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Efficient immune responses against Intimin and EspB of enterohaemorragic *Escherichia coli* after intranasal vaccination using the TLR2/6 agonist MALP-2 as adjuvant

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ABSTRACT

Mucosal vaccine formulations based on purified recombinant C_{280} γ-Intimin and EspB (*Escherichia coli* secreted protein B) from enterohaemorragic *E. coli* co-administered with a pegylated derivative of the TLR2/6 agonist MALP-2 (macrophage-activating lipopeptide) as adjuvant were evaluated in BALB/c mice. After intranasal vaccination, strong humoral and cellular immune responses were observed against C_{280} γ-Intimin and EspB. Sera of immunized mice inhibit bacterial haemolytic activity *in vitro*. Antigen-specific T-cell proliferation, IL-4, IL-2 and IFN-γ producing cells, and secretory IgA were mostly detected in animals receiving MALP-2 as adjuvant. These results suggest that C280 γ-Intimin and EspB are good candidate antigens to be incorporated into mucosal vaccines against this important pathogen.

1. Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) is the etiologic agent of diseases in both humans and animals, whose clinical spectrum includes diarrhoea, hemorrhagic colitis and haemolytic uremic syndrome (HUS), the leading cause of renal failure in children in Argentina and several other countries [1,2].

Many virulence factors of EHEC induce, fortunately, an immune response during the course of natural or experimental infections. Oral inoculation of calves and steers with *E. coli* O157:H7 promotes an increase in serum antibody titres against O157 lipopolysaccharide and neutralizing antibodies to verotoxin [3]. On the other hand, mice infected by *Citrobacter rodentium*, a bacteria showing virulence determinants and pathological effects in mice highly similar to those of EPEC in humans, develop an immune response against proteins encoded by a large chromosomal locus called LEE (locus of enterocyte effacement) [4] and they are resistant to bacterial reinfection [5]. LEE codes for a type III secretion system [6]; an outer membrane protein called Intimin, which is required for intimate attachment to host cells [7,8] and the secreted proteins EspA, EspD and EspB, which are required for signal transduction events leading to formation of the attachment and effacing lesions (A/E). While EspB recruits alpha-catenin at the EHEC adherence site by direct interaction, EspA forms a large filamentous organelle that is transiently present on the bacterial surface, which interacts with the host cell during the early stage of A/E lesion formation. EspA filaments may contribute to bacterial adhesion but, more importantly, they appear to be a component of a translocation apparatus and are essential for the translocation to host cells of EspB [9,10] and also of Tir, the receptor for Intimin [11]. Recently, Bretschneider et al. demonstrated that cattle respond serologically to Intimin and EspB of *E. coli* O157:H7 during the course of experimental infection [12].

Several strategies have been proposed to prevent EHEC infection in humans and animals. Potter et al. showed that subcutaneous immunization with type III secreted proteins decreased shedding of EHEC O157 by cattle [13]. EspB was demonstrated to be a potent antigen, whereas EspA induced only a modest responses [13]. Intimin was not tested as antigen in that work. It was also shown that antibodies against C-280 γ-Intimin, the C-terminal fragment of the protein that have main epitopes, prevent bacterial colonization by *C. rodentium* in mouse intestine [5]. In addition, when mice primed with Intimin and orally boosted with recom-

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Table 1
Oligonucleotides and PCR protocols

Protein	Accession number	Primers	Oligonucletide sequence (5′–3′)	Position in gene of the amplified fragment (nt)	Amplification protocol
EspB	U65681	espBup2	GGATCCATGAATACTATTGATTATAC	1	94 °C, 2 min (1 cycle); 94 °C, 1 min
		espBrev	AAGCTTTTAACCAGCTAAGCGAACCG	–945	50 °C, 1 min; 72 °C, 1 min (30 cycles)
C_{280} γ-Intimin	Z11541	ctereaeup	GGATCCCAAACCAAGGCCAGCATTAC	1962	94 °C, 1 min; 53 °C, 1 min; 72 °C, 1 min (30 cycles)
		Grev	AAGCTTTTATTCTACACAAACCGCATAGA	–2804	

