stomach and gut proteases, U-Omp19 co-administered by the oral route decreased the amount of digested Ag (OVADQ or casein-BODIPY) within cells from PPs at 2, 6 and 12 h ($P < 0.01$, $P < 0.001$, $P < 0.01$) and in MLNs at 6, 12 and 18 h post-vaccination ($P < 0.001$, $P < 0.01$, $P < 0.01$) (Figs. 4B, S1B and S2B fluorescence arbitrary units shown). In contrast, CT co-administration did not reduce the amount of degraded Ag in the inductive sites. Altogether these results indicate that U-Omp19 co-administration by the oral route can limit the susceptibility of the Ag to proteolysis *in vivo* at the gastrointestinal tract, increasing the half-life of the Ag within cells of the immune inductive sites: PPs and MLNs.

3.3. U-Omp19 induces the recruitment, activation and increases the amount of Ag within monocytes and DCs at PPs and MLNs after oral co-administration

Inducing an appropriate inflammatory response is crucial to drive an efficient adaptive immune response. In relation to this, single oral administration of Ag plus U-Omp19 induced DCs ($CD11c^+$) recruitment to PPs ($P < 0.01$) as well as monocytes ($CD11b^+$ $CD11c^-$) and $CD8\alpha^+$ DCs ($CD11c^+$ $CD8\alpha^+$) recruitment to MLNs ($P < 0.05$ at 4 h and $P < 0.01$ at 18 h post delivery) (Fig. S3). CT co-administration or aprotinin induced lower monocyte- and DC-recruitment (Fig. S3). Moreover, only U-Omp19 was able to induce the recruitment of a particular DC subtype: $CD11c^+$ $CD8\alpha^+$ DCs, known to stimulate Th1 responses and Ag crosspresentation to $CD8^+$ T cells [27] (Fig. S3).

Worth mentioning, U-Omp19 co-administered by the oral route increased the amount of Ag within monocytes and $CD11c^+$ $CD8\alpha^+$ DCs at PPs and MLNs (Fig. 4C). In agreement to the results shown in Figs. 1–3, the amount of digested Ag inside monocytes and $CD11c^+$ $CD8\alpha^+$ DCs from PPs partially decreased after oral U-Omp19 co-administration (Fig. S4). Furthermore, U-Omp19 oral administration induced *in vivo*

Fig. 1. (A) U-Omp19 presents sequence identity with other bacterial protease inhibitors of the inh family. Sequence alignment between *B. abortus* U-Omp19 and representative proteins belonging to the inh protease inhibitor family: *Pseudomonas aeruginosa* Alkaline protease inhibitor (APRin) Q03026, *Serratia marcescens* protease inhibitor Q54478 and *Erwinia chrysanthemi* protease inhibitor P18958. Similar residues are colored as the most conserved ones (according to BLOSUM62). Average BLOSUM62 score: Max: 3.0, Mid: 1.5, Low: 0.5. (see Supplementary materials and methods). (B) Superposition of known and modeled structures. Superposition of the three-dimensional structures of *Erwinia chrysanthemi* Inh (orange, PDB 1SMP) or *Pseudomonas aeruginosa* Alkaline protease inhibitor (green, PDB 1JIW) with *Brucella* U-Omp19 (purple, homology model obtained with the SWISS MODEL server). (C) U-Omp19 is a protease inhibitor. Protease inhibitor activity was determined using casein BODIPY-FL assay kit in which the increment in fluorescence is proportional to proteolytic activity. Pepsin, elastase, trypsin, α-chymotrypsin, carboxypeptidase A and B were pre-incubated in the optimal buffer for each enzyme for 1 h at different molar ratios with U-Omp19 (enzyme:U-Omp19 — 1:1, 1:5, 1:10 and 1:50 only with pepsin) or without U-Omp19 (1:0). A mammalian protease inhibitor cocktail was used as positive control and BSA and U-Omp16 as negative controls at 1:10 M ratio. Then, were incubated with 1 μg/ml casein BODIPY-FL for 1 h. Inhibitor activity is expressed as percentage of protease activity remaining when compared to condition 1:0.$^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$ *vs* 1:0 condition. U-Omp19 is stable at different pH and temperature conditions. (D) Far-UV CD spectra of U-Omp19 at pH 7.5 (full line), pH 2 (dotted line), pH 5 (dashed line) and pH 7.5 6 M guanidinium chloride (GdmCl) (dashed and dotted line). (E) Far-UV CD spectra of U-Omp19 were measured at 20 (black full line), 30, 40, 50, 60, 70, 80 and 90 °C (gray scaled full lines) and then back from 90 to 20 °C (black dashed line). The spectrum of denatured protein in buffer containing 6 M GdmCl is also shown (dotted line). (F) U-Omp19 activity after exposure to different pH. U-Omp19 was pre-incubated at room temperature for 1 h at different pH (2, 5, 7.5 and 8) and then adjusted to the optimum pH for α-chymotrypsin. Residual inhibitor activity was assessed as in (C) using α-chymotrypsin as model protease in a 1:10 M ratio (protease:U-Omp19) or without inhibitor (−). Inhibitor activity is expressed as percentage of protease activity remaining when compared to condition −. ($^{\star\star\star}P < 0.001$ *vs* −). (G) U-Omp19 activity after incubation at different temperatures. U-Omp19 was pre-incubated at 25–100 °C for 1 h and subsequently adjusted to room temperature. Residual inhibitor activity was assessed as in (F) using α-chymotrypsin or elastase as model proteases. Inhibitor activity is expressed as percentage of protease activity remaining when compared to condition −. ($^{\star\star\star}P < 0.001$ *vs* −). Results are shown as mean ± SEM and are representative of three independent experiments.

