

# Murine model of *Bacillus cereus* gastrointestinal infection

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*Bacillus cereus* is a spore-forming micro-organism responsible for foodborne illness. In this study, we focus on the host response following intragastric challenge with a pathogenic *B. cereus* strain (B10502) isolated from a foodborne outbreak. C57BL/6J female mice were infected by gavage with strain B10502. Controls were administered with PBS. Infection leads to significant modification in relevant immune cells in the spleen, Peyer's patches (PP) and mesenteric lymph nodes (MLN). These findings correlated with an increase in the size of PP as compared with uninfected controls. Histological studies showed that *B. cereus* infection increased the ratio of intestinal goblet cells and induces mononuclear cell infiltrates in spleen at 5 days post-infection. Evaluation of cytokine mRNA expression demonstrated a significant increase in IFN- $\gamma$  in MLN after 2 days of infection. The present work demonstrates that infection of mice with vegetative *B. cereus* is self-limited. Our findings determined relevant cell populations that were involved in the control of the pathogen through modification of the ratio and/or activation.

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## INTRODUCTION

The *Bacillus cereus* group constitutes a very homogeneous cluster of the genus *Bacillus*. This group, often called *B. cereus sensu lato*, comprises seven species: *Bacillus anthracis*, *B. cereus (sensu stricto)*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. cytotoxicus* (Guinebretière *et al.*, 2008).

*B. cereus (sensu stricto)* is a Gram-positive spore-forming micro-organism that is widely distributed in the environment. It is found in soil and plant surfaces and frequently contaminates foodstuffs (Stenfors Arnesen *et al.*, 2008).

*B. cereus* is responsible for foodborne illness and is associated with different pathologies such as emetic and diarrhoeic syndromes, endophthalmitis (Beecher & Wong, 1994; Moyer *et al.*, 2008; Durand, 2013), endocarditis (Thomas *et al.*, 2012; Barraud *et al.*, 2012), osteomyelitis, oral cavity infections (Kotiranta *et al.*, 1998), septicaemia, peritonitis, pneumonia, and meningitis (Rowan *et al.*, 2001; Lede *et al.*, 2011; Wright *et al.*, 2011; Miyata *et al.*, 2013). The nonintestinal infections mainly occur in immunocompromised patients (Rasko *et al.*, 2005; Slamti & Lereclus, 2005; Schoeni & Wong, 2005; Bottone, 2010) and drug users (Dancer *et al.*, 2002; McLauchlin *et al.*, 2002). *B.*

*cereus* infection usually leads to self-limited pathologies and it is not commonly reported, although severe and fatal cases have increased in recent years (Akiyama *et al.*, 1997; Dierick *et al.*, 2005; Mahler *et al.*, 1997; Wright *et al.*, 2011).

With respect to intestinal pathologies associated with *B. cereus*, two main syndromes have been reported: emetic and diarrhoeic. Whereas emetic syndrome is associated with cereulide, a thermostable dodecadepsipeptide (Agata *et al.*, 1995; Horwood *et al.*, 2004; Ehling-Schulz *et al.*, 2005), the diarrhoeic syndrome has been related to a plethora of extracellular factors with different biological effects (Asano *et al.*, 1997; Beecher & Wong, 2000; Kotiranta *et al.*, 2000; Lund & Granum 1997; Stenfors Arnesen *et al.*, 2008). Most of the secreted virulence factors are regulated by the PlcR regulon, which is the major virulence regulator in *B. cereus* (Gohar *et al.*, 2002; Slamti *et al.*, 2004). This regulation is relevant for the virulence in insect and mouse infections (Salamitou *et al.*, 2000).

It is known that *B. cereus* extracellular factors are able to produce different biological effects on cultured enterocytes (Caco-2 cells) and erythrocytes (Minnaard *et al.*, 2001). Moreover, direct bacteria–enterocyte interactions have been proposed as an important virulence factor (Rowan *et al.*, 2001; Minnaard *et al.*, 2004, 2007). Adherence combined with the ability of some strains to invade epithelial cells (Minnaard *et al.*, 2004, 2007) could be involved in diarrhoeic syndromes. Andersson *et al.* (1998) showed that,

Abbreviations: MLN, mesenteric lymph nodes; PP, Peyer's patches.

at least for some strains, *B. cereus* spores are able to germinate and produce enterotoxin when they are incubated with the target cells (Vero cells).

In the present study, we analysed the immunological and pathological characteristics of a mouse model infection induced by intragastric challenge with a pathogenic *B. cereus* strain (B10502). This strain is associated with a foodborne outbreak (Minnaard *et al.*, 2004). In previous *in vitro* studies using Caco-2 monolayers, we showed that *B. cereus* B10502 is a non-invading strain (Minnaard *et al.*, 2004). However, extracellular factors of strain B10502 lead to damage of cultured human enterocytes. This biological effect is related to the adhesion to eukaryotic cells (Minnaard *et al.*, 2004, 2007).

