microbial biotechnology

Microbial Biotechnology (2012) **5**(2), 283–294 doi:10.1111/j.1751-7915.2011.00316.x

Rectal single dose immunization of mice with *Escherichia coli* O157:H7 bacterial ghosts induces efficient humoral and cellular immune responses and protects against the lethal heterologous challenge

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Summary

Bacterial ghosts (BGs) have been applied through oral, aerogenic, intraocular or intranasal routes for mucosal immunization using a wide range of experimental animals. All these applications required a booster after primary immunization to achieve protective immunity against the lethal challenge. Here we report for the first time that a single rectal dose of BGs produced from enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 fully protects mice against a 50% lethal challenge with a heterologous EHEC strain given at day 55. BGs from EHEC O157:H7 were prepared by a combination of protein E-mediated cell lysis and expression of staphylococcal nuclease A guaranteeing the complete degradation of pathogen residual DNA. The lack of genetic material in the EHEC BGs vaccine abolished any potential hazard for horizontal gene transfer of plasmid encoded antibiotic resistance genes or pathogenic islands to the recipient's gut flora. Single rectal immunization using EHEC O157:H7 BGs without any addition of adjuvant significantly stimulated efficient humoral and cellular immune responses, and was equally protective as two immunizations, which indicates the possibility to develop a novel efficacious single dose mucosal EHEC O157:H7 BGs vaccine using a simplified immunization regimen.

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Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) belongs to the family of food-born pathogens associated with several life-threatening illnesses for human after contact with faeces of ruminants (Conlan *et al.*, 1999a; Verweyen *et al.*, 2000). The danger related to EHEC and the strength of EHEC strains pathogenicity were recently confirmed during 2011 German EHEC outbreak, which resulted in death of several dozen people and caused major economic losses in several countries of the EU (Hyde, 2011; Neumann *et al.*, 2011). Cattle represent a major reservoir for EHEC because of their capacity to harbour EHEC in the lymphoid follicle-dense mucosa at the terminal rectum (Hancock *et al.*, 1998; Verweyen *et al.*, 2000; Moxley *et al.*, 2009; McNeilly *et al.*, 2010; Vilte *et al.*, 2011). The predominant EHEC serotype affecting human is O157:H7 causing acute gastroenteritis that may be complicated by life-threatening systemic sequelae. EHEC O157:H7 is responsible for outbreaks of haemorrhagic colitis especially in the elderly, and the haemolytic uremic syndrome particularly in children (Su and Brandt, 1995). Moreover, EHEC O157:H7 also belongs to category B bioterrorism diseases/agents according to the classification of the National Institute of Health and National Institute of Allergy and Infectious Diseases. Person-to-person spread of the disease from close contact with primary cases is also considered as a source of infection. The use of antibiotics has not been recommended for treatment of patients suffering from EHEC infections by medical regulatory agencies. As there is no specific vaccine regimen against an EHEC infection at the moment, current treatment is limited largely to supportive care (Tarr *et al.*, 1988).

Prevention or reduction of EHEC O157:H7 levels in cattle by vaccination might help to minimize the clinical incidence of natural EHEC O157:H7 infections (WHO, 1999; Conlan *et al.*, 1999b). Moreover, vaccination of personal at risk could help to eliminate potential bioterrorism threat. The introduction of an EHEC vaccine in a

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combination with other diarrhoeal vaccines would also be of great benefit to prevent the spread of disease in children and travellers. Vaccine strategies related to the inhibition of the intestinal tract colonization by EHEC reflect the most promising way to prevent the infection (Lovett, 1998; Li *et al*., 2000; Funatogawa *et al*., 2002; Naylor *et al*., 2005; Mahajan *et al*., 2009; McNeilly *et al*., 2010; Farfan *et al*., 2011). Efficient vaccines against EHEC should be prepared from agents or particles possessing all important antigenic cell surface factors capable to inhibit the adherence of the pathogen to the mucosa (Conlan *et al*., 1999a; Li *et al*., 2000).

Bacterial ghosts (BGs) represent empty bacterial cell envelopes of Gram-negative bacteria developed as a novel carrier for DNA, antigen (Ag), enzymes, drugs and adjuvant system for the delivery of mucosal vaccines (Tabrizi *et al*., 2004; Eko *et al*., 2008; Lubitz *et al*., 2009; Kudela *et al*., 2010). Bacterial ghosts are produced by controlled expression of the cloned ϕX174 gene *E*, which results in protein E-mediated lysis of Gram-negative bacteria and the formation of empty bacterial cell envelopes with surface and structural components in a natural non-denatured form (Witte *et al*., 1992; Szostak *et al*., 1996; Eko *et al*., 1999). Inner and outer membrane structures including highly sensitive and fragile pili are well protected by this technology and remain intact as demonstrated for *Vibrio cholerae* BGs expressing the toxin co-regulated pilus (Eko *et al*., 2000). Because of the intact morphological, structural and antigenic surface components BGs can act as a natural adjuvant (Mayr *et al*., 2005a; Riedmann *et al*., 2007) and are able to induce a strong systemic and mucosal immune response (Jalava *et al*., 2002; 2003; Ekong *et al*., 2009). Moreover, BGs carrying multi-subunit Ags revealed the capacity to stimulate strong Ag-specific anamnestic systemic and mucosal immune responses after intramuscular administration. Furthermore, repeated challenging of mice (98 days after the primary infection and 86 days after the first challenge with Ag-specific pathogen) resulted in the recall of Ag-specific humoral and cellular immune responses indicating effective long-lasting protective immunity induced by the recombinant BGs platform (Eko *et al*., 2011).

The protein E-mediated lysis system was combined with the intracellular synthesis of a secondary lethal protein, the thermostable nuclease (E.C.3.1.4.7) of *Staphylococcus aureus* (SNUC), to improve the quality of BGs and exclude the presence of any bacterial DNA within the empty BG envelopes. The additional expression of SNUC in *E. coli* yielded into complete degradation of the DNA (Haidinger *et al*., 2003). This DNA degradation provides an additional safety feature for the EHEC BGs preparation as it avoids the presence of pathogenic islands or antibiotic resistance genes in the BGs vaccine.