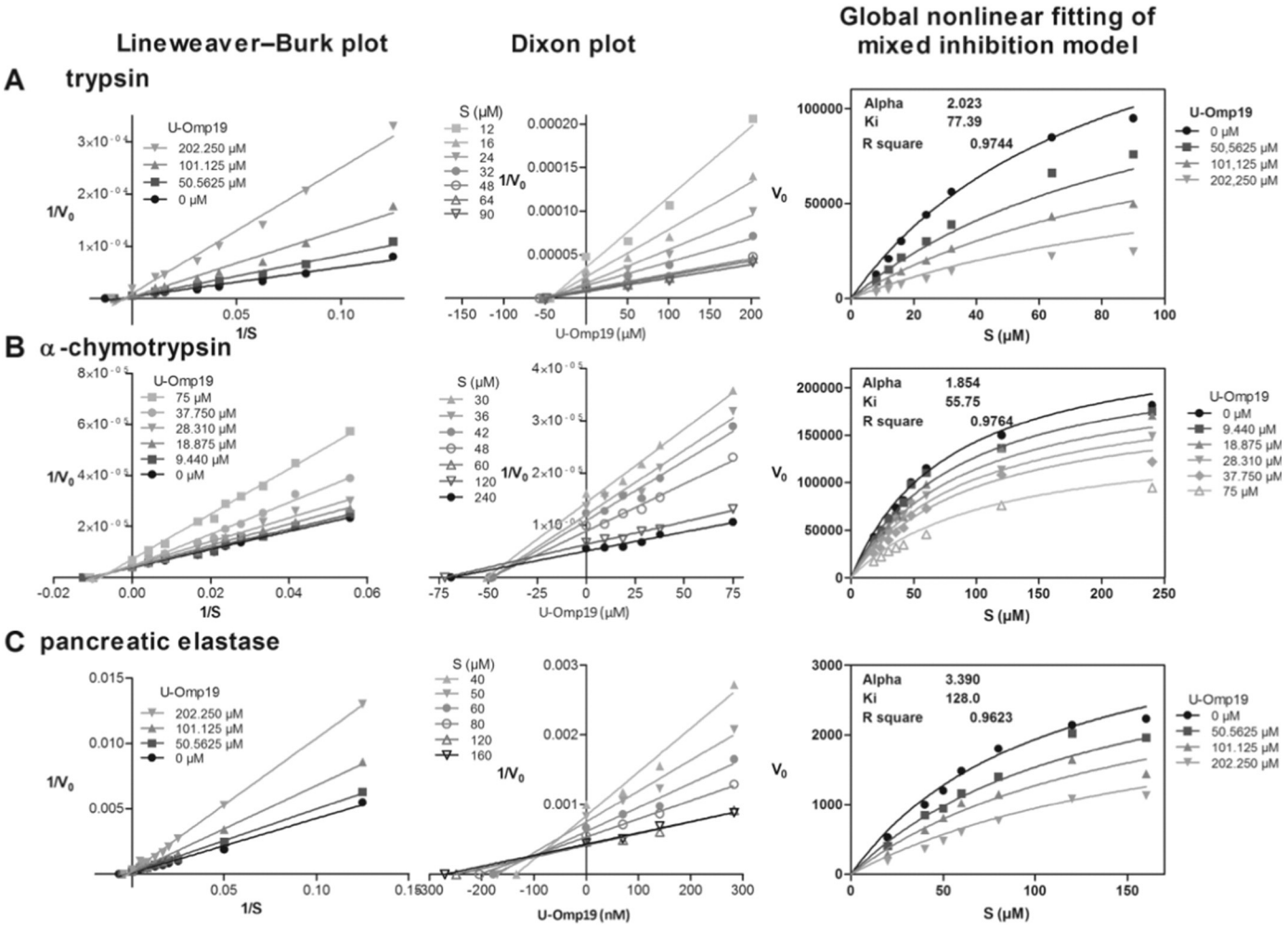


Fig. 2. Kinetics of U-Omp19's inhibition of (A) trypsin, (B) α-chymotrypsin and (C) pancreatic elastase. Samples of the specified enzyme preparations were treated with different concentrations of U-Omp19 before addition of the indicated specific substrate concentrations. Proteolytic activity was measured as the increment in fluorescence units of samples. Linear regressions of fluorescent units *versus* time (s) were used to calculate initial velocities (V_0) for each substrate concentration [S] and inhibitor concentration [U-Omp19] combination sample. Data were transformed into Lineweaver–Burk plots ($1/V_0$ *vs.* 1/[S]) (left panels) and Dixon plots ($1/V_0$ *vs.* [U-Omp19]) (middle panels). Best-fit lines were generated by linear regression analysis. Global nonlinear fit of data were used to calculate Ki and alpha values (right panels). Each panel is representative of at least three independent experiments.

the activation of DCs, as $CD8\alpha^+$ $CD11b^-$ $CD11c^+$ $MHCII^+$ DCs recruited to MLNs showed up-regulated expression of co-stimulatory molecules (CD80, CD40 and CD86) (Fig. 4D and supplementary materials and methods).

These results indicate that U-Omp19 induces the recruitment and activation of APCs to mucosal inductive sites and can increase Ag intracellular half-life in APCs.

3.4. Oral co-administration of U-Omp19 increases mucosal and systemic T lymphocyte adaptive immune responses

To study if oral delivery of OVA in the presence of U-Omp19 resulted in a significantly increased adaptive immune response, BALB/c mice were orally immunized with OVA plus i) buffer, ii) U-Omp19, iii) CT or iv) aprotinin. MLNs cells from OVA + U-Omp19 immunized mice produced significant amounts of IFN-γ and IL-17 in response to OVA ($P < 0.05$ *vs* OVA immunized mice, Fig. 5A). On the contrary, the same cells did not produce IL-4 or IL-10 after OVA *in vitro* stimulation (data not shown). Also, co-administration of OVA plus U-Omp19 induced OVA-specific IFN-γ at the spleens ($P < 0.05$). A slight but not statistically significant OVA-specific IL-17 production was evidenced only in the spleens of OVA + U-Omp19 immunized animals (Fig. 5A). OVA-specific IFN-γ producing $CD4^+$ and $CD8^+$ T cells were found in animals immunized with OVA plus U-Omp19 (Fig. 5B). In contrast, CT or aprotinin co-administration did not induce OVA-specific IFN-γ producing $CD4^+$ T or $CD8^+$ T cells nor IFN-γ or IL-17 secretion from stimulated MLNs and splenocytes (Fig. 5A–B). Similarly, animals immunized with OVA + U-Omp19 showed OVA-specific T cell responses *in vivo* (DTH assay) ($P < 0.05$) while animals immunized with OVA or OVA + CT did not (Fig. S5A). Also, oral co-administration of U-Omp19 or aprotinin induced a slight but not significant increase in anti-OVA IgA in feces, while CT + OVA did it ($P < 0.05$) (Fig. S5B). Important, anti-U-Omp19 IgA or anti-U-Omp19 IgG was not detected in any immunized group (Fig. S5B–C). U-Omp19 immunization alone did not elicit specific adaptive immune responses to OVA (Figs. S5A and S9).

