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A SopB-mediated immune escape mechanism of *Salmonella enterica* can be subverted to optimize the performance of live attenuated vaccine carrier strains

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Abstract

Salmonellae have evolved several mechanisms to evade host clearance. Here, we describe the influence on bacterial immune escape of the effector protein SopB, which is translocated into the cytosol through a type III secretion system. Wild type bacteria, as well as the *sseC* and *aroA* attenuated mutants exerted a stronger cytotoxic effect on dendritic cells (DC) than their SopB-deficient derivatives. Cells infected with the double *sseC sopB*, *phoP sopB* and *aroA sopB* mutants also exhibited higher expression of MHC, CD80, CD86 and CD54 molecules, and showed a stronger capacity to process and present an I-E^d-restricted epitope from the influenza hemagglutinin (HA) to $CD4^+$ cells from TCR-HA transgenic mice *in vitro*. The incorporation of an additional mutation into the *sopB* locus of the attenuated *sseC*, *phoP* and *aroA* mutants resulted in the stimulation of improved humoral and cellular immune responses following oral vaccination. The obtained results define a new potential immune escape strategy of this important pathogen, and also demonstrate that this mechanism can be subverted to optimize the immune responses elicited using *Salmonella* as a live vaccine carrier.

Keywords: Antigen Presentation; Bacterial infection; Dendritic Cells; Vaccination

Abbreviations: APC, antigen presenting cells, DC, dendritic cells; HA, hemagglutinin; SPI, *Salmonella* pathogenicity island; TTSS, type III secretion system.

1. Introduction

Most pathogenic microorganisms are either restricted to the mucosal membranes or need to transit across the mucosal barrier during the early steps of infection. Thus, the elicitation of mucosal immune responses after vaccination is highly desired. A potential approach to achieve this aim is the use of attenuated *Salmonella* as carrier for heterologous antigens [1].

The translocation of virulence factors into target cells plays a central role in the pathogenesis process. This virulence trait is linked to the presence of type III secretion systems (TTSS). The *Salmonella* pathogenicity island 1 (SPI-1) encodes a TTSS required for bacterial invasion of epithelial cells; whereas the SPI-2 codes for a TTSS which is necessary for bacterial intracellular growth [2]. *Salmonella* carrying mutations in the SPI-2-encoded TTSS are highly attenuated in virulence, showing reduced rates of intracellular survival and replication [3]. Oral vaccination with *Salmonella* carriers with a mutation in *sseC*, an effector protein secreted by SPI-2, resulted in the stimulation of strong immune responses [4]. Secreted effector proteins might facilitate *Salmonella* evasion of host clearance mechanisms. For example, bacteria can avoid the immune system by surviving and replicating within macrophages and dendritic cells (DC) [5]. Long-term residence and replication within host cells appear to be essential for virulence and chronic infection. Previous studies indicated that *Salmonella* occupies an altered vacuolar compartment within professional antigen presenting cells (APC) [6]. Bacteria can also trigger rapid apoptosis in macrophages through the SPI-1-encoded SipB protein [7]. However, *Salmonella* can also promote SPI-1-independent delayed apoptosis in macrophages. This process seems to require a functional SPI-2 TTSS [8]. We hypothesize that *Salmonella*-mediated effects on APC may lead to impaired presentation of bacterial antigens, which in turn may affect microbial clearance during natural infections.

The SPI-5-encoded SopB protein is secreted and translocated into the cytosol through the SPI-1-encoded TTSS. SopB has an inositol phosphate phosphatase activity and affects cytoskeletal rearrangement, host cell invasion and chloride homeostasis [9,10]. Inactivation of *sopB* reduces inflammatory responses and fluid secretion into the intestinal lumen [11]. However, *sopB* mutants show the same tissue distribution and promote similar chemokine/cytokine profiles as wild type strains [12]. The introduction of a SopB mutation in attenuated strains may reduce their cytotoxic potential without affecting their performance as vaccine carriers.

Thus, in the present study we evaluated the capacity of attenuated *Salmonella enterica* Serovar Typhimurium strains (i.e. *sseC*, *phoP* and *aroA*) and derivatives carrying a second mutation in the *sopB* locus to affect the activation, maturation and antigen presentation capacities of bone marrow-derived murine DC. The results suggest that *Salmonella* spp. could also escape the host immune system by a SopB-dependent mechanism, leading to impaired antigen presentation by DC. Immunization studies also showed that this escape mechanism can be subverted to optimize the performance of live vaccines, since the introduction of an additional *sopB* mutation in different attenuated carrier strains resulted in the stimulation of improved immune responses.