binant plants expressing C-280 γ-Intimin were challenged with EHEC, it was observed a reduction in bacterial shedding in faeces [14]. Other authors demonstrated that piglets are protected from EHEC O157:H7 colonization and intestinal damage by ingesting colostrum from Intimin-vaccinated females [15]. Agin et al. developed an isogenic rabbit enteropathogenic *E. coli* attenuated strain by truncating Intimin, which conferred protection against challenge with the wild type strain [16]. Furthermore, Stx2 holotoxin and Stx2B subunits have been also proposed as immunogens [17,18]. Animals immunized with Stx2 toxoid preparations are protected against Stx2 holotoxin challenge [19–21]. In contrast, other authors obtained humoral immune responses in 6-week-old calves vaccinated with recombinant Intimin, Efa-1 or EspA, but they could not demonstrate protection against O157:H7 challenge [22,23].

In this context, inhibition of intestinal colonization may be a good strategy for the development of human and bovine EHEC vaccines. However, the induction of mucosal immunity against LEE-encoded colonization factors needs mucosal adjuvants, because recombinant proteins are extremely poorly immunogenic when they are applied by oral or intranasal routes. Based on mentioned reports from other authors about the strong antigenicity of Intimin and EspB [3,5,12,15] and on the observation that antibodies from bovine colostrum recognized Intimin and EspB more than EspA [D. Vilte, in press in *Clinical and Vaccine Immunology*] we selected Intimin and EspB as antigens to generate mucosal immunity that could prevent the initial steps of EHEC colonization in the gastrointestinal tract. In the present work we determined the usefulness of a synthetic derivative of the macrophage-activating lipopeptide of 2 kDa from *Mycoplasma fermentans* (MALP-2) as adjuvant to generate mucosal immune responses directed to C-280 γ-Intimin and EspB of EHEC, as it was shown that MALP-2 is able to improve strong humoral and cell-mediated immunity against antigens co-administered by intranasal (i.n.) route [24–27].

2. Materials and methods

2.1. Strains and culture media

The *E. coli* strains DH5α and XL1-blue, which were used for cloning experiments, were grown on liquid or solid Luria–Bertani medium containing 125 μg/ml ampicillin. *E. coli* BL21(D3)/*p*-LysS was used for recombinant expression experiments, in these cases the culture medium was supplemented with 34 μg/ml chloramphenicol. The EHEC strains 146N4 (O157:H7) [28] and 97/23A (O26:H11) [29] were isolated from bovine faeces at slaughter and calf with bloody diarrhoea, respectively.

2.2. Cloning and expression procedures

The genes coding for EspB and C-280 γ-Intimin were obtained by PCR amplification from the *E. coli* strains 146N4 (O157:H7; γ-Intimin) and 97/23A (O26:H11; EspB) and cloned into the pGEM-T t/a cloning vector. The primers used for amplification contained 5' extensions with appropriate restriction sites and are shown in Table 1. The resulting inserts were then sub-cloned into the His_6-tagging pRSET-A expression vector and the ligations were used to transform the *E. coli* strain DH5α. After selection, the recombinant vectors containing the inserts of interest were then introduced into the production strain *E. coli* BL21(D3)/pLysS. Expression of the His_6-tagged proteins was achieved by induction with 0.5 mM IPTG of transformed *E. coli* BL21(D3)/pLysS cells grown in Luria Broth (OD_{600} = 0.6) supplemented with ampicillin (100 μg/ml). After 4 h of incubation at 37 °C, induced cells were washed, lysed with 6 M guanidine-HCl and sonicated. The His_6-tagged proteins were purified from the clarified lysates by affinity chromatography using a column of Probond™ Ni-chelating resin (Invitrogen, Carlsbad, CA). Proteins were eluted by washing with decreasing pH buffers containing 8 M urea, 20 mM sodium phosphate and 500 mM NaCl (binding buffer: pH 7.8, wash buffer: pH 6.3 and elution buffer: pH 4.5), under denaturing conditions. SDS-PAGE was carried out in a 12% gel, loading 1.5 μg of protein per lane. Proteins electrophoretically transferred to nitrocellulose sheets were detected by immunoblotting using either antigen-specific sera generated in mice or an anti-His monoclonal antibody diluted in PBS. Specifically bound antibodies in all filters were detected with anti-mouse IgG HRP-conjugate. The blots were revealed with 4-Cl-1-naphtol (data not shown). The LPS content of the protein preparations was determined using the HEK-Blue LPS Detection Kit (InvivoGen, San Diego, USA). The LPS content was below 40 pg/μg of recombinant protein.