The α4β7 integrin is critical for lymphocyte homing to the small intestine [28]. Oral delivery of OVA with U-Omp19 resulted in a significantly increased population of MLN $CD4^+$ and $CD8^+$ T cells with up-regulated α4β7 integrin expression 1 month after immunization in BALB/c mice. A similar degree of up-regulation was observed when OVA was co-administered with CT (Fig. S5D).

To study adaptive immune responses under a different genetic background, C57BL/6 mice were orally immunized with OVA, OVA + U-Omp19 or OVA + CT. One month later, OVA specific-Th1 and OVA-specific IFN-γ producing $CD4^+$ T and $CD8^+$ T cells were evidenced at the mucosal as well as systemic level, if U-Omp19 was co-

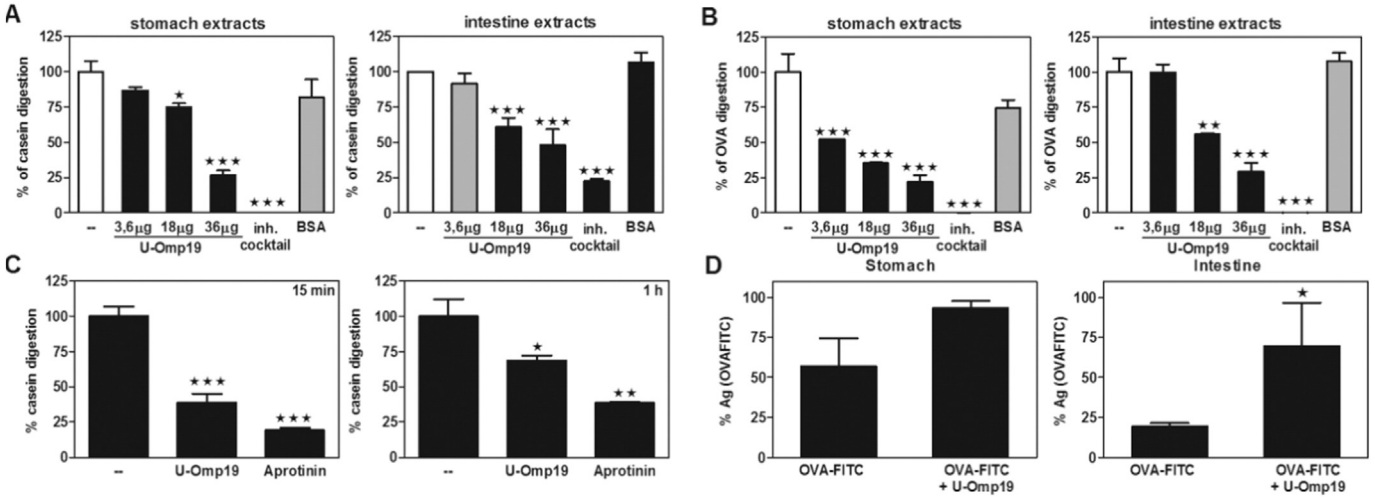


Fig. 3. U-Omp19 inhibits proteolytic activity of stomach and intestine extracts. Stomach and Intestine extracts from mice were pre-incubated with buffer (−), different amounts of U-Omp19 (3,6 µg; 18 µg or 36 µg/well), Inhibitor cocktail or BSA (36 µg/well) as negative control. Then, the mixtures were incubated with casein BODIPY-FL for 1 h (A) or with OVADQ for 4 h (B) and the fluorescence increment was determined. Results are presented as percentage of Casein or OVA digestion compared to the condition without inhibitor: −. (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ *vs* −). U-Omp19 partially inhibits Ag digestion *in vivo*. (C) Animals were fed with buffer, casein BODIPY-FL alone, casein BODIPY-FL plus U-Omp19 or casein BODIPY-FL plus aprotinin. Stomachs were removed 15 min and 1 h after oral delivery and Ag digestion was determined. Results are presented as percentage of Casein digestion compared to the group fed with casein BODIPY-FL alone. (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ *vs* −). (D) Animals were i.g. delivered with buffer, OVAFITC alone or OVAFITC plus U-Omp19. Stomachs and small intestines were removed 90 min after oral delivery and Ag fate was determined. Results are presented as percentage of OVA-FITC compared to maximum fluorescence intensity measured. Results are shown as mean ± SEM for each group ($n = 4$) and are representative of three independent experiments. (*$P < 0.05$ *vs* casein or OVA alone group).