*In vivo* studies of the effect of bacteria belonging to the *B. cereus* group were conducted mainly with *B. anthracis* spores. Studies included different routes of infection, e.g. intradermic, intravenous and intraperitoneal (Heninger *et al.*, 2006; Glomski *et al.*, 2007). Xie *et al.* (2013) developed a model of gastrointestinal infection with vegetative *B. anthracis* in complement-deficient mice. Vegetative cells were able to invade and grow within intestinal villi, with subsequent translocation to different organs. Interestingly, no immune activation was observed (Xie *et al.*, 2013). Since no *in vivo* models have been developed for *B. cereus*, the present study sought to gain further insight on the effect of *B. cereus* on a murine model of intestinal infection.

## METHODS

**Bacterial strains and culture conditions.** *B. cereus* strain B10502, isolated from a food-poisoning outbreak, was kindly provided by the Instituto Biológico Dr Tomás Perón (La Plata, Argentina). Micro-organisms were stored at  $-80^{\circ}\text{C}$  with 0.3 M sucrose as a cryoprotectant. Prior to the experiments, bacteria were cultured for 16 h at  $32^{\circ}\text{C}$  with agitation in brain heart infusion broth medium (Biorad Diagnostics) supplemented with 0.1% (w/v) glucose (BHIG). Afterwards, micro-organisms were inoculated (4%, v/v) in BHIG and incubated with agitation at  $32^{\circ}\text{C}$  for 3 h (mid-exponential phase). Bacteria were harvested by centrifugation (900 g for 10 min).

Bacterial concentration was estimated by optical density based on previously determined correlations with c.f.u. (data not shown). The bacterial suspension was then diluted in sterile PBS (0.144 g  $\text{KH}_2\text{PO}_4$   $\text{l}^{-1}$ , 0.795 g  $\text{Na}_2\text{HPO}_4$   $\text{l}^{-1}$ , 9 g  $\text{NaCl}$   $\text{l}^{-1}$ , pH: 7) to obtain the desired inoculum.

**Animals.** C57BL/6J female mice (6–8-weeks-old) were purchased from Facultad de Ciencias Veterinarias, UNLP (La Plata, Argentina). Each experimental group consisted of 5–7 mice housed in cages that were maintained at a controlled temperature and light cycle; the mice were fed with a balanced conventional diet. Animals were handled in accordance with international regulations for animal welfare.

**Infection.** Bacteria were administrated by intragastric gavage using a blunt end needle. Two different experiments were performed. For experimental protocol I mice were randomly allocated into different groups of five mice each. One group (control mice) received only sterile PBS and the other groups were challenged with  $1 \times 10^8$  *B. cereus* B10502 per mouse. Mice were euthanized by  $\text{CO}_2$  inhalation at 2 and 5 days after infection. For experimental protocol II each mouse

was infected with  $1 \times 10^9$  *B. cereus* B10502. After different times post-infection (30 min, 2 h 30 min, 4 h 30 min, 20 h and 2 days) mice were euthanized by  $\text{CO}_2$  inhalation. In both experiments, each animal received 100  $\mu\text{l}$  of the corresponding bacterial suspension or sterile PBS. Prior to infection, animals were starved for 16 h. Samples from different regions of the intestinal tract and mesenteric lymph nodes (MLN) were taken.

**Histological analysis.** The spleen and small intestine were aseptically removed for histological preparation. Samples were fixed in absolute ethanol, dehydrated and embedded in Histowax (Leica Microsystems) following the technique of Sainte-Marie (1962). Four-micron tissue sections were stained with haematoxylin–eosin. Small intestine samples were also stained with Alcian Blue–eosin for goblet cell staining. Villous-to-crypt ratios and presence of inflammatory cell infiltrates were assessed.

**Localization of *B. cereus*.** The presence of viable *B. cereus* was assessed in the stomach, duodenum, jejunum, ileum, caecum, faeces, Peyer's patches (PP), MLN and spleen. Stomach and intestinal luminal contents were collected by washing with sterile PBS. The spleen, PP and MLN were aseptically removed. Afterwards samples were mechanically disrupted and centrifuged, and cells were lysed with distilled water (Pérez *et al.*, 2007). One millilitre samples were inoculated in 9 ml BHIG for growth assessment. One hundred microlitres were spread out on nutritive agar. Both broth and agar were supplemented with polymyxin B (0.01 g  $\text{l}^{-1}$ ). After incubation at  $32^{\circ}\text{C}$  for 24 h positive cultures were streaked on nutritive agar.