2.3. Immunization schedule

Groups (n = 5) of 6–8-week-old female BALB/c (H-2d) mice were purchased from Harlan Winkelmann GmbH (Borchen, Germany) and treated in accordance with local and European Community guidelines. Animals were immunized by i.n. route on day 1, 7 and 21 with antigen (20 μg) alone or co-administered with 5 μg of a pegylated derivative of the TLR2/6 agonist MALP-2 (10 μl/nostril) [26]. Pegylated MALP-2 was used, since the pegylation increases the solubility and stability of the molecule.

2.4. Evaluation of antigen-specific IgG responses

Antibody titres were determined by ELISA as previously described [30]. Briefly, 96-well Nunc-Immuno MaxiSorp assay plates (Nunc, Roskilde, Denmark) were coated with antigen at 1 μg/ml in 0.05 M carbonate buffer (pH 8.2) per well. Serial twofold dilutions of sera in PBS with 1% BSA and 0.05% Tween 20 were added (100 μl/well), and plates were incubated for 2 h at 37 °C. After washing, biotinylated chain-specific goat anti-mouse IgG (Sigma Chemie, Deisenhofen, Germany) was added as secondary antibody. Plates were incubated for an additional 1 h at 37 °C. After four washes, 100 μl of peroxidase-conjugated streptavidin (Sigma Chemie) was added to the wells and plates were incubated at 37 °C for 30 min. After four washes, reactions were developed with ABTS in 0.1 M citrate-phosphate buffer (pH 4.35) containing 0.01% H_2O_2. Endpoint titres were expressed as the reciprocal log2 of the last dilution, which gave an optical density at 405 nm of 0.1 units above the values of the negative controls after 30 min of incubation.

2.5. Evaluation of antigen-specific secretory IgA

The amount of total and antigen-specific IgA present in broncho-alveolar and intestinal lavages was determined by ELISA as previously described [30]. To establish the IgA standard curve, plates coated with goat anti-mouse IgA (Sigma Chemie) as capture antibody were further incubated with serial dilutions of purified mouse IgA (Dianova, Hamburg, Germany). As secondary antibody, biotinylated goat anti-mouse IgA (Sigma Chemie) was used, plates were developed as described above. To compensate for variations in the efficiency of recovery of secretory antibodies between animals, the results were normalized and expressed as end point titre of antigen-specific IgA with respect to 1 μg of total IgA present in the sample.

2.6. Red blood cell (RBC) lysis assay

The inhibitory capacity of sera from immunized mice on the haemolytic activity exhibited by TTSS-encoding *E. coli* strains was evaluated as previously described [31]. In brief, the EPEC E2348/69 strain was grown in LB broth overnight at 37 °C without shaking and then diluted 1:100 into Dulbecco's modified Eagle medium (DMEM) (lacking phenol red; Gibco-BRL). A pool of sera from one group and 2 ml of the diluted bacterial suspension were incubated for 1 h at 37 °C. In turn, red blood cells were separated by centrifugation from fresh defibrinated sheep blood, washed three times with 10 mM PBS pH 7.4 and resuspended at 5% in PBS. Then, 2 ml of the EPEC E2348/69 suspension preincubated with sera was mixed with 2 ml of 5% suspension of RBC in PBS and further incubated for 3 h at 37 °C under a 5% CO_2 atmosphere in 12-well plates. The suspension was removed from the plates and centrifuged at 12,000 × *g* for 1 min. Supernatants were monitored for the presence of released haemoglobin by measuring the optical density at 543 nm. The *E. coli* EPEC E2348/69 *escN* strain, a mutant that does not synthesize the TTSS and it does not produce lysis, was used as a negative control. The positive control consisted of the same incubation but without the addition of sera.