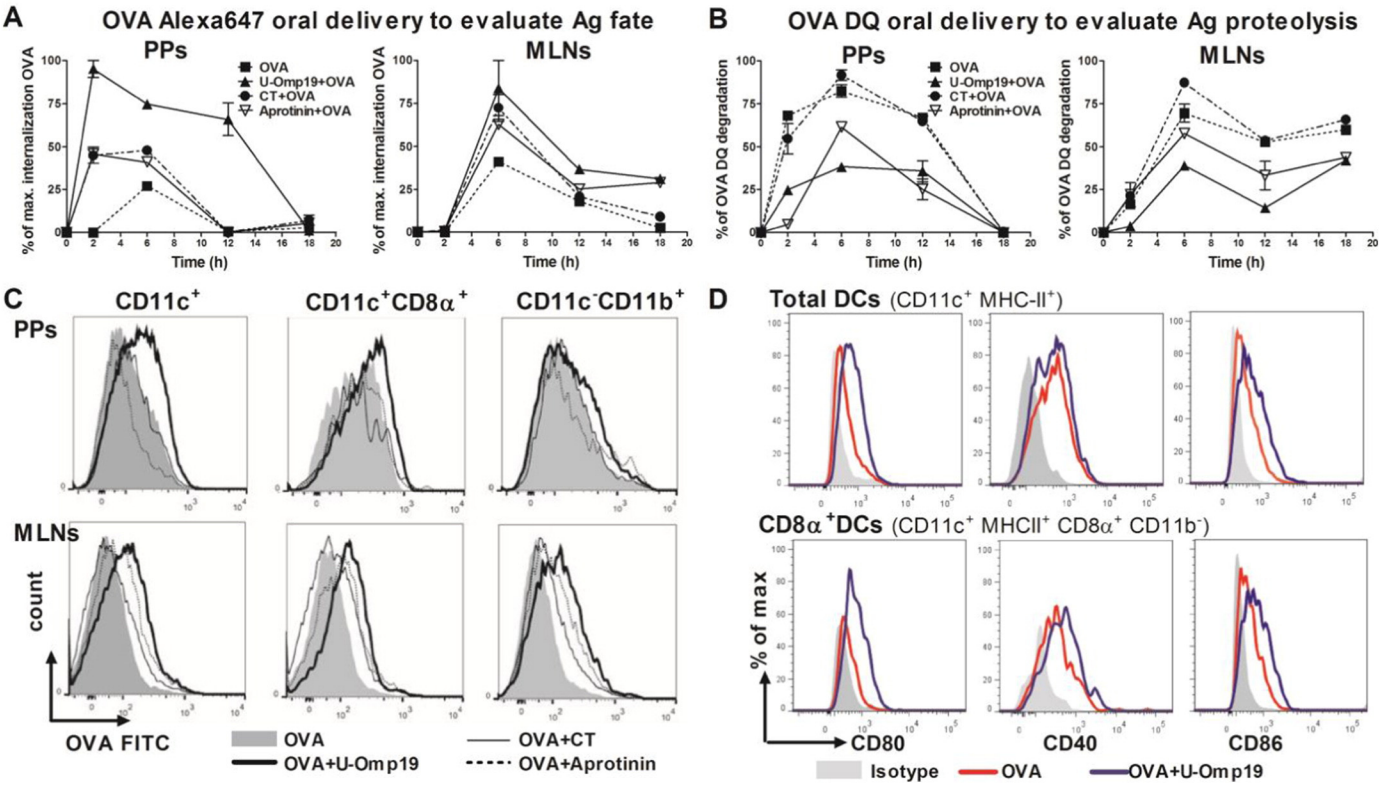


Fig. 4. U-Omp19 oral co-administration with an Ag limits Ag proteolysis and increases the amount of Ag within cells at PPs and MLNs. Mice ($n = 3$/group) were i.g. administered once with a mixture of OVA Alexa Fluor-647 (OVAAF647) and OVADQ plus i) buffer, ii) U-Omp19, iii) cholera toxin (CT), iv) aprotinin or buffer alone as a control. At different time points post delivery PP or MLN cell suspensions were obtained and washed with PBS to eliminate extracellular Ag and Ag presence or proteolysis was determined in 1×10^6 cells by detection of OVA Alexa 647 (A) or OVADQ (B) fluorescence respectively. Fluorescence increase is proportional to the amount of Ag in the case of OVAAF647 while it is proportional to Ag degradation in the case of OVADQ. Results are expressed as % of max. internalization of OVA or % of OVA degradation (mean ± SEM). Representative of three independent experiments. (C) Oral delivery of U-Omp19 increases the amount of Ag within APCs *in vivo*. Ag presence was determined by flow cytometry in $CD11c^+$, $CD11c^+$ $CD8\alpha^+$ and $CD11c^-$ $CD11b^+$ cells at PPs and MLNs 4 h after single oral delivery of OVAFITC plus i) buffer, ii) U-Omp19, iii) CT or iv) aprotinin. Results are presented as representative histograms obtained for each group ($n = 3$/group) and are representative of two different experiments. (D) U-Omp19 induces activation of APCs *in vivo*. Flow cytometric analysis of CD80, CD40 and CD86 expression by MLN $CD11c^+$ $MHC\text{-}II^+$ and $CD11c^+$ $MHC\text{-}II^+$ $CD8\alpha^+$ DCs after single oral delivery of i) OVA or ii) OVA + U-Omp19 at 18 h post-administration. Results are presented as representative histograms obtained for each group ($n = 3$/group) and are representative of two different experiments.

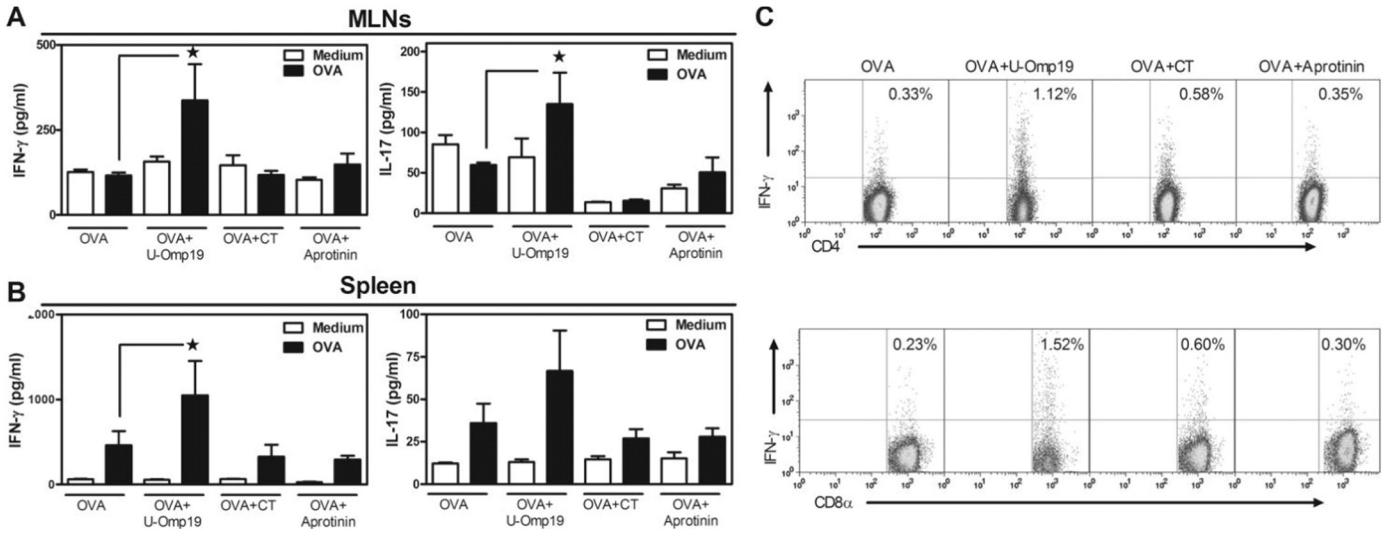


Fig. 5. Oral immunization of U-Omp19 with Ag increases mucosal and systemic Ag-specific Th1–Th17 immune responses in BALB/c. Cytokine (IFN-γ and IL-17) production determined by ELISA on supernatants harvested 5 days after stimulation of (A) MLN and spleen cells with OVA or complete medium. Cells were obtained 3 weeks after the last boost of animals ($n = 6$/group) orally immunized with OVA, OVA + U-Omp19, OVA + CT or OVA + aprotinin. Results are shown as mean ± SEM for each group and are representative of 3 independent experiments. (★$P < 0.05$ *vs* OVA group). (B) Spleen cells from immunized mice were stimulated *in vitro* with OVA for 23 h, last 5 h cells were treated with brefeldin A. Then, cells were stained with specific antibodies anti-CD4, anti-CD8, fixed, permeabilized and stained intracellularly with anti-IFN-γ for flow cytometry analysis. Results are presented as percentage of IFN-γ producing T cells and are representative of 3 independent experiments.

administered orally with OVA ($P < 0.01$ *vs* OVA immunized mice) (Fig. S6). These results indicate that U-Omp19 has a similar immune adjuvant effect in BALB/c and C57BL/6 mice.