**Flow cytometry analysis.** The immune-cell population in the spleen, PP and MLN were evaluated by flow cytometry (Medrano *et al.*, 2011). The following mAbs were used: FITC-conjugated anti-mouse CD4 (clone GK 1.5), phycoerythrin (PE)-conjugated anti-mouse CD8 (clone CT-CD8b), FITC-conjugated anti-mouse/human CD45R (B220) (clone RA3-6B2), PE-conjugated anti-mouse MHC class II (I-A/I-E) (clone M5/114.15.2), biotin-conjugated anti-mouse F4/80 antigen-pan macrophage marker (BM8-100), biotin-conjugated anti-mouse CD11c (clone N418) and PE-conjugated anti-mouse CD86 (B7-2) (clone PO3.1). The corresponding isotype controls were used. The antibodies were purchased from eBioscience and used according to the manufacturer's instructions. Spleen, PP and MLN were aseptically removed from animals infected with  $1 \times 10^8$  *B. cereus* B10502 per mouse. Cell suspensions were obtained by mechanical disruption of tissue and collected in ice-cold PBS. Samples were centrifuged for 5 min (250 g) at  $4^{\circ}\text{C}$  and suspended in PBS supplemented with 2% (v/v) heat-inactivated fetal bovine serum (PAA Laboratories). Red blood cells in spleen samples were lysed with sterile water. The concentration of viable cells was determined by Trypan blue exclusion. A total of  $5 \times 10^5$  cells per tube were washed twice with PBS, 2% fetal bovine serum, centrifuged for 5 min at 250 g and incubated for 30 min on ice with 50  $\mu\text{l}$  of the corresponding specific antibody. Cells were then washed with PBS and fixed in 1% (w/v) paraformaldehyde in PBS. Unlabelled control cells were also processed for each sample. All samples were analysed with a FACScalibur flow cytometer (Becton and Dickinson) and CellQuest software (BD Biosciences). A total of 10 000 events per sample were collected.

**Real-time PCR.** Mice infected according to experimental protocol II were euthanized at different times after infection: 30 min, 2 h 30 min, 4 h 30 min, 20 h and 2 days. MLN and PP were aseptically removed, RNA extraction was performed using the Illustra Illustra RNA spin RNA mini isolation kit (GE Healthcare) following RNA extraction protocol LG according to the manufacturer's instructions, and RNA concentration was measured by optical density at 260 nm. Reverse transcription was conducted by using random primers and MMLV-reverse transcriptase (Promega), and the resulting cDNA was

amplified using IQ SYBR Green PCR mix (Bio-Rad Laboratories). Real-time PCR was performed following the manufacturer's protocol using an iCycler thermal cycler (Bio-Rad). The housekeeping gene  $\beta$ -actin was used as control. The specificity of the PCR was verified by melting curves. The relative difference calculation using the  $\Delta C_t$  method has been described elsewhere (Rumbo *et al.*, 2004; Anderle *et al.*, 2005; Smaldini *et al.*, 2012; Moreno *et al.*, 2013). Primers specific for mouse were designed using the software Primer Express (Applied Biosystems):

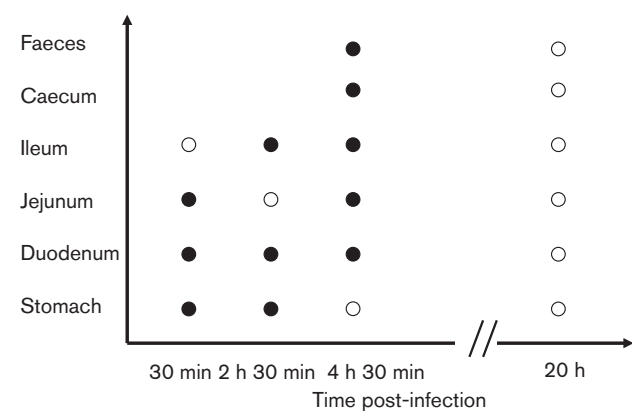
CXCL2fwd CCCTCACAGGAAGAACCAAA, CXCL2rev CACATCAGGTACGATCCAGGC; TNF- $\alpha$ fwd CATCTTCTAAAAATTCGAGTGACAA, TNF- $\alpha$ rev CCTCCACTGGTGGTTTGCT; IL-6fwd GTTCTCTGGGAAATCGTGGAAA, IL-6rev AAGTGCATCATCGTTGTTCTATACA; IL-10fwd CATTGAAATTCCTGGGTGAGA, IL-10rev TGCTCCACTGCCTTGCTCTT; IL-12p40fwd GCAAAGAAACATGGACTTGAAGTTC, IL-12p40rev CACATGTCAGTCCCGAGAGT; INF- $\gamma$ fwd TGGCATAAGATGTGGAAGAAAAGAG, INF- $\gamma$ rev TGCAAGATTTTCATGTCACCAT;  $\beta$ -Actinfwd CGTCATCCATGGCGAACTG,  $\beta$ -Actinrev GCTTCTTGCAGCTCCTTCGT.