2. Material and methods

2.1 *Construction of* Salmonella *derivatives*

Restriction and modification enzymes were purchased from New England Biolabs (Frankfurt, Germany). The *aroA* and *sseC* deletions were introduced by chromosomal gene replacement into the *Salmonella* strain ATCC 14028 using the suicide vector pCVD442 [13]. The 5'-flanking regions of *aroA* (1147 bp) and *sseC* (1678 bp) were PCR-amplified using the oligonucleotides 0787 and 0788, and 1406 and 1405 (Table 1), cut with *Sal*I and *Xba*I, and cloned into pCVD442. The 3'-flanking regions of *aroA* (640 bp) and *sseC* (1547 bp) were amplified using the oligonucleotides 0789 and 1021, and 0096 and 1407, cut with *Xba*I and *Sac*I, and cloned into pCVD442 attached to the 5'-flanking regions. Chromosomal deletions were introduced as previously described [13]. The mutation in *phoP* was created by generalized phage transduction using SL1344 *phoP*::Tn10d-tet (C. Hueck) as donor. The *sopB* insertion mutant was created by conjugation. A 198 bp *sopB* fragment was PCR-amplified using the oligonucleotides 1427 and 1428 and cloned into the *Kpn*I site of a pGP704 derivative in which the ampicillin resistance gene was replaced by a chloramphenicol resistance cassette (pKRG9). The pKRG9-sopB vector was then transformed into the *Escherichia coli* strain S17.1 [14] and subsequently transferred into the *Salmonella* recipient strains. For antigen presentation studies, bacteria were transformed with the pBlueScript-SK+/- derivative pLS879, which encodes amino acids 95-144 and 507-527 of the hemagglutinin (HA) from the influenza virus (A/pR/8/34, Cambridge strain) under the control of an *in vivo*-activated promoter. For immunization studies the mutant strains were transformed with the plasmid pLS1000, which codes for β-galactosidase as model antigen under the control of a *S. typhi* promotor that is activated upon infection [15].

2.2 *Experimental growth conditions*

For antigen presentation studies *Salmonella* grown in Brain heart infusion broth supplemented with ampicillin (100 μg/ml) and/or chloramphenicol (50 μg/ml) were pelleted (3000 x *g*) and resuspended in Dulbecco modified Eagle medium (DMEM, Gibco BRL, Karlsruhe) supplemented with 10% fetal calf serum (FCS, Greiner). For immunization studies bacteria transformed with pLS1000 were grown in Luria Bertani broth supplemented with ampicillin.

2.3 *Infection of DC*

Bone marrow-derived DC from BALB/c mice (Harlan Winkelmann, Borchen) were prepared as previously described [16], resuspended in DMEM with GM-CSF ($5x10^4$ U/ml) and seeded in 6-well plates ($5x10^6$ cells/well). Bacteria were added at a multiplicity of infection 10:1 and plates were incubated for 2 h. After washing, cells were incubated with gentamycin (100 μg/ml; Sigma) for 22 h and further analyzed by flow cytometry or used as APC.

2.4 *Flow cytometric analysis*

Staining of DC was performed as previously described [16], using FITC-labeled monoclonal antibodies against MHC-I (SF1-1.1), MHC-II (AMS-32.1), CD80 (16-10A1), CD86 (GL1), CD40 (3/23) and CD54 (3E2), and PE-labeled anti-CD11c (HL3) from BD Pharmingen. Apoptotic and necrotic cells were stained using Alexa Fluor 488-conjugated annexin V (MoBiTec) and 7- Amino-Actinomycin D (BD Pharmingen), respectively. FACS analysis was performed using a FACSort and the CellQuest software (Becton Dickinson, San Jose, CA) with gating on CD11c positive cells (acquisition of 50,000 events).

2.5 *Antigen presentation tests*

Spleens from the TCR-HA transgenic mice, which express T cell receptors (TCR) specific for the 111-119 peptide from the influenza HA in the context of I-E^d [17], were used as source for T cells. Infected DC were added in graded doses to T cells ($2x10^5$ cells/well) in quadruplicates, in a final volume of 200 µl of RPMI1640 (10% FCS, 2 mM L-glutamine and 50 µM ß-mercaptoethanol). Proliferation of T cells was measured by [^{3}H]-thymidine uptake [16].

2.6 *Immunization studies*

Female BALB/c mice (n=5) were orally immunized on day 1, 14 and 28 with $1x10^9$ colony forming units of *Salmonella*. Animals were sacrificed on day 38, and sera and intestinal lavages were tested for ß-galactosidase- or lipopolysaccharide-specific antibodies by ELISA [18]. $CD8^+$ T cells enriched by magnetic depletion using anti-mouse CD4 antibodies (RM4.4, BD Pharmingen) and Dynabeads (Dynal, Oslo) were incubated in triplicates ($1x10^5$ cells /well) with a peptide (5 µg/µl) corresponding to the MHCI-restricted (L^d) epitope (TPHPARIGL) from the ß-galactosidase and the number of antigen-specific IFNγ-secreting cells was determined by ELISPOT (Becton Dickinson).