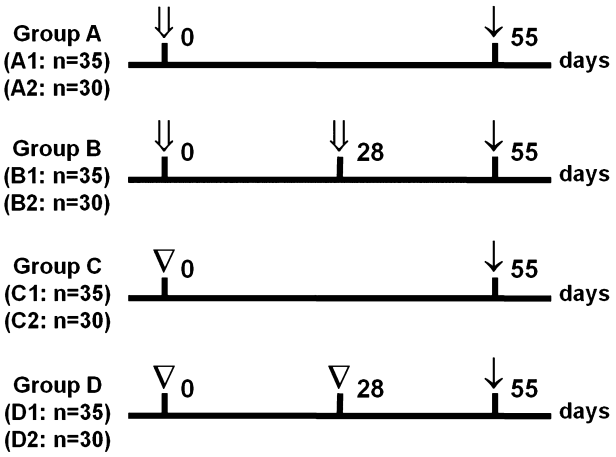

Fig. 1. Scheme for immunization and bacterial pathogen challenge. Mice were immunized *per rectum* with 1 mg of EHEC O157:H7 (N°CIP 105282) BGs once (group A) or twice (group B), and with PBS once (group C) or twice (group D) serving as controls for groups A and B respectively. All mice were challenged with live heterologous EHEC O157:H7 (N°CIP 103571) on day 55. ⇓, immunization with EHEC O157:H7 (N°CIP 105282) BGs; ∇, placebo-immunization with PBS; ↓, challenge with 1×10^8 live heterologous EHEC O157:H7 strain N°CIP 103571.

The aim of this study was to evaluate the efficiency of EHEC BGs as a rectal vaccine in mice using a single and two dose BGs vaccine regimens. Obtained results showed that the single dose rectal administration of EHEC O157:H7 BGs stimulated efficient humoral and cellular immune responses, and exhibited 100% protection of vaccinated mice against a LD50 challenge with a heterologous EHEC strain. These results demonstrate the potential to develop a novel type of BGs vaccines for rectal administration and exhibit a safe and efficient vaccination regimen applicable for adults and especially for children.

Results

Rectal administration of EHEC O157:H7 BGs protects immunized mice from heterologous challenge

EHEC O157:H7 BGs were prepared from strain N°CIP 105282 under standard conditions as described previously (Mayr *et al*., 2005b). No surviving bacteria were found within the washed, freeze-dried BGs after 6 days under enrichment growth conditions in an amount of 10 mg EHEC O157:H7 BGs corresponding to 10 times the immunization dose per mouse. Mice from group A and B were rectally immunized with 1 mg freeze-dried EHEC O157:H7 BGs through a soft polyethylene catheter at day 0 or at day 0 and day 28 (booster) respectively (Fig. 1). These mice did not change their behaviour and did not show any signs of illness during the observation period until the challenge (day 55) with live 1×10^8 heterologous

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Table 1. Faecal shedding of EHEC from immunized and control mice.

	D0	D28	D55[e]	D56	D57	D58	D59	D60	D61	D62	D63	D69	D76
A[a]	–	–	–	+	+	+	–	–	–	–	–	–	–
B[b]	–	–	–	+	+	+	–	–	–	–	–	–	–
C[c]	–	–	–	+	+	+	+	+	+	+	–	–	–
D[d]	–	–	–	+	+	+	+	+	+	+	–	–	–

a. Mice immunized with EHEC ghosts once (day 0).
b. Mice immunized with EHEC ghosts twice (day 0, 28).
c. Mice immunized with PBS once (control for group A).
d. Mice immunized with PBS twice (control for group B).
e. Heterologous challenge with live EHEC O157:H7.
–, No faecal shedding of EHEC.
+, Detection of faecal EHEC shedding.

EHEC O157:H7 (strain N°CIP 103571). No faecal shedding of EHEC was detected in all mice before the heterologous oral challenge. Immunization with EHEC O157:H7 BGs led to EHEC shedding within the time period 1–3 days post the challenge. By contrast, mice immunized with placebo excreted EHEC O157:H7 up to 7 days post heterologous challenge. Thereafter, no EHEC O157:H7 shedding was detected for all surviving mice until day 21 post heterologous challenge when all mice were killed (Table 1). A clear difference in the survival rate (Table 2) was observed for the groups immunized with EHEC O157:H7 BGs compared with the groups that received only placebo. All mice (100%) from the group A2 (single rectal immunization with EHEC O157:H7 BGs at day 0) and the group B2 (two rectal immunizations with EHEC O157:H7 BGs at day 0 and day 28) survived after the heterologous challenge with viable EHEC O157:H7 (strain N°CIP 103571). Sixteen mice (53.3%) survived in both control groups C2 and D2 immunized only with placebo ($P < 0.001$). The behaviour and health conditions of mice were monitored daily after the heterologous challenge until all mice were killed. Disease manifestations appeared in both control groups within the time period 4–7 days after the heterologous challenge and increased in severity from slowing of activity to no stimulus reaction, anorexia and convulsions before death.

Immunization with EHEC O157:H7 BGs stimulates specific humoral immune responses

Titres of specific IgA and IgG antibodies against EHEC O157:H7 in sera and intestinal lavage samples were analysed by ELISA using EHEC O157:H7 BGs as Ag (Fig. 2). Presence of IgG antibodies was found in sera of mice immunized with EHEC O157:H7 BGs (Fig. 2A) but not in intestine samples (data not shown), whereas IgA antibodies were found in both sera (Fig. 2B) and intestine samples (Fig. 2C and D). No specific antibodies were found in both control groups of mice immunized with placebo before heterologous challenge with viable EHEC O157:H7 (strain N°CIP 103571). Mice in group A1 (single rectal immunization with EHEC O157:H7 BGs at day 0) showed the highest level of IgG in sera at day 35 post immunization and stayed at the level determined until day 62. Mice in group B1 (two rectal immunizations with EHEC O157:H7 BGs at day 0 and day 28) exhibited increased IgG titres in sera after the booster immunization (day 49 post the first immunization and day 21 after the second immunization with EHEC O157:H7 BGs), which decreased thereafter. The booster immunization of mice with EHEC O157:H7 BGs (group B1) stimulated significantly higher production of IgG in sera (Day 49 and Day 55) compared with IgG levels detected in sera of mice

Table 2. Survival rate among immunized and control mice after infection with *E. coli* O157:H7.