**Statistical analysis.** Results were compared by means of a two-tailed Student's *t*-test (InfoStat version 2008; InfoStat).  $P < 0.05$  was considered significant.

## RESULTS AND DISCUSSION

### Localization of *B. cereus* B10502 in infected mice

Fig. 1 shows the progress of *B. cereus* along the digestive tract. As expected, shortly after inoculation (30 min), viable bacteria were found at proximal localizations (i.e. the stomach, duodenum and jejunum). At 2 h 30 min micro-organisms were also found in the ileum. At 4 h 30 min viable micro-organisms reached the caecum and faeces, but stomach samples were negative. Twenty hours after inoculation all the samples were negative. Non-infected mice were negative for *B. cereus* in all the samples and time points analysed (data not shown). Results are in agreement with published studies on gastrointestinal transit of markers such



**Fig. 1.** Localization of *B. cereus* B10502 in the gastrointestinal tract. The graph indicates the presence of *B. cereus* B10502 in a time-course infection. Different samples were analysed by plate counts. Experimental protocol II was used. ●, Presence of *B. cereus* in the sample; ○, *B. cereus* not found in the sample.

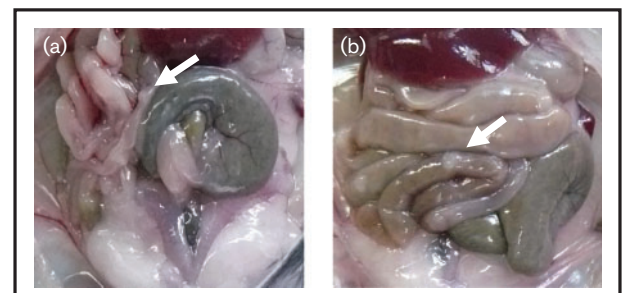
as charcoal or radiopaque balls, which were found in faeces a few hours after administration (Marona & Lucchesi, 2004; Myagmarjalbuu *et al.*, 2013). Lack of viable *B. cereus* in faeces 20 h after inoculation indicates that bacteria follow the same kinetics as inert transit markers, although these findings do not necessarily exclude colonization or bacterial translocation (see below).

*In vitro* survival studies in simulated gastric conditions demonstrated that *B. cereus* vegetative forms are able to survive passage through the acidic conditions of the stomach (Wijnands *et al.*, 2009). However, it was shown that survivors represent only around 10% of the initial inoculum (Ceuppens *et al.*, 2012). In addition, survival was higher in the stomach after food ingestion than after food deprivation (Clavel *et al.*, 2004).

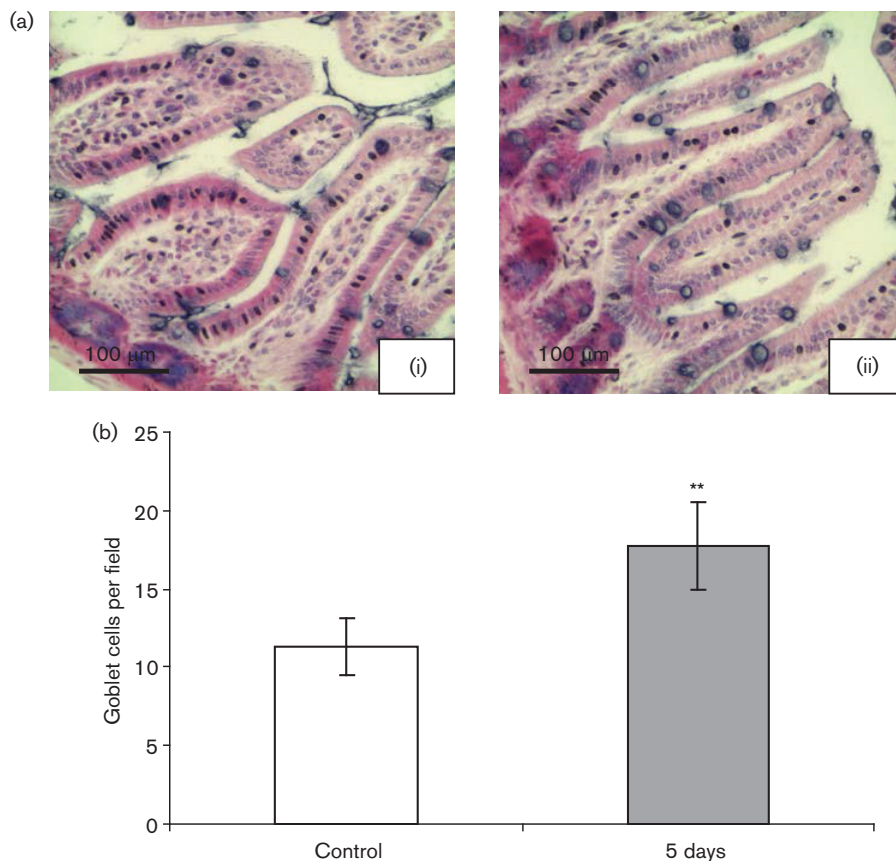
Our results show a transient passage of vegetative bacteria through the gastrointestinal tract. Even though a high proportion of the bacterial population was inactivated, this finding does not rule out that low numbers of micro-organisms could be present adhered to the epithelium or internalized into the host's cells.