2.7. Proliferation of spleen cells after in vitro stimulation

Spleen cell suspensions were adjusted to 5×10^6 cells/ml in RPMI medium and seeded (100 μl/well) in a flat-bottom 96-well microtiter plate (Nunc), which were then incubated for 4 days in the presence of different concentrations of antigens in triplicates. During the final 18 h of culture, 1 μCi [^{3}H]thymidine (Amersham International, Freiburg, Germany) was added to each well. Cells were harvested on paper filters (Filtermat A; Wallac, Freiburg, Germany) by using a cell harvester (Inotech, Wohlen, Switzerland), and the amount of [^{3}H]thymidine incorporated into the DNA of proliferating cells was determined by scintillation counter (Wallac 1450, Micro-Trilux). The results are expressed as the ratio between values (average of triplicates) from stimulated and non-stimulated samples (stimulation index).

2.8. Measurement of IFN-γ, IL-2 and IL-4 production by ELISPOT

To determine the amount of IFNγ, IL-2 and IL-4 secreting cells, the murine IFNγ, IL-2 and IL-4 ELISPOT kits (BD Pharmingen) were used according to the manufacturer's instructions. In brief, flat bottomed 96-well plates with a 0.45 μm hydrophobic high protein binding immobilin-*p*-membran were coated with the corresponding capture antibody (anti-IFNγ, anti-IL-2 or anti-IL-4) and stored over night at 4 °C. To remove unbound capture antibodies plates were washed once and unspecific binding sites were saturated by incubating with blocking solution for 2 h at room temperature (RT). Afterwards, splenocytes (1×10^6/well) were incubated at 37 °C in an atmosphere containing 5% CO_2 for 24 h (IFN-γ) or 48 h (IL-2 and IL-4) in the absence or presence of either C280 γ-Intimin or EspB protein (20 μg/ml). As negative control, cells were cultured in RPMI complete without stimulants. For the stimulation of 0.5×10^6 cells/well, ConA (positive control) was diluted in RPMI (final concentration 5 μg/ml). After the incubation time, plates were washed twice with deionised water including a soaking for 5 min followed by three washes using wash buffer (0.05% Tween 20/PBS). To detect the captured cytokines, the corresponding biotinylated detection antibody was added and incubated for 2 h at RT. After additional three washing steps, horseradish peroxidase (HRP) was added and plates were incubated for 1 h at RT. After final four washing steps, spots were developed for 5–60 min using substrate solution (333.3 μl of AEC stock solution +10 ml 0.1 mM acetate solution +5 μl H_2O_2). The reaction was stopped by washing the plates with deionised water. After drying the plates for 2 h at RT in the dark, the results are expressed as spot forming units (SPUs) for 1×10^6 spleen cells. The spots were counted using the automated ELISPOT ImmunoSpot S4 Analyzer (CTL-Europe GmbH, Aalen, Germany) and analyzed using the C.T.L. ImmunoSpot image analyzer software v3.2. The spots produced by non-re-stimulated cells served as background and were subtracted from the spots produced by the re-stimulated cells. Only ratios stimulated versus non-stimulated spots above >2 were further analyzed.

2.9. Statistical analysis

Statistical analyses were performed with the Student's *t*-test and the Mann–Whitney rank sum test. Differences were considered at statistic significant at $P < 0.05$.

3. Results

3.1. Efficient elicitation of antigen-specific antibody responses in serum and mucosal secretions after intranasal immunization with C280 γ-Intimin and EspB containing vaccine formulations

All vaccine formulations were well tolerated by the animals, which did not show significant alterations in the weight, food intake or general behaviour (data not shown). To determine the level of humoral immune responses to EspB and C_{280} γ-Intimin proteins, antigen-specific serum IgG was measured by ELISA. Intranasal immunization of mice with C_{280} γ-Intimin co-administered with MALP-2 induced a rapid raise in specific IgG antibodies in sera, even after the first boost (Fig. 1). In contrast, when mice were immunized with protein alone, antibody titres were increased only after a second boost and the titres were 2.5-fold lower ($P \leq 0.001$). Similar results were observed in sera of mice vaccinated with EspB. Again, co-administration of MALP-2 stimulated increased EspB-specific antibody production, even after the first boost ($P \leq 0.001$). Nevertheless, EspB-specific antibody titres were considerably lower than those obtained after immunization with C_{280} γ-Intimin (Fig. 1). In order to get an idea about the biological activity of the stimulated antibody responses, we evaluated the ability of sera from vaccinated animals to neutralize *in vitro* the type III secretion system-dependent RBC lysis. The obtained results showed that pooled sera from animals immunized with EspB- and C_{280} γ-Intimin inhibited by 60% and 65% the lysis of erythrocytes, respectively.