	Number of surviving mice/Number of dead mice				
Group	3 days after challenge	5 days after challenge	10 days after challenge	21 days after challenge	Survival rate (%)
A2[a]	30/0	30/0	30/0	30*/0	100
B2[b]	30/0	30/0	30/0	30*/0	100
C2[c]	30/0	22/8	16/14	16/14	53.3
D2[d]	30/0	21/9	16/14	16/14	53.3

a. Mice immunized with EHEC ghosts once (day 0).
b. Mice immunized with EHEC ghosts twice (day 0, 28).
c. Mice immunized with PBS once (control for group A2).
d. Mice immunized with PBS twice (control for group B2).
*$P < 0.001$.

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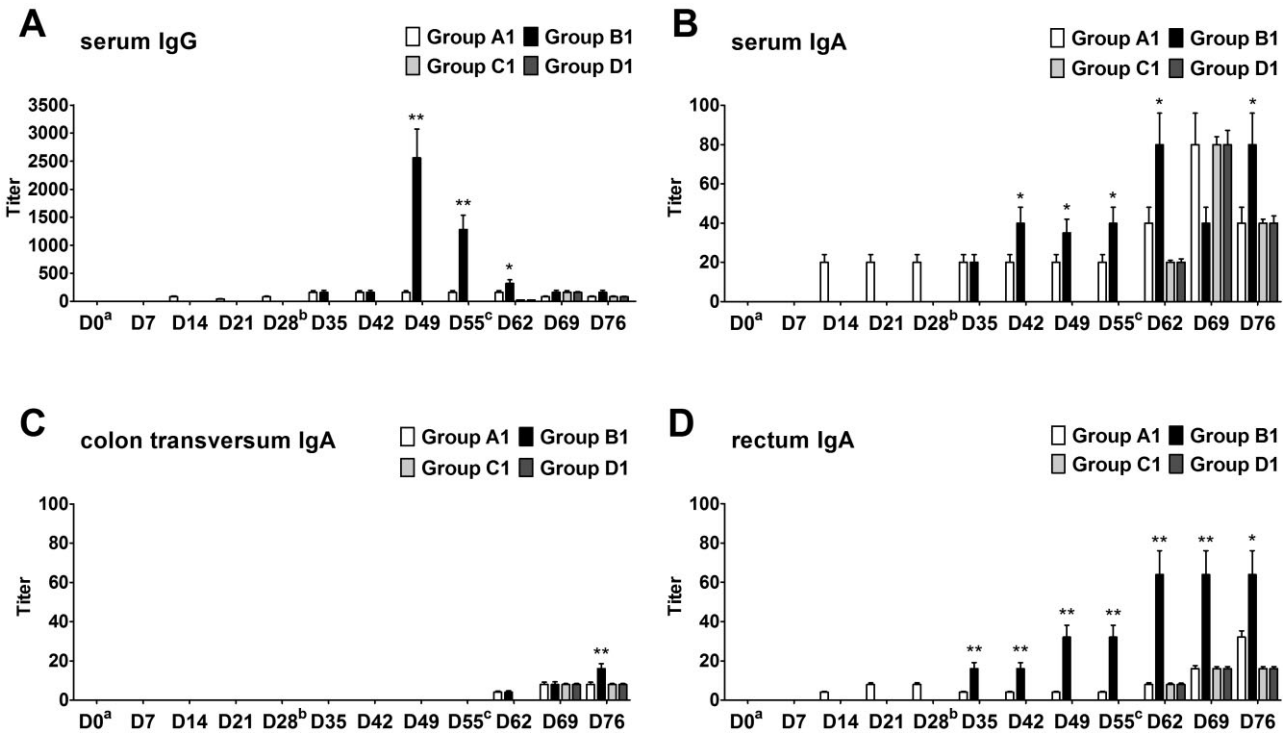

Fig. 2. Antibody titres in mice after the immunization with EHEC O157:H7 (N°CIP 105282) BGs *per rectum* and the heterologous oral challenge with live *E. coli* O157:H7 (N°CIP 103571). Each value represents the mean of antibody titres obtained from three mice: serum IgG antibody titres against EHEC O157:H7 BGs (A), serum IgA antibody titres against EHEC O157:H7 BGs (B), colon transversum IgA antibody titres against EHEC O157:H7 BGs (C), and rectal IgA antibody titres against EHEC O157:H7 BGs (D). Mice were immunized as described in Fig. 1. Data represent the mean of three independent experiments ± SD. *P*-values < 0.05 were considered significant and are indicated with asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). a, day of the first immunization in all groups; b, day of the second immunization in groups B1 and D1; and c, day of the challenge.

which received only the single dose mucosal EHEC O157:H7 BGs vaccine (group A1) (Fig. 2A). The IgA titres in sera of mice from group A1 stayed constant until the oral heterologous challenge with viable EHEC O157:H7, while in group B1 mice titres increased after the second immunization and were more substantially after the challenge with live bacteria (Fig. 2B). No presence of EHEC-specific IgG and IgA was detected in sera of mice from both control groups (C1 and D1) until day 7 after the heterologous challenge (day 62). Mice from the control groups surviving the heterologous challenge showed a specific antibody titre increase in both serum IgG and IgA (Fig. 2A and B).

Three different parts from the collected intestine samples, namely duodenum, colon transversum and rectum were used to determine specific humoral immune responses after immunization with EHEC O157:H7 BGs within the intestinal area. The Ag-specific IgA antibodies inside the duodenum of all tested groups were not detected at any time point of the immunization scheme (data not shown). The colon transversum IgA antibodies were found only at day 7 after the heterologous challenge with live bacteria (day 62) (Fig. 2C). The highest titres of Ag-specific IgA in the rectum of mice from group A1 (single rectal immunization with EHEC O157:H7 BGs at day 0) were found between day 8 and 28, but then the level of antibodies dropped to half until the day of the heterologous challenge with live bacteria (Fig. 2D). Similar results were observed in group B1 until the second immunization with EHEC O157:H7 BGs, but the booster immunization increased the level of Ag-specific IgA in rectum four times. Moreover, the titres of Ag-specific IgA in rectum after the heterologous challenge of mice from group B1 increased twofold (Fig. 2D). The increased IgA titres in rectum after the heterologous challenge with live EHEC O157:H7 were detected in all mice including control groups (C1 and D1) (Fig. 2D).