### Histological analysis

We analysed the effect of infection on different regions of the intestinal tract and other relevant organs. Infected mice showed larger PP than control mice. This could be appreciated macroscopically at 2 and 5 days after infection (Fig. 2). Histological sections of small intestine showed a significant ( $P < 0.005$ ) increase of mucus-producing cells (Goblet cells) with no evidence of villous widening, shortening or blunting (Fig. 3). There are several mechanisms involved in the maintenance of barrier function; i.e. integrity of epithelial cells, tight and adherens junctions, and secretions from specialized cells. Goblet cells are key players of the barrier function through the secretion of mucus as well as proteins involved in epithelial repair and function such as Trefoil factor 3 and RELM- $\beta$  (Kim & Ho, 2010). The increase in the number of goblet cells suggests



**Fig. 2.** Abdominal cavity. Gastrointestinal infection of mice with  $1 \times 10^9$  *B. cereus* B10502 after 5 days of infection showing induction of more voluminous PP (b) compared with the PP observed in control mice (a). The arrows indicate the PP.



**Fig. 3.** Goblet cells. (a) Goblet cells in the small intestine stained with Alcian Blue–eosin in (i) mice from the control group and (ii) mice infected with *B. cereus*, after 5 days of infection. (b) Number of goblet cells per field in the small intestine epithelium in uninfected control mice (white bar) and in mice 5 days post-infection (grey bar). Error bars represent SD. The experiment was carried out following protocol II. \*\*Significant differences (*t*-test,  $P < 0.005$ ) in the number of Goblet cells as compared to the control.

that there is a response of the intestinal mucosa following *B. cereus* infection, although no dramatic effects were observed on the intestinal epithelium.

Histological sections of spleen stained with haematoxylin–eosin showed differences between infected and control animals. Indeed, evidence of cell infiltration was found in spleens of infected mice 5 days after infection (Fig. 4).

Overall, the observed biological effects on different localizations could be due to the direct interaction of the bacteria with the host's cells (Minnaard *et al.*, 2004) or the release of intracellular bacterial toxins after lysis (Henderson *et al.*, 1999), although effects related to the host's immune response cannot be ruled out.

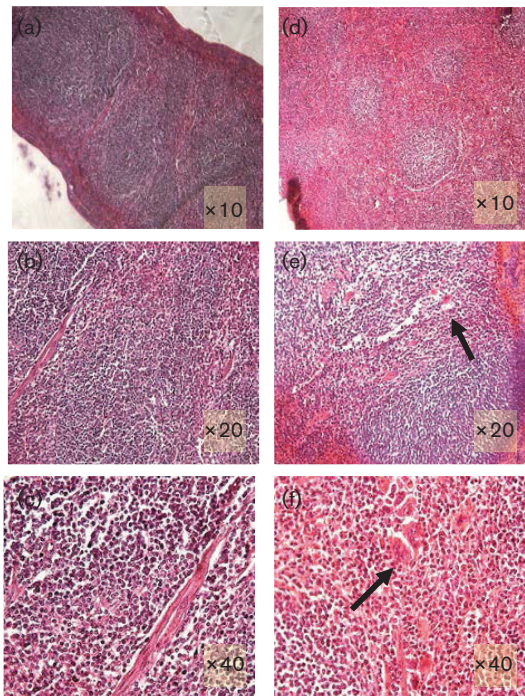
### Effect on the balance of immune-cell populations

**T cells.** T cell populations (CD4<sup>+</sup> and CD8<sup>+</sup>) were evaluated by flow cytometry in the PP, MLN and spleen of control and infected mice. The ratio of CD8<sup>+</sup> cells decreased in MLN at 2 and 5 days post-infection, whereas

there was an increase of this cell population in the spleens of the infected mice as compared with non-infected mice (Table 1).