2.7 *Statistical analysis*

The statistic significance of the observed differences was evaluated by the X^2- or the Student's t-test (SigmaStat 3.1; Systat). Differences were considered significant at $P < 0.05$.

3. Results

3.1 Salmonella sopB *derivatives exhibit an impaired cytotoxic effect on infected DC*

To evaluate the effect of *S. enterica* derivatives on DC viability, cells infected with *Salmonella* for 22 h were analyzed by flow cytometry after staining with Alexa Flour 488-conjugated annexin V and 7- Amino-actinomycin D gating on $CD11c^+$ cells. Non-infected DC showed 67.5% of viable cells, 23.7% of apoptotic cells and only 8.9% of necrotic cells (Table 2). After infection with wild type *Salmonella*, the percentage of apoptotic cells was similar (23.9%). However, the proportion of viable and necrotic cells was reduced (1.2%) and increased (74.9%), respectively. Similar results were observed after infection with either the *sseC* mutant strain (1.6, 22.9 and 75.5% of viable, apoptotic and necrotic cells, respectively) or the *aroA* derivative (2.0, 23.6 and 74.4%). In contrast, an increased percentage of viable and apoptotic cells ($P < 0.05$) was observed after infection with the *sseC sopB* (4.7 and 37.7%, respectively) or the *aroA sopB* double mutant (9.3 and 37.1%).

3.2 *DC infected with the* sopB *mutant showed improved expression of activation markers*

To evaluate the influence of *Salmonella* infection on DC maturation and activation, immature cells were infected with the bacterial strains and further analyzed by flow cytometry. In comparison to control cells, DC infected with wild type *Salmonella* showed an up-regulated expression of adhesion (*e.g.*, CD40), co-stimulatory (*e.g.*, CD80, CD86), and MHC-II molecules (Fig. 1 and Table 3). Similar results were observed when attenuated *sseC* and *phoP* mutants were used (Fig. 1 and Table 3). Interestingly, cells infected with the double mutants *sseC sopB* or *phoP sopB* exhibited a significantly ($P < 0.05$) higher expression of CD54, CD80, CD86 and MHC-I molecules than DC infected with the *sseC* and *phoP* single

mutants. Similar results were observed when DC were infected with the *aroA* and *aroA sopB* derivatives (data not shown).

3.3 *DC infected with* Salmonella sopB *mutants exhibited an improved capacity to present antigens to T cells*

Our data demonstrated that an additional mutation in the *sopB* locus does not only result in a reduced bacterial cytotoxicity on DC, but also in an improved expression of activation markers which are critical for their antigen presentation functions. Thus, we investigated the influence of the *sopB* mutation on the *in vitro* capacity of infected DC to present antigens to specific T cells. DC were infected with *Salmonella* carrying a plasmid coding for a polypeptide encompassing amino acids 95-144 and 507-527 of the HA from the influenza virus. Then, infected DC were co-cultured with T cells from the TCR-HA transgenic mice, which have a TCR specific for the HA peptide 111-119.

DC infected with the *Salmonella sseC* derivative stimulated the proliferation of antigen-specific T cells, yielding about 4-fold higher [^{3}H]-thymidine incorporation values to those observed using non-infected DC (Fig. 2, A). When DC were infected with the *sseC sopB* double mutant, a significant increase in T cell activation was observed with respect to that obtained using as APC DC infected with the *sseC* mutant ($P< 0.05$). Similar results were obtained using DC infected with the *phoP* and the *phoP sopB* mutants (Fig. 2, B). These results demonstrate that antigens delivered by SopB deficient *Salmonella* were more efficiently processed and presented to antigen-specific T cells by infected DC *in vitro*.

3.4 *Attenuated* Salmonella *strains carrying an additional mutation in the* sopB *locus stimulate more efficient immune responses after oral vaccination*

DC infected with the *sseC sopB* mutant exhibited enhanced antigen presentation functions, thus we evaluated the capacity of this strain as vaccine carrier. Mice were orally immunized with $1x10^9$ colony forming units of *Salmonella* strains mutated either in the *sseC* or the *sseC sopB* loci expressing the model antigen β-galactosidase. Due to its toxicity, immunization using the single mutant *sopB* was omitted. Sera from animals vaccinated with the *sseC sopB* mutant showed anti-lipopolysaccharide and anti-β-galactosidase IgG responses similar to those observed in mice receiving the *sseC* mutant (not shown). However, incorporation of the *sopB* mutation resulted in the stimulation of stronger secretory IgA responses (Fig. 3, A). Interestingly, when the subclass distribution of β-galactosidase-specific serum IgG was analyzed, it was clear that immunization with the *sseC sopB* mutant led to a dominant IgG1 response (*i.e.*, Th2-biased), whereas vaccination with the *sseC*-deficient strain yielded an IgG2a-dominated pattern (Th1-biased, Fig. 3, B) [19]. This is in agreement with the improved secretory IgA responses detected in these animals.