Rectal vaccinations with EHEC O157:H7 BGs induce Ag-specific IFN-γ secreting cells and stimulate proliferation of Ag-specific cells

To evaluate the efficacy of cellular immune response stimulated by rectal immunization with EHEC O157:H7 BGs, the capacity of spleen cells to produce IFN-γ in response to stimulation with EHEC O157:H7 BGs was determined using ELISPOT assay (Fig. 3). A single rectal immunization with EHEC O157:H7 BGs (group A1)

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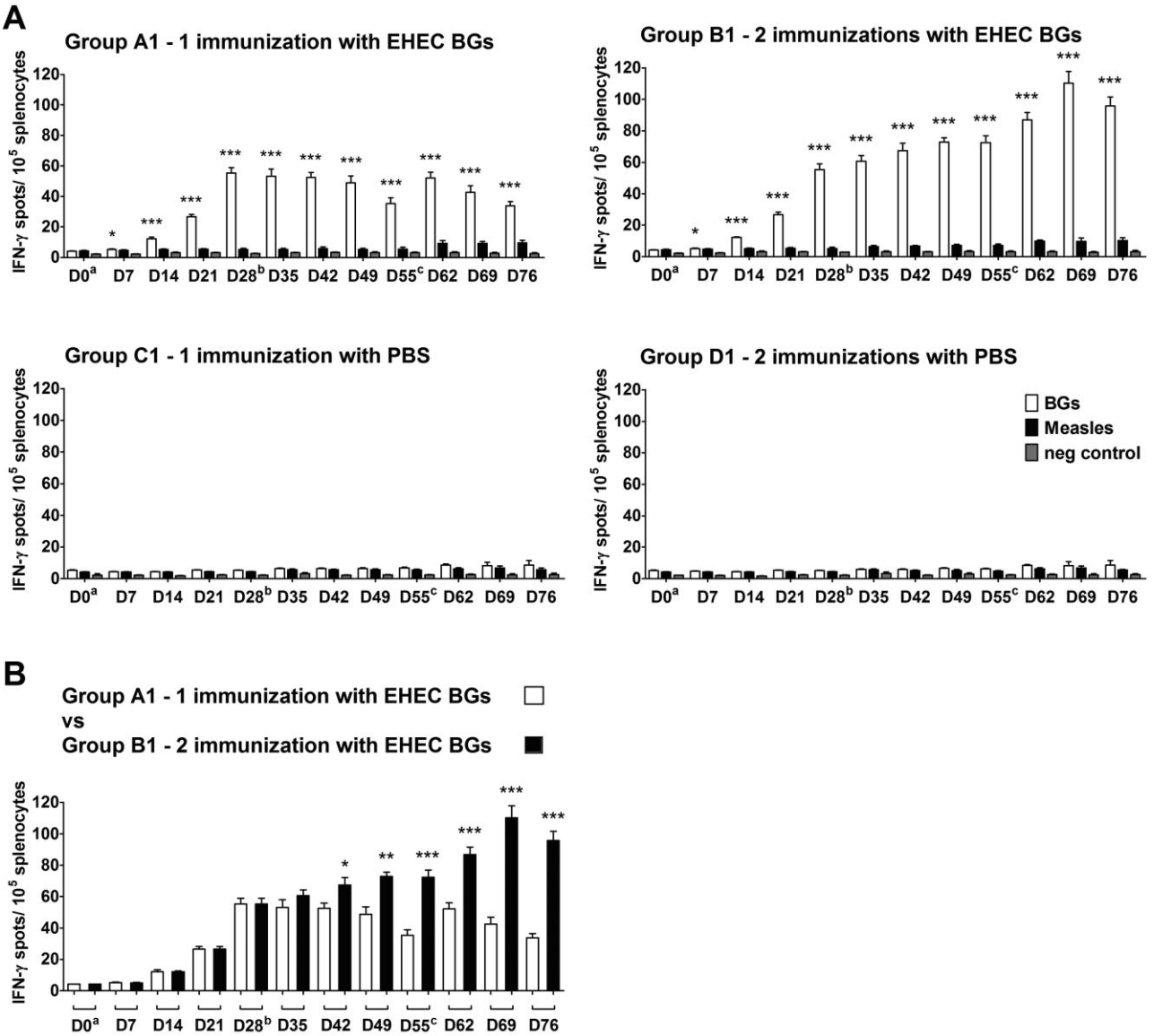

Fig. 3. Cellular immune responses stimulated in mice after the immunizations with EHEC O157:H7 (N°CIP 105282) BGs. Mice were immunized as described in Fig. 1. Spleen cells (1×10^5 per well) were incubated for 16 h in the presence of EHEC O157:H7 BGs (specific Ag); measles inactivated Ag (strain Edmonston, non-specific Ag) or as a negative control spleen cells were left without stimulation. The numbers of IFN-γ-producing cells were determined by ELISPOT assays. Rectal immunization with EHEC O157:H7 BGs effectively increased the number of IFN-γ-producing spleen cells after recognition of specific Ag (A). The stimulatory effect of the boost immunization was detected on day 42 (14 days after the second immunization) and remained significant until the end of experiment (B). Data represent the mean of three independent experiments ± SD (three mice per time point). *P*-values < 0.05 were considered significant and are indicated with asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). a, day of the first immunization in all groups; b, day of the second immunization in groups B1 and D1; c, day of the challenge.