Some strains of *B. cereus* have demonstrated the ability to gain access to intracellular compartments of epithelial cells (Minnaard *et al.*, 2004). It is known that there is a correlation between the intracellular localization of micro-organisms and immune response. Indeed, it has been demonstrated that access of *Listeria monocytogenes* to different cellular compartments leads to a shift in CD8 T cell response (Bahjat *et al.*, 2006). This response occurs even when the micro-organism fails to enter and multiply within the cytosol (Bahjat *et al.*, 2006). The above findings could be related to the results of the histological analysis shown in Fig. 4, since an increase of cytotoxic T cells in the spleen could account for the cell infiltrates found at 5 days post-infection.

The increase of CD8<sup>+</sup> T cells in the spleen and the concomitant decrease in MLN is compatible with a recruitment of T cells by the spleen. These findings correlated



**Fig. 4.** Spleen microphotography. Representative images generated from haematoxylin–eosin stained sections from the spleens of the mice. (a), (b) and (c) correspond to control mice (magnification  $\times 10$ ,  $\times 20$  and  $\times 40$ , respectively); (d), (e) and (f) correspond to infected animals at 5 days post-infection using  $1 \times 10^8$  *B. cereus* per mouse (magnification  $\times 10$ ,  $\times 20$  and  $\times 40$ , respectively). Arrows indicate the presence of infiltrated cells.

with cell infiltrates in the spleen, but it is intriguing how a micro-organism that did not shown evidence of systemic dissemination could lead to this effect. Certainly this issue

**Table 1.** Percentages of T cells ( $CD4^+$  and  $CD8^+$ ) in the PP, MLN and spleen of infected mice (2 and 5 days post-infection) and untreated (control) mice

Values were calculated from the gates of lymphocytes in the Forward Scatter (FSC) versus Side Scatter (SSC) plots, and represent the means of five determinations  $\pm$  SD. \*Significant differences (*t*-test,  $P < 0.05$ ) as compared with control mice. PI, Post-infection.

Sample	Group	$CD4^+$ cells	$CD8^+$ cells
PP	Control	$8.2 \pm 2.8$	$3.4 \pm 0.4$
	2 days PI	$5.5 \pm 0.9$	$3.4 \pm 0.3$
	5 days PI	$7.4 \pm 2.4$	$3.5 \pm 0.2$
MLN	Control	$19.7 \pm 3.9$	$31.7 \pm 6.1$
	2 days PI	$21.9 \pm 2.9$	$24.7 \pm 6.6$
	5 days PI	$21.9 \pm 4.4$	$20.4 \pm 5.6^*$
Spleen	Control	$5.6 \pm 1.3$	$12.3 \pm 1.1$
	2 days PI	$6.6 \pm 1.4$	$16.3 \pm 2.2^*$
	5 days PI	$6.7 \pm 1.6$	$14.8 \pm 2.5$

encourages further studies. In contrast, no differences in the ratio of  $CD4^+$  cells were found between control and infected mice (Table 1).

**B cells.** Consistent with the above results, infection with strain B10502 led to changes in other spleen-cell populations. As shown in Table 2, a significant decrease ( $P < 0.05$ ) in  $B220^+$  cells was observed in the spleen at 5 days post-infection. These results could suggest a migration of these cells from the spleen due to infection. No changes in the expression of MHCII were found in any of the analysed samples (data not shown).

**Dendritic cells ( $CD11c^+/CD86^+$ ).** Percentages of dendritic cells ( $CD11c^+$ ) expressing the co-stimulatory marker CD86 were assessed in the PP, MLN and spleen. As for other immune markers studied, the main changes were found in the spleen where a significant decrease of double positive  $CD11c^+/CD86^+$  cells was observed at day 2 post-infection ( $P < 0.05$ ) (Table 3). In addition, there was a trend ( $P = 0.07$ ) of a decrease in the ratio of activated dendritic cells in MLN after 5 days of infection (Table 3) but no other changes were observed (Table 3). Since no changes in the total number of dendritic cells were observed (data not shown), we concluded that infection of mice with strain B10502 leads to a decrease in the activation of antigen presenting cells. This hypothesis correlates with the demonstrated ability of a key enzyme of the *B. cereus* group (phosphatidylinositol phospholipase C, PI-PLC) to decrease the expression of the co-stimulatory marker CD86 in dendritic cells (Zenewicz *et al.*, 2005).