Together these results indicate that oral co-administration of this bacterial protease inhibitor with Ag increases mucosal and systemic Ag-specific Th1, Th17 and $CD8^+$ T cell immune responses.

3.5. U-Omp19 co-administered orally with bacterial Ags increases Ag-specific mucosal IgA and systemic IgG responses

OVA-specific antibody (Ab) mucosal responses were difficult to achieve using U-Omp19 (Fig. S5B–C). Thus, to test U-Omp19's capacity to elicit Ab responses we decided to use other Ags, such as Shiga Toxin (Stx2) from *E. coli*, CT from *Vibrio cholera* or a heat killed extract from *Salmonella typhimurium* (HKS). There was a significant increase ($P < 0.01$) in anti-ST IgA in feces when ST was orally co-administered with U-Omp19 (Fig. 6A and supplementary material and methods). Serum anti-ST IgG responses were not induced in any immunized group (Fig. S7A). Oral co-administration of U-Omp19 with CT (as Ag) induced increased ($P < 0.05$ *vs* CT alone) anti-CT IgA in feces and IgG in sera (Fig. 6B). Also, U-Omp19 oral co-administration with HKS increased anti-HKS fecal IgA and serum IgG compared with oral delivery of HKS alone (Fig. 6C). Anti-U-Omp19 serum IgG or fecal IgA were not detected in any immunized group (Fig. S7B–C).

Altogether these results indicate that U-Omp19 co-administered orally with pathogen-derived Ags can increase Ag-specific mucosal IgA and systemic IgG responses.

3.6. U-Omp19 in an oral vaccine formulation induces protection against oral challenge with a virulent parasite

Besides and considering that *Toxoplasma gondii* is able to infect the host through the gut mucosa and Th1 responses are known to be important in protection [29], these prompted us to study *T. gondii* infection model for vaccination using U-Omp19. Thus, the efficacy of oral immunization with a recombinant protein GRA4 plus U-Omp19 was evaluated. C3H/HeN mice were orally immunized with i) PBS, ii) GRA4, iii) GRA4 + U-Omp19 or iv) GRA4 + CT. Three weeks after the last boost, mice were challenged orally with Me49 tissue cysts and individual parasite levels were assessed 1 month later. An important decrease in the brain cyst burden ($P < 0.05$) together with a reduction in serum of liver damage associated enzymes (AST, LDH and ALT) and an increase ($P < 0.05$) in GRA4-specific IgA and DTH was observed in GRA4 + U-Omp19 immunized mice as compared to the control groups (Fig. 7A–C).

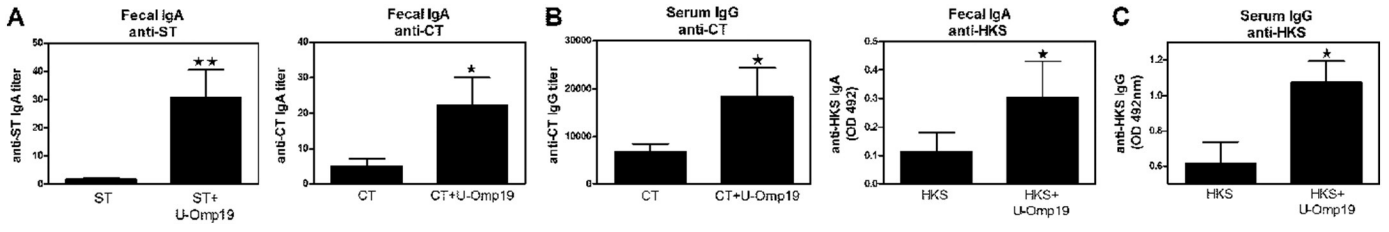


Fig. 6. U-Omp19 co-administered orally with bacterial Ags can increase mucosal Ag-specific IgA and serum Ag-specific IgG responses. BALB/c mice were orally immunized with PBS, Ag alone (CT, HKS or ST) or the Ag + U-Omp19. (A) ST-, (B) CT- and (C) HKS-specific IgG and IgA were determined in sera and in feces respectively 2 weeks after last immunization. Results are expressed as mean Ab titers or OD 492 nm ± SEM for each group ($n = 5$–6/group) and are representative of three independent experiments (★$P < 0.05$ and ★★$P < 0.01$ *vs* Ag alone group).