significantly stimulated production of IFN-γ by spleen cells after recognition of specific Ag as early as 7 days after the immunization (Fig. 3A). The IFN-γ production reached maximal levels 28 days after the first EHEC O157:H7 BGs rectal administration and remained unchanged for additional 21 days. Although the number of IFN-γ-producing cells slightly decreased on day 55, the heterologous challenge with live bacteria stimulated spleen cells to produce the same level of IFN-γ as was observed within the time period 28–49 days after immunization. The number of splenocytes producing IFN-γ after the heterologous challenge did not change significantly up to day 76 (Fig. 3A). Similar results were detected within the group B1 after the second immunization with EHEC O157:H7 BGs until the heterologous challenge (Fig. 3A). The booster immunization of mice resulted in significantly increased number of

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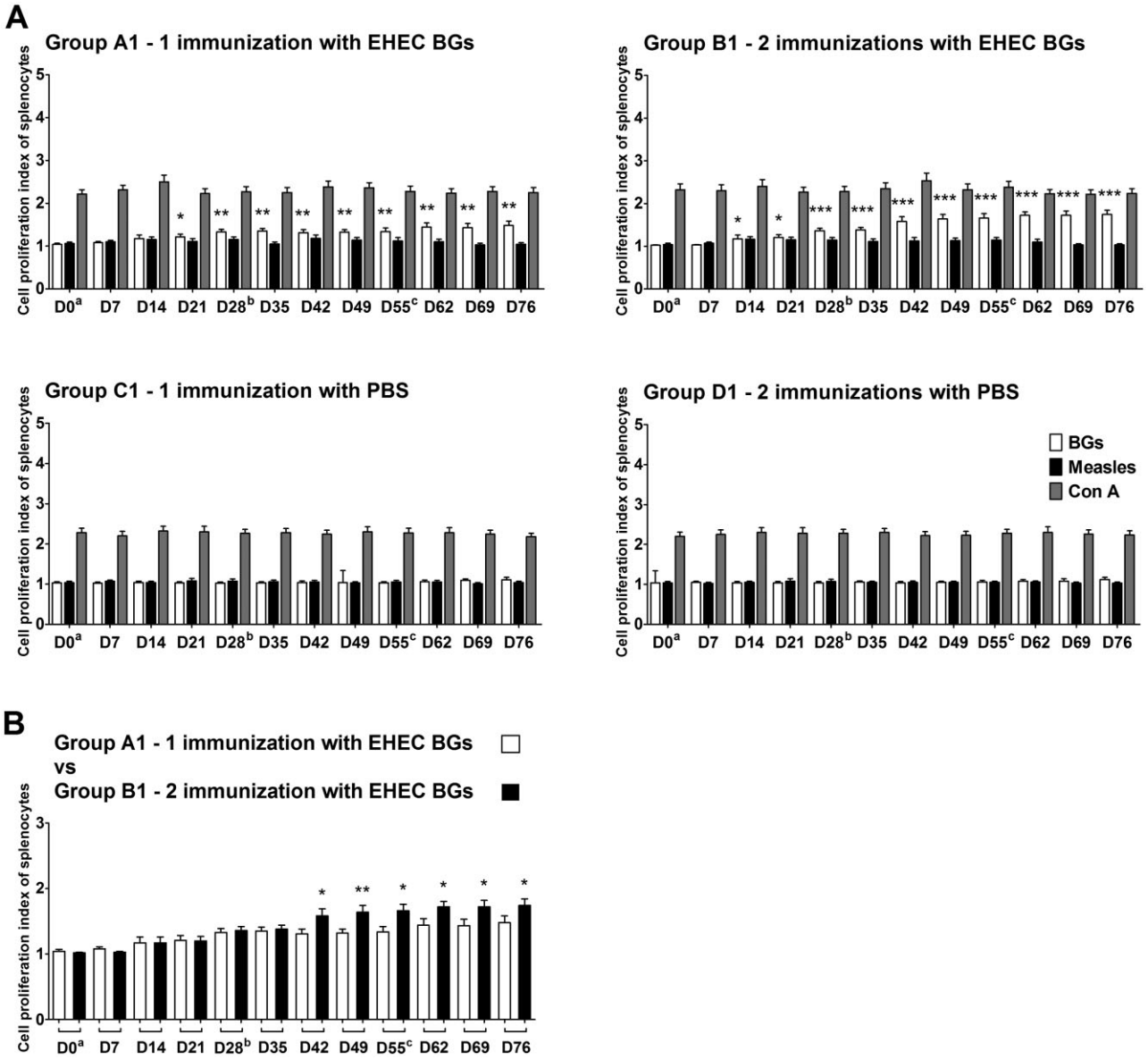

Fig. 4. Stimulation of Ag-specific cell proliferation after the immunizations of mice with EHEC O157:H7 (N°CIP 105282) BGs. Mice were immunized as described in Fig. 1. Proliferation of Ag-specific cells was assessed after 4 days of *in vitro* restimulation of spleen cells in the presence of EHEC O157:H7 BGs (specific Ag); measles inactivated Ag (strain Edmonston, non-specific Ag) or as a negative control spleen cells were left without stimulation (A). The stimulatory effect of the boost immunization significantly enhanced proliferation of Ag-specific spleen cells compared with proliferation of Ag-specific spleen cells observed after the single immunization with EHEC O157:H7 BGs (B). Data represent the mean of three independent experiments ± SD (three mice per time point). *P*-values < 0.05 were considered significant and are indicated with asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). a, day of the first immunization in all groups; b, day of the second immunization in groups B1 and D1; c, day of the challenge.

IFN-γ secreting spleen cells detected 14 days after the second immunization and also after the heterologous challenge with live bacteria compared with the number of IFN-γ-producing splenocytes observed after a single rectal immunization with EHEC O157:H7 BGs (Fig. 3B). No significant changes in IFN-γ production after stimulation with specific Ag were observed within both control groups (C1 and D1) immunized with placebo. The challenge with heterologous live EHEC O157:H7 slightly increased the number of IFN-γ-producing spleen cells but without statistical significance (Fig. 3A).