We further evaluated the elicitation of mucosal responses in vaccinated animals. To this end, the sIgA response was determined in broncho-alveolar and intestinal lavages. The strongest sIgA production was observed in broncho-alveolar lavages of vaccinated animals. However, high levels of sIgA were only stimulated

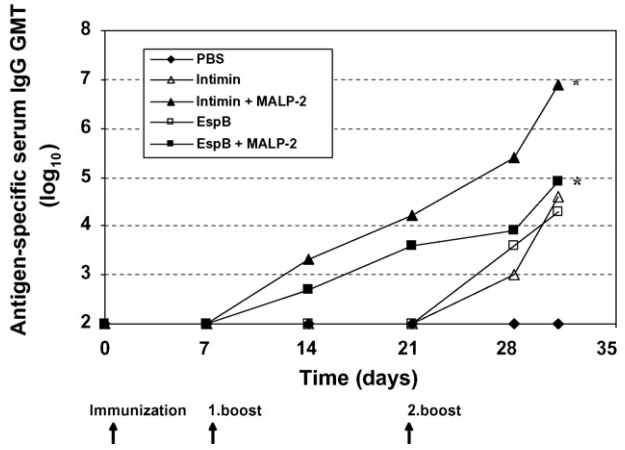

Fig. 1. Humoral immune responses in mice vaccinated with the C280 γ-Intimin and EspB protein of EHEC. Kinetics of the antigen-specific IgG titres in sera of vaccinated mice. The standard error of the mean (S.E.M.) was in all cases lower than 10% of the values. The obtained results are statistically significant when compared with the values in animals vaccinated with antigen alone at $^{*}P \leq 0.001$.

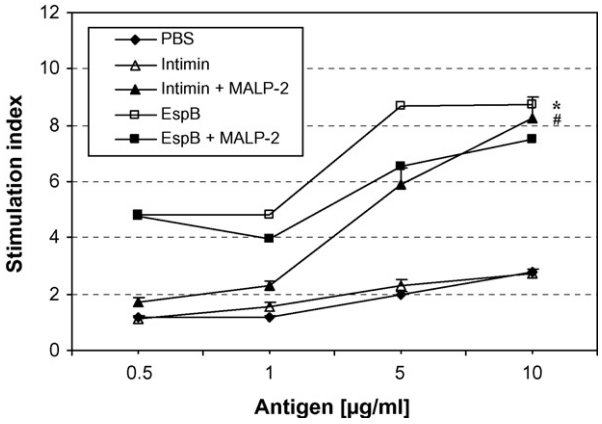

Fig. 3. Cellular immune responses stimulated in vaccinated animals. Results are expressed as the ratio between values (average of triplicates) from stimulated and non-stimulated samples (stimulation index). S.E.M. is indicated by vertical lines. The obtained results are statistically significant when compared with the values in the animal groups receiving the vaccine formulations without adjuvants at either $^{\#}P \leq 0.01$ (C280 γ-Intimin) or $^{*}P \leq 0.05$ (EspB).