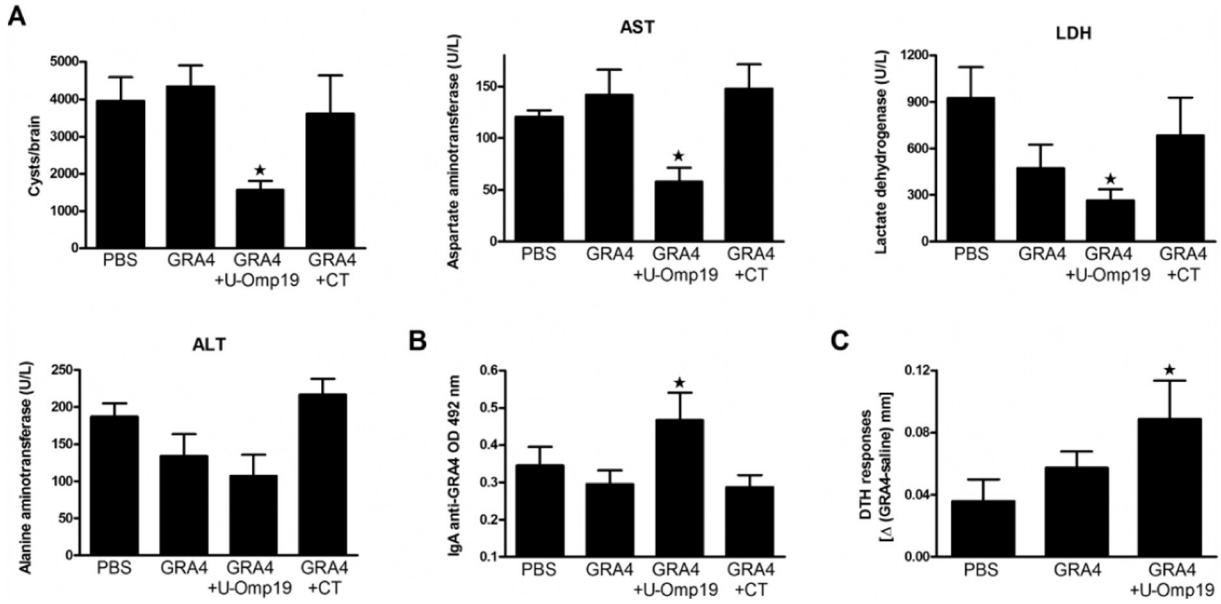


Fig. 7. U-Omp19 in an oral vaccine formulation reduces parasite levels after oral challenge with *Toxoplasma gondii*. C3H/HeN mice were orally immunized with PBS, GRA4, GRA4 + U-Omp19 or GRA4 + CT and orally infected with *T. gondii* tissue cysts three weeks later. (A) Mean number of cysts per brain were counted 1 month after challenge. Activity of the specified enzymes (AST, LDH and ALT) was measured in serum obtained 8 days post challenge. (B) GRA4 specific IgA Abs in feces were determined 3 weeks after the last immunization. Results are shown as mean OD at 492 nm ± SEM for each group (C) DTH response to GRA4 assessed two weeks after the last immunization. Results are shown as mean increment in the hind footpad between right and left foot ± SEM for each group (n/group = 6–8), measured 72 h later. ($^{\star}P < 0.05$ *vs* PBS or GRA4 group).

These results together indicate that U-Omp19 could be a useful constituent of oral vaccine formulations against infectious diseases.

4. Conclusions and discussion

There is no vaccine that is more amenable to mass immunization in field settings than oral vaccine. Yet, there is a relative paucity of oral vaccines in current medical practice. The greatest threat to peptide and protein oral administration lies in the small intestine, which contains large quantities of peptidases. For example, luminal digestion in the duodenum by trypsin and chymotrypsin can convert almost half of the ingested protein to trichloroacetic acid-soluble material within 10 min, 60–70% of which is in the form of small peptides with 2–6 amino acids that cannot bind MHC molecules [26].

As demonstrated in this work, we have completed proof of concept studies that indicate that a *Brucella* spp. protein would be a useful component in an oral vaccine formulation because: i) it bypasses the hostile environment of the gastrointestinal tract inhibiting stomach and gut proteases and consequently increases the half-life of co-administered Ags at the inductive sites: PPs and MLNs while ii) it induces the recruitment of APCs, activation and Ag internalization. Besides, it reduces the amount of digested Ag within APCs at inductive sites. Consequently, mucosal as well as systemic Ag-specific immune responses, Abs, Th1, Th17 and $CD8^+$ T cells are enhanced if U-Omp19 is orally co-administered with the Ag.

The inhibition of proteolysis and induction of immune responses has also been demonstrated using BALB/c, C57BL/6 (Fig. S6 and Supplementary material) or C3H/HeN mice further indicating that U-Omp19 can be used under different genetic backgrounds. Moreover, in this work U-Omp19's usefulness was evaluated using model Ags such us OVA and casein as well as bacterial and parasite derived Ags.

As U-Omp19 possesses the capability of partially inhibiting the destruction of the Ag, less Ag would be needed to induce protection, which could lower vaccine costs. Interestingly, this protease inhibitor is pH and thermally stable; these are important attributes that will allow it to work at the low pH from the stomach and its use in vaccines avoiding cold chain. We believe that if U-Omp19 would not be pH stable nor be able to inhibit stomach and gut proteases immune properties can never been seen. This would explain why at present most mucosal adjuvants are given intranasal and not oral to avoid their digestion before reaching immune cells.

Being U-Omp19 a protein, the observed effect on gastrointestinal proteases should be short term and reversible because U-Omp19 -when present in the gut- will be finally digested by the proteases newly secreted from the pancreas to the intestine. In fact, we have demonstrated that U-Omp19 effect on *in vivo* inhibition of proteolysis is transient. Moreover, the amount of *in vivo* inhibition is not total, allowing digestion, which would lower the chances of potential adverse effects of U-Omp19 in vaccine formulations. Of note, there were no visible adverse effects in mice or histopathological changes in PPs, MLNs and intestines from U-Omp19 orally immunized mice (Fig. S8). Worth mentioning, antibody responses against U-Omp19 were not detected in any immunized group. This was reported in our previous work using U-Omp19 alone as Ag [14] and in the present work where U-Omp19 was orally co-administered with the Ag. This is of great importance because it was described for other adjuvants and delivery systems that antibody responses against their self may inhibit subsequent utility. Hence these results would support repeated use of U-Omp19 contained in different vaccine formulations without anticipating this problem.

In this work U-Omp19's mechanism of action *in vitro* on main gut proteases was characterized for the first time and results indicated that U-Omp19 inhibits trypsin, chymotripsin and pancreatic elastase by a mixed non-competitive mechanism of inhibition and with a K_i in the μM range. Representative proteins belonging to inh protease inhibitor family as is U-Omp19 from *Brucella* spp. are: *P. aeruginosa* Alkaline protease inhibitor (APRin), *S. marcescens* protease inhibitor (SmaPI) and *E. chrysanthemi* protease inhibitor (Inh). It is probable that these inhibitors protect bacteria from proteolysis during export of self proteases [30]. Until present there are few studies on these types of protease inhibitors (family inh I38), and all of them were shown to inhibit their cognate — self — or heterologous bacterial proteases [15,30]. However, while belonging to other family of protease inhibitors there is a known broad spectrum protease inhibitor from bacteria — from the entomopathogenic bacterium *Photorhabdus luminescens* — that can inhibit also mammalian proteases like trypsin and elastase [17] as is the case for U-Omp19 from *Brucella* spp. Despite we have demonstrated that

U-Omp19 is able to inhibit *in vitro* protease activity of intestine extracts from large animals like pigs (Fig. S10) we believe that new systems including micro-, nanoparticles and enteric capsules may have significant potential to increase U-Omp19 capacity to work in larger animals using advanced combined formulations in the future.