**Macrophages ( $F4/80^+$ ) and MHCII expression.** Changes in the macrophage population following *B. cereus* infection showed a different pattern as compared with other relevant cell populations. Indeed, whereas other cell populations

**Table 2.** Percentages of  $B220^+$  cells in the PP, MLN and spleen of infected mice (2 and 5 days post-infection) and untreated (control) mice

$B220^+$  cells were selected from the gates of lymphocytes in the Forward Scatter (FSC) versus Side Scatter (SSC) plots. Values represent the means of five determinations  $\pm$  SD. \*Significant differences (*t*-test,  $P < 0.05$ ) as compared with control mice. PI, Post-infection.

Sample	Group	$B220^+$ cells
PP	Control	$67.9 \pm 3.2$
	2 days PI	$62.4 \pm 4.4$
	5 days PI	$67.1 \pm 3.5$
MLN	Control	$33.8 \pm 5.3$
	2 days PI	$42.5 \pm 9.9$
	5 days PI	$36.8 \pm 10.3$
Spleen	Control	$46.4 \pm 3.8$
	2 days PI	$45.3 \pm 2.5$
	5 days PI	$35.0 \pm 4.6^*$

**Table 3.** Percentages of activated dendritic cells (CD11c<sup>+</sup>/CD86<sup>+</sup>) in the PP, MLN and spleen of infected mice (2 and 5 days post-infection) and untreated (control) mice

Cells of the different samples were double labelled to analyse the percentage of activated dendritic cells (CD11c<sup>+</sup>/CD86<sup>+</sup>). Values represent the means of five determinations  $\pm$  SD. \*Significant differences (*t*-test,  $P < 0.05$ ) as compared with control mice. PI, Post-infection.

Sample	Group	CD86 <sup>+</sup> DC <sup>†</sup>
PP	Control	23.5 $\pm$ 11.2
	2 days PI	19.7 $\pm$ 5.3
	5 days PI	32.3 $\pm$ 14.3
MLN	Control	54.1 $\pm$ 12.4
	2 days PI	47.3 $\pm$ 1.9
	5 days PI	37.5 $\pm$ 14.3
Spleen	Control	43.3 $\pm$ 1.3
	2 days PI	34.2 $\pm$ 1.3*
	5 days PI	44.7 $\pm$ 3.0

<sup>†</sup>Percentages of CD86<sup>+</sup> in the total population of CD11<sup>+</sup> cells.

were modified in the spleen, there were no changes in the ratio of macrophages in this organ. Instead, there was an increase of F4/80<sup>+</sup> cells in the PP, a main site of interaction between bacteria and the host, at day 2 post-infection (Table 4). At day 5 post-infection, F4/80<sup>+</sup> cells with high expression of MHCII showed a significant increase in the PP ( $P < 0.05$ ). Interestingly, F4/80<sup>+</sup>/MHCII<sub>high</sub> spleen cells also were increased at day 5 post-infection.

Taken together, the above findings indicate that intestinal infection with *B. cereus* stimulates cells in the PP, thus leading to macroscopic changes in size as well as an increase of ratio and activation of F4/80<sup>+</sup> cells. In the spleen, the increase of activated macrophages correlates with the altered structure observed in the histological analysis.

**Table 4.** Macrophages (F4/80<sup>+</sup>) and MHCII expression

MHCII expression was analysed as low and high expression in F4/80<sup>+</sup> cells. Values represent the means of five determinations  $\pm$  SD. \*Significant differences (*t*-test,  $P < 0.05$ ) as compared with untreated (control) mice. PI, Post-infection.

Sample	Group	F4/80 <sup>+</sup>	F4/80 <sup>+</sup> MHCII low	F4/80 <sup>+</sup> MHCII high
PP	Control	9.8 $\pm$ 1.4	51.4 $\pm$ 0.7	30.9 $\pm$ 0.6
	2 days PI	16.4 $\pm$ 1.8*	49.5 $\pm$ 4.7	34.5 $\pm$ 11.7
	5 days PI	10.1 $\pm$ 0.7	47.2 $\pm$ 7.9	43.5 $\pm$ 9.2*
MLN	Control	4.9 $\pm$ 1.1	33.7 $\pm$ 4.8	58.0 $\pm$ 6.6
	2 days PI	4.4 $\pm$ 1.9	41.5 $\pm$ 9.4	50.7 $\pm$ 9.1
	5 days PI	8.5 $\pm$ 4.2	42.6 $\pm$ 6.5	50.3 $\pm$ 9.4
Spleen	Control	9.8 $\pm$ 0.5	32.8 $\pm$ 4.4	47.6 $\pm$ 1.3
	2 days PI	10.7 $\pm$ 2.5	38.1 $\pm$ 6.0	42.7 $\pm$ 9.9
	5 days PI	10.1 $\pm$ 0.7	28.8 $\pm$ 3.6	54.0 $\pm$ 3.8*