after intranasal immunization with C280 γ-Intimin and EspB co-administered with MALP-2 (Fig. 2). The obtained values were significantly increased respect to those of mice receiving the antigen alone ($P \leq 0.008$ and $P = 0.03$, respectively). In contrast, the antigen-specific sIgA titres obtained in the intestine of the vaccinated animals were significantly lower than those measured in the mucosal surface of the lung. Only immunization with C280 γ-Intimin co-administered with MALP-2 stimulated strong sIgA production in the intestine (Fig. 2). In contrast, animals receiving C_{280} γ-Intimin alone or any of the EspB-based formulations showed considerable lower amounts of antigen-specific sIgA in the intestines, that was not statistically different between groups immunized with or without co-administration of the adjuvant. Interestingly, when evaluating the levels of antigen-specific IgG transudated in mucosal secretions Intimin-specific IgG has been detected only in mice receiving C_{280} γ-Intimin co-administered with MALP-2, whereas no differences could be observed when immunizing with EspB with or without co-administration of MALP-2. Furthermore, only immunization with C280 γ-Intimin co-administered with MALP-2 stimulated a strong IgG production in the intestine. No antigen-specific IgG has been detected in intestinal mucosa of mice receiving C_{280} γ-Intimin alone or any of the EspB-based formulations (data not shown). However, the amounts of antigen-specific IgG in the lavages were up to 2000 times lower compared to those of sIgA.

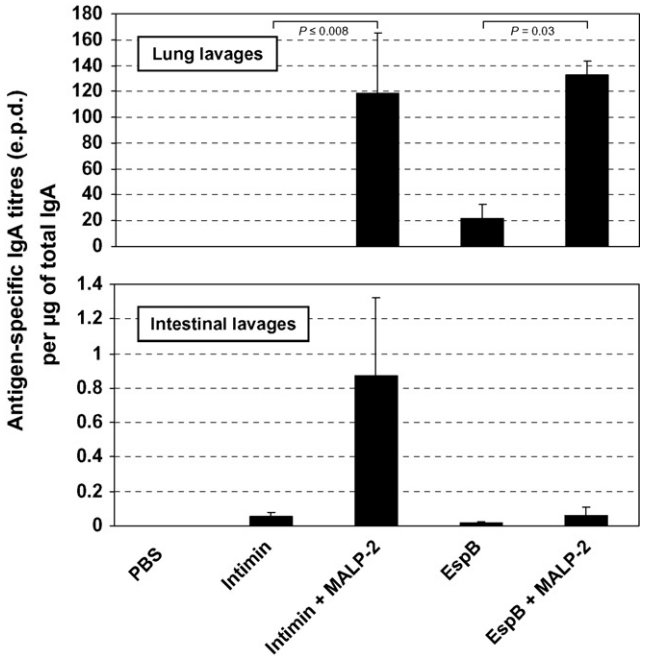

Fig. 2. Mucosal immune responses in mice vaccinated with the C280 γ-Intimin and EspB protein of EHEC. Antigen-specific IgA antibodies were measured in broncho-alveolar and intestinal lavages of immunized mice. Results are expressed as the end point dilution of antigen-specific IgA respect to 1 μg of total IgA. S.E.M. is indicated by vertical lines. The obtained results are statistically significant when compared with the values of those vaccination groups receiving antigen alone at $P \leq 0.008$ or $P \leq 0.03$, respectively.

In conclusion, co-administration of the mucosal adjuvant MALP-2 not only efficiently increases antigen-specific serum antibody production but also enhances the mucosal responses at the local level.

3.2. *Efficient elicitation of antigen-specific cellular immune responses after intranasal immunization with the C280 γ-Intimin and EspB containing vaccine formulations*

The stimulation of an efficient and long lasting immune response requires the induction of a T-helper immune response. Thus, we have evaluated cellular immune responses following intranasal immunization with the C280 γ-Intimin and EspB protein containing vaccine formulations. To this end, we measured the proliferative responses of splenocytes after *in vitro* restimulation with the corresponding antigen. The strongest proliferative responses were observed with splenocytes of mice immunized with EspB protein. Interestingly, co-administration of MALP-2 seems to significantly reduce the proliferative potential of EspB-specific lymphocytes ($P < 0.05$). In contrast, significantly enhanced C280 γ-Intimin-specific cellular responses were only obtained when the antigen was co-administered with MALP-2 ($P < 0.01$; Fig. 3).