Although there are studies that postulate the usefulness of mammalian protease inhibitors like aprotinin for the co-administration of oral drugs [31,32] and before oral immunization [33] to our knowledge there are no published reports describing the use of a bacterial protease inhibitor in oral vaccine formulations. Until present most studies on the immunomodulatory properties of protease inhibitors were conducted on eukaryotic protease inhibitors [34–36] and some viral as well as filarial nematodes protease inhibitors [35,37] that were found to be immunoregulatory and anti-inflammatory. For example, a serine leucocyte proteinase inhibitor targets monocytes, which in turn inhibit $CD4^+$ T cell proliferation and Th1 cytokine secretion [36,38]. In fact, our results demonstrated that a eukaryotic protease inhibitor like aprotinin can limit gastrointestinal digestion of the co-administered Ag but cannot increase immune responses, this last particularity is important for its application on oral delivery of drugs where immune reactions must be avoided. In contrast, a bacterial protease inhibitor inhibits digestion and increases mucosal and systemic Abs, Th1, Th17 and $CD8^+$ immune responses.

The natural route of infection and the need for a Th1-biased response [29] make *T. gondii* strong candidate for oral vaccination with the novel adjuvant U-Omp19. Consequently, in this work we confirmed U-Omp19's usefulness in an oral subunit vaccine formulation against oral *T. gondii* infection. Nowadays, most used and approved adjuvants are associated with the induction of Th2 and humoral responses [39]. As U-Omp19 induces Abs, Th1, Th17 and $CD8^+$ T cell mucosal immune responses, the discovery of this novel type of molecules: protease inhibitor/adjuvant lays the groundwork for new directions in mucosal vaccine design against infectious diseases [3,40].

Acknowledgments

We would like to thank Dr. Emir Salas-Sarduy, Dr. Juan José Cazzulo, Dra. Vanina Alvarez and Dr. Joaquin J. B. Cannata (IIB-UNSAM, Argentina) for their scientific advises and help on kinetic assays and identification of protease inhibitor mechanism of action.

This work was supported by grants from the Bill and Melinda Gates Foundation through the Grand Challenges Explorations Initiative (phase I OPP1017298, OPP1060394 and phase II OPP1119024); from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT-Argentina): PICT 2010 No. 1163, PICT 2006 No. 1670, ANPCyT/CNPq PICT 2008 No. 18 (to JC).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2015.10.011.