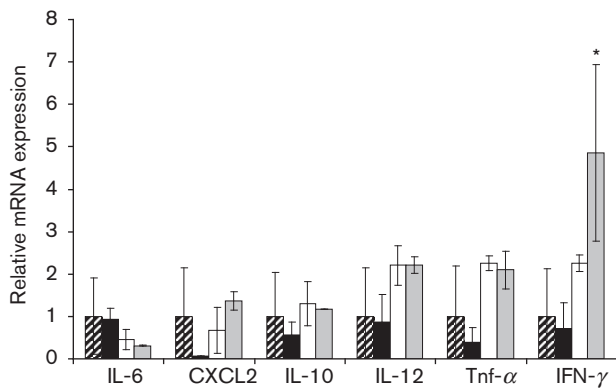
**Relative mRNA expression of cytokines.** After infection with  $1 \times 10^9$  micro-organisms per mouse, the mRNA expression of cytokines was analysed by Real-Time Quantitative PCR (qRT-PCR). IFN- $\gamma$  mRNA expression was significantly increased ( $P < 0.05$ ) when compared to the control at 2 days post-infection in MLN (Fig. 5). There were slight increases in IL-12 mRNA (trend at 20 h  $P = 0.18$ ; trend at 2 days  $P = 0.28$ ) and TNF- $\alpha$  mRNA (trend at 20 h  $P = 0.14$ ; trend at 2 days  $P = 0.35$ ) expression when 20 h and 2 days MLN post-infection samples were analysed (Fig. 5). Other cytokines studied, i.e. IL-6, CXCL2 and IL-10, were not modified in MLN. In the PP, no changes in the levels of mRNA of the cytokines studied were found (data not shown).

INF- $\gamma$  is related to macrophage activation and to an increase in MHCII expression on the surface of antigen presenting cells. This is associated with antigen processing and presentation, and a Th1 immune response (Murphy *et al.*, 2007). Our findings are in agreement with a sequential response following mouse infection with *B. cereus*. This response encompasses the activation in the expression of chemical mediators such as IFN- $\gamma$  and the modification of cell populations relevant for the immune response.

Studies of cytokine expression after *B. cereus* infection have been conducted mainly with cultured cells. Infection of macrophages with *B. cereus* showed TNF- $\alpha$ , IL-6 and IL-1 $\beta$  production in addition to microbicide factors (Huttunen *et al.*, 2003). In the *B. cereus* group, *in vivo* studies have been conducted mainly with *B. anthracis*. In a study on a murine model of *B. anthracis* gastrointestinal infection, Xie *et al.* (2013) demonstrated that infection preferentially localized in the small intestine and did not lead to immune activation. These findings are in agreement with the immunosuppressive effects of anthrax lethal toxin (Xie *et al.*, 2013).

## Conclusion

The present work shows that gastrointestinal infection with *B. cereus* B10502 vegetative cells induces an immune



**Fig. 5.** Relative mRNA expression of cytokines in MLN. Real-Time Quantitative PCR (qRT-PCR) of IL-6, CXCL2, IL-10, IL-12, TNF- $\alpha$  and INF- $\gamma$  in MLN. Control (striped bars) and infected mice were analysed. Samples of infected mice were studied at different times post-infection: 4 h 30 min (black bars), 20 h (white bars) and 2 days (grey bars). Error bars represent SD.  $\beta$ -actin was used to normalize gene expression. \*Significant differences ( $t$ -test,  $P < 0.05$ ) in the mRNA relative expression as compared to the control.

response in the host. Although self-limiting, the intestinal infection with *B. cereus* modifies the balance of relevant cell populations in different regions of the intestinal mucosa and immune-associated tissues. Our results show that following infection, the host orchestrates an immune response mediated by soluble factors (i.e. IFN- $\gamma$ ) and that this response includes the spleen as the target organ where the main changes in cell populations take place.

In contrast with murine gastrointestinal anthrax, which leads to dramatic effects including intestinal haemorrhage, oedema, systemic dissemination and death, infection with *B. cereus* led to a transient passage of the micro-organism through the digestive tract. Although limited, this host-microbe interaction is enough to trigger a Th1 response that includes modification of the ratio of cell populations in relevant tissues, as well as to a diminution of the ability for antigen presentation. This shift in the immune status of the host following *B. cereus* infection could modify the response to other intestinal micro-organisms that concomitantly are found together with *B. cereus* in the intestine. It is worth noting that strain B10502 was unable, *in vitro*, to invade enterocytes (Minnaard *et al.*, 2004) and that invasive strains could lead to a very different outcome. Although other studies are necessary, our model of a *B. cereus* intestinal infection constitutes a promising approach for elucidating the mechanisms involved in the interaction of *B. cereus* with the host, as well as for gaining further insight on the differences between members of the *B. cereus* group.

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