To analyze the stimulated immune responses in more detail, we have also evaluated cytokine production by spleen-derived lymphocytes after restimulation with the corresponding antigen. When the number of IFN-γ producing cells was determined by

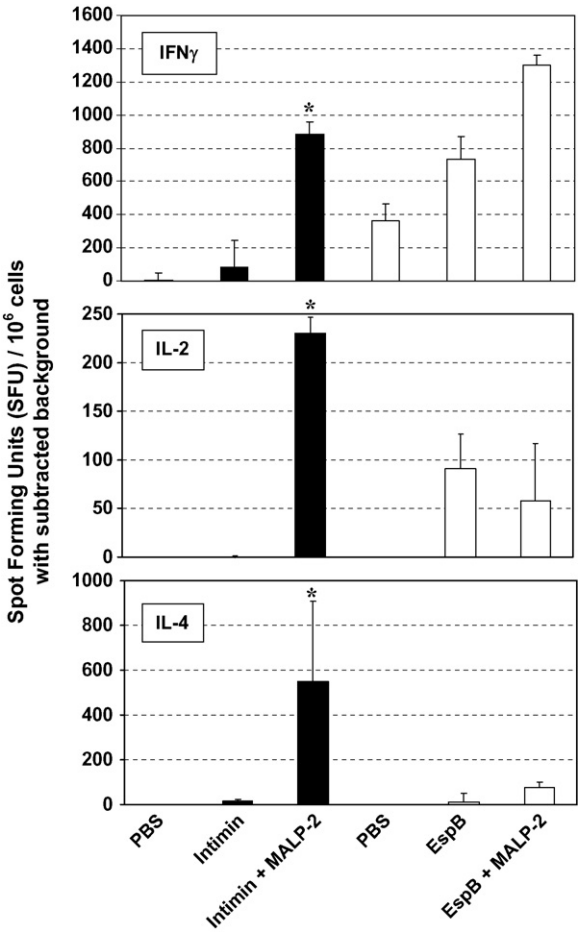

Fig. 4. Characterization of cytokine producing cells stimulated by the Intimin and EspB proteins. Spleen-derived lymphocytes were cultured for 16 h with different concentrations of the protein. INF-γ, IL-2 and IL-4 production was determined by ELISPOT. The S.E.M. is indicated by vertical lines. Results are expressed as SFU per 10^6 cells above background. The observed differences were statistically significant in comparison with the vaccination group receiving C280 γ-Intimin alone (*) at $P \leq 0.001$ (INF-γ), $P \leq 0.001$ (IL-2) and $P \leq 0.006$ (IL-4).

ELISPOT, it was observed that the adjuvant has a stimulatory effect for both antigens. Thus, the level of this cellular subpopulation was statistically significant increased when C280 γ-Intimin was co-administered with MALP-2 ($P < 0.001$). Although the incorporation of the adjuvant in the vaccine formulation with EspB resulted in an additional increment of IFN-γ producing cells, no statistically significant differences could be observed respect to cells from mice receiving EspB alone (Fig. 4). In addition, immunization with EspB resulted in the stimulation of similar levels of IL-2-producing cells, independently of the presence of the adjuvant in the formulation. In contrast, C280 γ-Intimin only stimulated high levels of IL-2 producing cells when co-administered with MALP-2 ($P < 0.001$; Fig. 4). The same was true for IL-4-producing cells, which were increased only in mice receiving C280 γ-Intimin with MALP-2 ($P < 0.006$), whereas no significant differences were observed in animals receiving the EspB formulations (Fig. 4). In conclusion, while co-administration of MALP-2 in both vaccine formulations was needed to efficiently increase antigen-specific humoral responses, cellular responses were only significantly increased in mice receiving C280 γ-Intimin co-administered with MALP-2.