References

[1] I.M. Belyakov, J.D. Ahlers, What role does the route of immunization play in the generation of protective immunity against mucosal pathogens? J. Immunol. 183 (2009) 6883–6892.
[2] C.J. Davitt, E.C. Lavelle, Delivery strategies to enhance oral vaccination against enteric infections, Adv. Drug Deliv. Rev. (2015).
[3] R.L. Coffman, A. Sher, R.A. Seder, Vaccine adjuvants: putting innate immunity to work, Immunity 33 (2010) 492–503.
[4] L.C. Freytag, J.D. Clements, Mucosal adjuvants, Vaccine 23 (2005) 1804–1813.
[5] D.R. Hill, L. Ford, D.G. Lalloo, Oral cholera vaccines: use in clinical practice, Lancet Infect. Dis. 6 (2006) 361–373.
[6] E.B. Norton, L.B. Lawson, L.C. Freytag, J.D. Clements, Characterization of a mutant *Escherichia coli* heat-labile toxin, LT(R192G/L211A), as a safe and effective oral adjuvant, Clin. Vaccine Immunol. 18 (2011) 546–551.
[7] Q. Zhu, J. Talton, G. Zhang, T. Cunningham, Z. Wang, R.C. Waters, J. Kirk, B. Eppler, D.M. Klinman, Y. Sui, S. Gagnon, I.M. Belyakov, R.J. Mumper, J.A. Berzofsky, Large intestine-targeted, nanoparticle-releasing oral vaccine to control genitorectal viral infection, Nat. Med. 18 (2012) 1291–1296.
[8] R. De Smet, T. Demoor, S. Verschuere, M. Dullaers, G.R. Ostroff, G. Leclercq, L. Allais, C. Pilette, M. Dierendonck, B.G. De Geest, C.A. Cuvelier, Beta-glucan microparticles are good candidates for mucosal antigen delivery in oral vaccination, J. Control. Release 172 (2013) 671–678.
[9] H. Shima, T. Watanabe, S. Fukuda, S. Fukuoka, O. Ohara, H. Ohno, A novel mucosal vaccine targeting Peyer's patch M cells induces protective antigen-specific IgA responses, Int. Immunol. 26 (2014) 619–625.
[10] T. Azegami, Y. Yuki, H. Kiyono, Challenges in mucosal vaccines for the control of infectious diseases, Int. Immunol. 26 (2014) 517–528.
[11] L.B. Lawson, E.B. Norton, J.D. Clements, Defending the mucosa: adjuvant and carrier formulations for mucosal immunity, Curr. Opin. Immunol. 23 (2011) 414–420.
[12] G.H. Giambartolomei, A. Zwerdling, J. Cassataro, L. Bruno, C.A. Fossati, M.T. Philipp, Lipoproteins, not lipopolysaccharide, are the key mediators of the proinflammatory response elicited by heat-killed *Brucella abortus*, J. Immunol. 173 (2004) 4635–4642.
[13] K.A. Pasquevich, S.M. Estein, C. Garcia Samartino, A. Zwerdling, L.M. Coria, P. Barrionuevo, C.A. Fossati, G.H. Giambartolomei, J. Cassataro, Immunization with recombinant *Brucella* species outer membrane protein Omp16 or Omp19 in adjuvant induces specific $CD4^+$ and $CD8^+$ T cells as well as systemic and oral protection against *Brucella abortus* infection, Infect. Immun. 77 (2009) 436–445.
[14] K.A. Pasquevich, A.E. Ibanez, L.M. Coria, C. Garcia Samartino, S.M. Estein, A. Zwerdling, P. Barrionuevo, F.S. Oliveira, C. Seither, H. Warzecha, S.C. Oliveira, G.H. Giambartolomei, J. Cassataro, An oral vaccine based on U-Omp19 induces protection against *B. abortus* mucosal challenge by inducing an adaptive IL-17 immune response in mice, PLoS One 6 (2011) e16203.
[15] S. Letoffe, P. Delepelaire, C. Wandersman, Characterization of a protein inhibitor of extracellular proteases produced by *Erwinia chrysanthemi*, Mol. Microbiol. 3 (1989) 79–86.
[16] U. Baumann, M. Bauer, S. Letoffe, P. Delepelaire, C. Wandersman, Crystal structure of a complex between *Serratia marcescens* metallo-protease and an inhibitor from *Erwinia chrysanthemi*, J. Mol. Biol. 248 (1995) 653–661.
[17] K.E. Wee, C.R. Yonan, F.N. Chang, A new broad-spectrum protease inhibitor from the entomopathogenic bacterium *Photorhabdus luminescens*, Microbiology 146 (Pt 12) (2000) 3141–3147.
[18] B.S. Sheridan, L. Lefrancois, Isolation of mouse lymphocytes from small intestine tissues, Curr. Protoc. Immunol. (2012) (Chapter 3, Unit3 19).
[19] D. Grdic, R. Smith, A. Donachie, M. Kjerrulf, E. Hornquist, A. Mowat, N. Lycke, The mucosal adjuvant effects of cholera toxin and immune-stimulating complexes differ in their requirement for IL-12, indicating different pathways of action, Eur. J. Immunol. 29 (1999) 1774–1784.
[20] A.M. Mowat, A.M. Donachie, S. Jagewall, K. Schon, B. Lowenadler, K. Dalsgaard, P. Kaastrup, N. Lycke, CTA1-DD-immune stimulating complexes: a novel, rationally designed combined mucosal vaccine adjuvant effective with nanogram doses of antigen, J. Immunol. 167 (2001) 3398–3405.
[21] M.V. Delpino, M.I. Marchesini, S.M. Estein, D.J. Comerci, J. Cassataro, C.A. Fossati, P.C. Baldi, A bile salt hydrolase of *Brucella abortus* contributes to the establishment of a successful infection through the oral route in mice, Infect. Immun. 75 (2007) 299–305.
[22] D. Jankovic, M.C. Kullberg, C.G. Feng, R.S. Goldszmid, C.M. Collazo, M. Wilson, T.A. Wynn, M. Kamanaka, R.A. Flavell, A. Sher, Conventional T-bet(+)Foxp3(−) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection, J. Exp. Med. 204 (2007) 273–283.
[23] L.Y.M. Del, I. Farran, M.L. Becher, V. Sander, V.R. Sanchez, V. Martin, J. Veramendi, M. Clemente, A chloroplast-derived *Toxoplasma gondii* GRA4 antigen used as an oral vaccine protects against toxoplasmosis in mice, Plant Biotechnol. J. 10 (2012) 1136–1144.
[24] K. Arnold, L. Bordoli, J. Kopp, T. Schwede, The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling, Bioinformatics 22 (2006) 195–201.
[25] R.A. Copeland, Evaluation of Enzyme Inhibitors in Drug Discovery : A Guide for Medicinal Chemists and Pharmacologists, Wiley-Interscience, Hoboken, N.J., 2005.
[26] R.I. Mahato, A.S. Narang, L. Thoma, D.D. Miller, Emerging trends in oral delivery of peptide and protein drugs, Crit. Rev. Ther. Drug Carrier Syst. 20 (2003) 153–214.
[27] B. Pulendran, H. Tang, T.L. Denning, Division of labor, plasticity, and crosstalk between dendritic cell subsets, Curr. Opin. Immunol. 20 (2008) 61–67.
[28] L. Lefrancois, L. Puddington, Intestinal and pulmonary mucosal T cells: local heroes fight to maintain the status quo, Annu. Rev. Immunol. 24 (2006) 681–704.
[29] G.S. Yap, A. Sher, Cell-mediated immunity to *Toxoplasma gondii*: initiation, regulation and effector function, Immunobiology 201 (1999) 240–247.
[30] T. Kantyka, N.D. Rawlings, J. Potempa, Prokaryote-derived protein inhibitors of peptidases: a sketchy occurrence and mostly unknown function, Biochimie 92 (2010) 1644–1656.
[31] M. Werle, B. Loretz, D. Entstrasser, F. Foger, Design and evaluation of a chitosan–aprotinin conjugate for the peroral delivery of therapeutic peptides and proteins susceptible to enzymatic degradation, J. Drug Target. 15 (2007) 327–333.
[32] T.W. Wong, Design of oral insulin delivery systems, J. Drug Target. 18 (2010) 79–92.
[33] H. Mirchamsy, M. Hamedi, G. Fateh, A. Sassani, Oral immunization against diphtheria and tetanus infections by fluid diphtheria and tetanus toxoids, Vaccine 12 (1994) 1167–1172.
[34] J.M. Sallenave, Secretory leukocyte protease inhibitor and elafin/trappin-2: versatile mucosal antimicrobials and regulators of immunity, Am. J. Respir. Cell Mol. Biol. 42 (2010) 635–643.
[35] P.I. Bird, J.A. Trapani, J.A. Villadangos, Endolysosomal proteases and their inhibitors in immunity, Nat. Rev. Immunol. 9 (2009) 871–882.

[36] D. Guerrieri, N.L. Tateosian, P.C. Maffia, R.M. Reiteri, N.O. Amiano, M.J. Costa, X. Villalonga, M.L. Sanchez, S.M. Estein, V.E. Garcia, J.M. Sallenave, H.E. Chuluyan, Serine leucocyte proteinase inhibitor-treated monocyte inhibits human CD4(+) lymphocyte proliferation, Immunology 133 (2011) 434–441.
[37] A. Lucas, L. Liu, E. Dai, I. Bot, K. Viswanathan, G. Munuswamy-Ramunujam, J.A. Davids, M.Y. Bartee, J. Richardson, A. Christov, H. Wang, C. Macaulay, M. Poznansky, R. Zhong, L. Miller, E. Biessen, M. Richardson, C. Sullivan, R. Moyer, M. Hatton, D.A. Lomas, G. McFadden, The serpin saga; development of a new class of virus derived anti-inflammatory protein immunotherapeutics, Adv. Exp. Med. Biol. 666 (2009) 132–156.
[38] V.A. Guazzone, D. Guerrieri, P. Jacobo, R.J. Glisoni, D. Chiappetta, L. Lustig, H.E. Chuluyan, Micro-encapsulated secretory leukocyte protease inhibitor decreases cell-mediated immune response in autoimmune orchitis, Life Sci. 89 (2011) 100–106.
[39] S.G. Reed, S. Bertholet, R.N. Coler, M. Friede, New horizons in adjuvants for vaccine development, Trends Immunol. 30 (2009) 23–32.
[40] R.W. Titball, Vaccines against intracellular bacterial pathogens, Drug Discov. Today 13 (2008) 596–600.