Moreover, the single rectal administration of EHEC O157:H7 BGs (group A1) significantly stimulated proliferation of Ag-specific cells already 21 days after immunization (Fig. 4A). The slightly increased proliferation of responder cells was detected in group B1 (two rectal

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immunizations EHEC O157:H7 BGs at day 0 and day 28) (Fig. 4A) compared with group A1 (single rectal immunization with EHEC O157:H7 BGs at day 0), but a significant difference was only observed 14 days after the second immunization (day 42 after the first immunization) and remained unchanged until the end of experiment (Fig. 4B). Mice from the control groups immunized with placebo (C1 and D1) showed only minor differences in the cell proliferation indexes of spleen cells within the whole observation period (Fig. 4A).

Discussion

Enterohaemorrhagic *Escherichia coli* belongs to the group of the most important pathogens of food-borne diseases causing severe harms, especially in children and the elderly (Pennington, 2010). EHEC express potent toxins known as Verocytotoxin or Shiga toxins which cause systemic damage of the vascular endothelium leading to haemorrhagic colitis and kidney damage. Moreover, the EHEC toxin may cause the thrombocytopenic purpura and the haemolytic uremic syndrome leading to a fatal kidney failure (Karmali *et al*., 1983; Su and Brandt, 1995; Paton and Paton, 1998). Specially children are more susceptible to these damages, partly due to higher toxin receptor levels in the kidney (Quantrell *et al*., 2004). Efficient therapy of EHEC infections still does not exist and treatment with conventional antibiotics is not recommended because of increasing rate of drug resistance as well as the use of anti-diarrhoeal medication should be avoided. Several alternative strategies how to eliminate lethal impact of bacterial toxins were described proposing the use of harmless or dead bacteria genetically engineered to express specific toxin receptors and hence to block binding of toxins to their natural receptors. However, acceptance of the treatment of human with genetically manipulated microorganisms even prepared from a harmless and probiotic strains is still controversial (Paton *et al*., 2001; 2006).

Cattle represent the major reservoir for EHEC harbouring the microbe in the intestinal tract particularly in the lymphoid dense mucosa in the terminal rectum (Verweyen *et al*., 2000; Naylor *et al*., 2003). Contamination of meat during slaughter, dairy products in farms, water, vegetables and fruits contaminated with manure or faeces of infected animals followed by insufficient hygiene processes allows entering of the pathogen to the human food chain and leads to major disease outbreaks with harmful consequences (Orskov *et al*., 1987; Locking *et al*., 2001; O'Brien *et al*., 2001; Olsen *et al*., 2002; Naylor *et al*., 2005; Hussein, 2007). The recent EHEC 2011 outbreak in Germany causing casualties and enormous economic losses brought back to attention the eminent need for the development of a novel efficient vaccine against EHEC and other food-borne diseases.

Several vaccination strategies against EHEC were systematically investigated using various immunization routes, different EHEC-specific antigens and were performed in a wide range of experimental animals (Sheng *et al*., 2006; Moxley *et al*., 2009; Smith *et al*., 2009; Cai *et al*., 2010; McNeilly *et al*., 2010; Farfan *et al*., 2011; Vilte *et al*., 2011; Vande Walle *et al*., 2011a).

Systemic immunizations of cattle with bacterial proteins actively participating in EHEC colonization considerably elicited serum and mucosal IgG and IgA antibody responses against bacterial proteins, and significantly reduced shedding of EHEC from orally challenged animals (McNeilly *et al*., 2008; Vilte *et al*., 2011). Rectal inoculation of sheep with EHEC O157:H7 induced cellular immune response, but no humoral immune response leading to protection of infected animals was detected (Vande Walle *et al*., 2011b). Similarly oral immunization of sheep with Stx-negative EHEC O157:H7 stimulated both humoral and cellular immune responses but was not able to fully protect animals after the challenge with the homologous strain (Vande Walle *et al*., 2011a). By contrast, oral administration of EHEC O157:H7 BGs using mice model stimulated both humoral and cellular immune responses with mixed Th1/Th2 type immune response. Moreover, high protection rates were observed against the lethal challenge after single dose immunization (56%; 86%) and these rates increased significantly after one booster (84%; 93.3%). These two separate studies were performed independently in two different laboratories (Mayr *et al*., 2005b; Cai *et al*., 2010).

Here we demonstrate for the first time that a single rectal immunization of mice with EHEC O157:H7 BGs results in complete protection against a live heterologous sub-lethal challenge. A single dose vaccine is highly desirable for all recipients particularly for children in whole world, but even more in developing countries. EHEC infections and diarrhoeal diseases in general are known to occur most frequently in children of less than 4 years of age (Pennington, 2010). Within this age group, the rectal route is frequently used for administration of drugs and represents one of the most desirable routes of immunization. The mucosal immunization has several advantages over the conventional parenteral route, as it is safer, less expensive and easier to distribute in developing countries. It is easily accessible even by untrained personnel and has the capacity to induce both efficient systemic and mucosal Ag-specific immune responses. The intestine contains lymphoid tissues with lymphoepithelial structures involved in the induction of mucosal immune responses after uptake of Ags within the intestinal tract (Brandtzaeg *et al*., 1998). It was shown previously that the routes of immunization induced higher immune responses at sites proximal to the site of induction than at distal sites after immunization of mice with

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a recombinant *Salmonella typhimurium* vaccine and concluded that rectal immunization might be necessary to protect the rectal mucosa (Hopkins *et al*., 1995). Furthermore, rectal administration of Ags in human could induce an efficient local immune response in the rectum with very high levels of antibodies at the site of immunization, therefore the generation of large proportions of specific IgA in the rectum clearly requires local administration of the vaccine (Kozlowski *et al*., 1997).

EHEC O157:H7 was shown to colonize the terminal rectum in the bovine host that is characterized by a lymphoid follicle-dense mucosa (Naylor *et al*., 2003; Nart *et al*., 2008). Also in sheep and humans this region is rich in lymphoid follicles and there is some evidence that EHEC O157:H7 attaches specifically at the follicle-associated epithelium of human Peyer's patches (Quantrell *et al*., 2004). Many factors affecting the colonization of EHEC including pili, outer membrane proteins, type three secretion system, H7 flagella, and others represent primary targets for the development of vaccines capable to stimulate efficient mucosal humoral and cellular immune responses able to entirely prevent pathogen colonization (Mahajan *et al*., 2009; McNeilly *et al*., 2010; Vande Walle *et al*., 2011a; Vilte *et al*., 2011). Unaltered surface structures present on BGs in their original state functionally contribute to efficient induction of both innate and adaptive immune responses against the bacterium used for BGs generation (Walcher *et al*., 2004; Abtin *et al*., 2010; Kudela *et al*., 2010). Stimulation of immune responses against several surface Ags influencing EHEC colonization at the same time might have a cumulative effect and would improve protection against the colonization of intestinal mucosa by pathogens.