4. Discussion

The incidence of EHEC infections varies by age group, with the highest incidence occurring in children aged less than 15 years-old (0.7 cases per 100,000 in the United States). Up to 85% of the cases are food-borne and up to 20% of EHEC infections progress to the severe sequelae, HUS, the leading cause of acute renal failure in children. Furthermore, up to 40% of patients with HUS develop long-term renal dysfunction, and about 3–5% of patients even die during the acute phase of the disease. Until now, there is neither a specific treatment for HUS available nor a vaccine in the market to prevent disease. In addition, antibiotic therapy does not represent a valid alternative, since it might lead to the release of the bacterial intracellular toxin cargo, thereby resulting in even more severe symptoms. This and the occurrence of massive outbreaks make EHEC infection a public health problem of serious concern (Centers for Disease Control and Prevention: http://www.cdc.gov/ecoli/2007/october/103107.html). For this reason, it is essential to develop new strategies to fight infections caused by EHEC, not only in humans but also in the main reservoir, cattle. In the past, many authors have studied the antigenicity of different EHEC proteins (e.g., Intimin, EspA, EspB and Shiga toxins), but mainly following systemic immunization protocols, since strong humoral responses against these proteins were observed in HUS patients and EHEC or EPEC infected children [32–34]. However, a prerequisite for optimizing vaccine design is the establishment of strategies to evoke strong mucosal immune responses, since the stimulation of an efficient local response against EHEC is highly desirable. This may allow blocking the infection and colonization process at a very early stage, thereby reducing the risk of microbial transmission to other susceptible hosts. However, recombinant antigens are generally only poor immunogenic when administered by mucosal route. Therefore, to increase the efficacy of potential vaccine antigens adjuvants have to be included. MALP-2 was demonstrated to be able to significantly enhance both humoral and cellular immune responses against antigens co-administered by mucosal route [26,30,35,36]. MALP-2 promotes the activation and maturation of dendritic cells, thereby improving their capacity to process and present antigens to naïve and antigen-specific T-cells. Interestingly, the composition and activity of the proteasome is also modified, by fostering a switch to immune proteasome [37]. In addition, MALP-2 activates other key immune cells, such as macrophages and B cells. Furthermore, when administered by the i.n. route, MALP-2 promotes a local microenvironment which is conductive to antigen processing [27]. Therefore, we decided to evaluate if MALP-2 can be of use in order to improve the immunogenicity of EHEC antigens.

Our results demonstrated that strong humoral, mucosal and cellular immune responses against C280 γ-Intimin and EspB are stimulated when co-administered with a pegylated derivative of MALP-2 by the i.n. route. The strongest antibody responses were stimulated by C280 γ-Intimin followed by EspB, with high titres even after the first boost. Furthermore, the produced antigen-specific antibodies were able to inhibit the haemolytic activity of the enteropathogenic *E. coli* strain E2348/69. At mucosal level, strong antigen-specific IgA production was observed in the lungs, but only when the proteins were co-administered with the adjuvant. Interestingly, in intestinal lavages only C280 γ-Intimin was able to stimulate increased amounts of sIgA. However, the levels of antigen-specific sIgA in the gut were lower when compared to those in the lungs. Nevertheless, there is no general consensus on whether sIgA is a key effector mechanism during the course of natural infections in human or cattle [38]. In fact, when analyzing the levels of antigen-specific IgG transudated into the mucosa, we also detected low titres of C280 γ-Intimin- and EspB-specific antibodies.

While increased levels of C280 γ-Intimin-specific IgG were stimulated only after co-administration of MALP-2, no differences in the values of EspB-specific IgG could be observed after immunizing with our without adjuvant. In addition, no EspB-specific IgG was detected in the gut of immunized mice.

To stimulate strong and long lasting humoral immune responses, efficient T helper responses are needed. Thus, we analyzed the potential of EspB and C280 γ-Intimin to stimulate cellular immune responses. The strongest proliferative response was stimulated by EspB with or without co-administration of MALP-2, followed by C280 γ-Intimin co-administered with MALP-2. In contrast, immunization with C280 γ-Intimin alone failed to stimulate cellular proliferation. Co-administration of MALP-2 was also more efficient in stimulating IL-2, IL-4 and IFN-γ production, with the exception of IL-2 when EspB was used as antigen.

In conclusion, C280 γ-Intimin seems to be more potent in order to stimulate immunity. Furthermore, in case of EspB the potentiating effect of MALP-2 on the stimulated cellular responses seems to be limited. However, remains to be elucidated if the observed differences in immunogenicity of the two proteins are due to the intrinsic physicochemical properties of the molecules, their immunological properties or their specific interaction with immune cells. Nevertheless, the data presented here demonstrate that efficient immune responses against C280 γ-Intimin and EspB can be obtained at both systemic and mucosal levels after i.n. immunization using the MALP-2 derivative as adjuvant. These promising results suggest that this mucosal vaccine formulation might be of use to protect cattle from colonization by EHEC O157 and warrant further studies.

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