In contrast to conventional non-living bacterial vaccines BGs provide all inner and outer membrane antigenic structures, the entire BGs surface remains in the native form with all intact structures of their living counterparts (Hensel *et al*., 2000; Tabrizi *et al*., 2004; Lubitz *et al*., 2009). Therefore, administration of a single non-living BG envelope type behaving as a package containing numbers of important Ags responsible for colonization would simplify the whole vaccination process and save time and costs necessary for selection and testing of potential candidate Ags. The presence of bacterial lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria and other antigenic and strain-specific structures enhances maturation of Ag-presenting cells and directly participates in the induction of humoral and cellular immune responses against the specific pathogen used for generation of BGs (Haslberger *et al*., 2000).

Although no detailed analysis was performed yet to determine the specific membrane subcomponents of EHEC BGs responsible for the induction of protective immune responses in mice, the data obtained during the study investigating the rapid clearance of the challenge bacteria indicate that several BG's surface antigens stimulate efficient production of antibodies capable to block the adherence of pathogenic bacteria to the intestinal target cells. Several studies showed that anti-O157 LPS antibodies protect against EHEC O157:H7 induced illnesses (WHO, 1999; Conlan *et al*., 1999a). Moreover, specific bacteriocidal titres directed against the LPS of BGs made from *V. cholerae* have been also reported (Eko *et al*., 2000).

The novel EHEC O157:H7 BGs candidate vaccine was produced by coexpression of gene *E* and SNUC from two different plasmids. No live cells were detected in enrichment cultures of DNA-free EHEC O157:H7 BGs samples used for the mouse trial with 10 times the amount of the immunization dose. These facts also demonstrate the safety of the freeze-dried non-living EHEC candidate vaccine what was likewise confirmed in the study investigating the use of EHEC O157:H7 BGs as an oral vaccine (Mayr *et al*., 2005b). Oral administration is a very promising way for future vaccine applications as the oral immunization route mimics the natural infection of EHEC and the BGs prepared from EHEC strain O157:H7 have been able to elicit protective immunity in contrast to a parenteral immunization with a glycoconjugated candidate vaccine (Conlan *et al*., 1999b). However, it is still an open question whether the denaturation processes by stomach acid and proteolytic enzymes can impair the quality of BGs administered by this route and hence affect the grade of required immune response. Immunization via the lymphoepithelial structures at the rectal mucosal might avoid this challenge arising during the oral vaccine administration. Moreover, the mucosal and parenteral routes of immunization may result in uptake by various types of Ag-presenting cells and thereby elicit different qualities of Ag-specific immune responses. The superiority of rectal immunization was also demonstrated by more efficient protection against the challenge with a virulent *M. tuberculosis* strain after immunization of newborn mice via rectal route compared with subcutaneous vaccination (Lagranderie *et al*., 2002). Traditionally prepared non-living full cell or subunit bacterial vaccine preparations have been shown to be less immunogenic when delivered orally (Vande Walle *et al*., 2011a). Mucosal adjuvant need to be added to these vaccine formulations to elicit protective immunity against enteric pathogens even though the negative side effects of adjuvants are well known (Spickler and Roth, 2003). Additional advantage of the BG system is that the vaccine is independent of cold chain and does not need the addition of adjuvants for the induction of relevant mucosal immune responses. Together with BGs stability at room temperature, easy storage and handling makes the BG system the most suitable for the

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use in developing countries (Jalava *et al*., 2003; Walcher *et al*., 2004; Riedmann *et al*., 2007).

In summary, this study reports that a single immunization with EHEC BGs is equally protective as double immunizations against the lethal challenge in mice. Moreover, after a single rectal immunization the mucosal immunity was adequately stimulated and provided high protection. Raised antibody levels and IFN-γ production in mice immunized twice indicate that mild natural infections with the pathogen or related bacteria will confer long lasting protective immunity. BG as candidate vaccine and carrier of foreign viral and/or bacterial Ag are currently under development as multivalent vaccines against diarrhoeal diseases of human. EHEC BGs as carrier of subunit vaccine combined with rectal immunization might lead to new improved non-living bacterial vaccines with excellent safety properties and high immunological potential.

Experimental procedures

Bacterial strains, plasmids and production of EHEC O157:H7 BGs

EHEC serotype O157:H7 strain N°CIP 103571 and strain N°CIP 105282 (both expressing Stx1 and Stx2) were obtained from the Collection de l'Institut Pasteur, Paris, France. Growth conditions of EHEC O157:H7 (N°CIP 105282, pML1, pSNUCIQ3) and production of BGs has been described previously (Mayr *et al*., 2005b). Briefly, plasmid pML1 carrying the lysis gene *E* under the transcriptional control of the phage λ P_R/*cI857*-promoter/operator system was co-transformed together with plasmid pSNUCIQ3 carrying the gene for SNUC under the transcriptional control of the synthetic lac promoter A1-04/03 into EHEC O157:H7 (strain N°CIP 105282). EHEC O157:H7 BGs (N°CIP 105282) were produced in a 10 l fermenter (Medorex, Bovenden, Germany) with a stirring rate of 350 r.p.m. and 3.5 l of air per min. No antifoam was added and pH values were stable in the range 6.5–7.5. To induce SNUC expression, 2 mM Isopropyl-β-D-thiogalactoside was added to the EHEC cultures at OD_{600} of 0.3. Protein E-mediated lysis was induced 45 min later by temperature shift from +28°C to +42°C. For full activity of the expressed nuclease 1 mM $MgCl_2$ and 10 mM $CaCl_2$ were added 90 min after induction of lysis. Growth and lysis of the bacteria were monitored by measuring the optical density (OD). Samples were taken at various time points during the growth, lysis and nuclease treatment and viable cell were determined using a spiral-plater (WASP-system; Don Whitley Scientific limited, West Yorkshire, UK). Samples were serially diluted in 0.85% NaCl, inoculated to LB agar plates and incubated at +28°C overnight. The BGs were collected by centrifugation 6 h after lysis induction and washed 3 times with 0.85% NaCl solution (with 1/3, 1/6 and finally with 1/12 of the starting culture volume). The final pellet was resuspended in distilled water (20 ml) and stored at −80°C. Samples were lyophilized for about 24 h using a Lyolab B (LSL Secfroid, Aclens, Switzerland) lyophilisator and stored at +4°C until further use.

Experimental animals

Inbred mice (BALB/c, male, 4 weeks old) were obtained from the vivarium of the State Research Centre of Virology and Biotechnology 'Vector' (Koltsovo, Novosibirsk region, Russia). Mice were placed in individual cages with autoclaved food and water available ad libitum.

Rectal vaccination of mice with EHEC O157:H7 BGs and challenge scheme

Mice were divided into four groups A, B, C and D with different schemes of immunization as summarized in Fig. 1. All mice were deprived of food 24 h before the rectal immunization and the challenge. Twenty minutes before immunization all mice were given an enema with 100 μl PBS, pH ~ 7.4, applied through a soft polyethylene catheter *per rectum*. Ten minutes before the challenge, 30 μl of PBS, pH 7.4, was given orally to all mice. On day 0, mice from group A divided into two subgroups (A1-35 mice and A2-30 mice), were immunized with 1 mg of freeze-dried EHEC O157:H7 BGs strain N°CIP 105282 (corresponding to 4.8×10^9 non-living BGs) in a volume of 30 μl PBS applied through a soft polyethylene catheter *per rectum*. Group B mice were divided into two subgroups (B1-35 mice and B2-30 mice) and were immunized twice, on day 0 and day 28, with the same dose as described for group A. Group C mice (control for group A), divided into two subgroups (C1-35 mice and C2-30 mice), were immunized with 30 μl of PBS on day 0 and mice from group D (control for group B), divided into two subgroups (D1-35 mice and D2-30 mice), were immunized with 30 μl of PBS, on day 0 and day 28. On day 55, all mice were orally challenged with 1×10^8 live EHEC O157:H7 heterologous strain N°CIP 103571. The dose of challenge was assessed as 'sub-lethal' killing about 50–75% of the BALB/c mice and determined in our previous experiments (data not shown). Blood samples for antibody determinations were taken from the orbital sinus of the mice at various time points from subgroups A1, B1, C1 and D1 under methoxyflurone anaesthesia. Intestinal lavage samples as well as spleen samples were collected after the mice were killed by decapitations as described previously (Ignatyev *et al*., 1995). Three mice from each group A1, B1, C1 and D1 were used for each time point starting on day 0 and continue in weekly intervals after the first immunization. The intestine samples were presented as the segments of duodenum, rectum and colon transversum taken in an equal length of 2.5 cm. Intestinal lavage was obtained as described earlier (Douce *et al*., 1998). Briefly, solid material was gently removed and the entire gut was washed through twice with 1.5 ml of 0.1% BSA in PBS containing 1 mM PMSF, which acts as a protease inhibitor. Mice from subgroups A2, B2, C2 and D2 were used as controls of mortality after the challenge. All mice were killed on day 76 by CO_2.

Infection confirmation

All mice were examined for the presence of live EHEC in faeces before challenge, and on days 1, 2, 3, 4, 5, 6, 7, 8, 14 and 21 post challenge using *E. coli* O157:H7 diagnostic test

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(Oxoid, Basingstoke, Hampshire and England) following the manufacturer's instructions.

Antibody determinations

The presence of specific IgA and IgG antibodies against EHEC O157:H7 BGs in sera, duodenum, rectum and colon transversum samples was determined separately by enzyme-linked immunosorbent assay (ELISA) (Douce *et al*., 1998). Each well of the microtitre plate (MaxiSorp Surface Assay Plates, Nunc Brand Products, Denmark) was coated with 0.5 μg EHEC O157:H7 BGs (N°CIP 105282) and antibodies against the EHEC O157:H7 BGs were measured with ELISA as previously reported (Mayr *et al*., 2005b).

IFN-γ ELISPOT assays

Spleen cells (1×10^5 cells per well) were incubated with BGs prepared from EHEC O157:H7 strain N°CIP 105282 (specific Ag) at concentration 0.5 ng μl^{-1}. Cells incubated with measles inactivated Ag (strain Edmonston; non-specific Ag; 0.5 ng μl^{-1}) and cells without stimulation served as controls. The number of IFN-γ-producing cells was determined by Mouse IFN-γ ELISpot Kit (R&D Systems, Inc, Minneapolis, USA) following the manufacturer's instructions.

Cell proliferation assay

Spleen cells (1×10^5 cells per well) were cultured in 100 μl of RPMI as described previously (Ignatyev *et al*., 1995). Cells were incubated with BGs prepared from EHEC O157:H7 strain N°CIP 105282 (specific Ag) at concentration 5 μg ml^{-1}, measles inactivated Ag (strain Edmonston; non-specific Ag; 2 μg ml^{-1}) or with Con A (5 μg ml^{-1}) used as positive control for 96 h at +37°C in a 5% CO_2 humidified atmosphere. XTT and PMS (Sigma, USA) were added as previously described (Scudiero *et al*., 1988; Ignatyev *et al*., 1995) for the final 8 h of incubation and the absorbance was measured at 450 nm (reference 690 nm). Results were calculated as the ratio between the control (spleen cells left without any activation) OD and a sample OD, and are presented as an index.

Statistical analysis

Obtained results were analysed by GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). The statistical significance of the difference between two groups was evaluated by Student's *t*-test or chi-squared test. Differences were considered to be significant with $P < 0.05$.

Acknowledgements

This work has been supported by BIRD-C GmbH&CoKG, Kritzendorf, Austria.

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