We also evaluated the capacity of $CD8^+$ T cells to produce IFNγ in response to a peptide encompassing a MHC class I-restricted immunodominant epitope of β-galactosidase. Approximately 7.5-fold more IFNγ-secreting cells were detected in the animals vaccinated with the double mutant *sseC sopB* than in mice immunized with *sseC* (Fig. 3, C). Spleen cells recovered from animals after immunization with the *sseC sopB* mutant strain also produced more IL12 than cells from mice vaccinated with the *sseC* mutant after *in vitro* stimulation (117 versus 13 pg/ml).

To analyze the influence on immune responses resulting from the incorporation of an additional mutation in the *sopB* locus on other attenuated *Salmonella* strains, animals were vaccinated with the *aroA* and *aroA sopB* or *phoP* and *phoP sopB* derivatives of ATCC 14028. The obtained results demonstrated that the incorporation of the *sopB* mutation

resulted in the elicitation of 3.3- and 2.4-fold stronger antibody responses against β-galactosidase, respectively (Fig. 3, D).

4. Discussion

Attenuated *Salmonella* strains have been widely used as vaccine carriers for heterologous antigens. After oral administration, bacteria invade the intestinal mucosa, preferentially through the M cells. Then, they reach the sub-epithelial dome of Peyer's patches, confronting the extensive network of resident macrophages and DC. However, *Salmonella* seem to be mainly taken up by $CD18^{+}$ phagocytes (including DC) in a M cell-independent pathway, which allows the transit across the intestinal epithelium of even non-invasive *Salmonella*. In this context, DC located in the lamina propria open tight junctions between epithelial cells, sending dendrites into the gut lumen to sample bacteria [20]. In contrast to the M cell-dependent invasion, this alternative pathway does not require the virulence proteins that are translocated by the SPI-1 TTSS.

Because of the critical involvement of DC in *Salmonella* uptake and dissemination, it is essential to elucidate if wild type and attenuated *Salmonella* strains can affect their biological functions. On the one hand, APC may offer bacteria a potential niche in which they can survive and replicate. On the other hand, *Salmonella* can also trigger cell death in macrophages and DC, thereby affecting the antigen presentation process [6,21]. Therefore, these two mechanisms might contribute to the immune escape of this pathogen. Rapid activation of programmed cell death in macrophages depends on the SPI-1-encoded TTSS, which is active in *Salmonella* displaying an invasive phenotype [6,7]. However, macrophages can be also killed in a delayed SPI-1-independent manner, when *Salmonella* express the SPI-2-encoded TTSS [8].

Our studies demonstrated that *Salmonella* defective in the synthesis of the SPI-2-encoded effector protein SseC promote apoptosis in infected DC to a similar extent to the wild type parental bacteria. This is particularly clear when *Salmonella* were grown until they

reach the exponential growth phase, in which the SPI-1-encoded TTSS is mainly active. On the other hand, DC infected with a *Salmonella sseC* mutant carrying an additional mutation in the SPI-1-translocated effector protein SopB showed both increased membrane integrity and viability. These results can be explained, at least in part, by differences in the infective capacity of the *Salmonella* mutants. In accordance to previous studies we observed a similar invasiveness in DC for wild type *Salmonella* and its attenuated derivatives [22,23]. Concerning the *sopB* mutant, there are contrasting data in the literature, which suggest a non affected invasiveness in DC [24,25] and impaired survival in macrophages [26]. In our hands, a significantly reduced number of viable intracellular bacteria was observed when DC were infected with the double mutants (data not shown).

The effector protein SopB is delivered into the cytosol, where it acts as an inositol phosphatase. It was shown that this protein affects different processes, such as the rearrangement of cytoskeletal proteins, host cell invasion and chloride homeostasis [9,10]. SopB is associated with the host cell membrane via hydrophobic interactions and is required for the elimination of phosphatidylinositol-4,5-biphosphate from invaginating host cell membranes [27]. The formation and fusion of *Salmonella*-containing vacuoles is also influenced by SopB [26]. Due to its broad spectrum of substrate specificities, the identity of the *in vivo* relevant substrates and the exact molecular functions of SopB in the pathogenesis process are still a matter of discussion. However, our results suggest that a deficient production of SopB results in reduced bacterial survival within DC and increased viability and functionality of APC. This hypothesis is further supported by the findings of Knodler et al., who observed an anti-apoptotic activity of SopB in *Salmonella*-infected epithelial cells [25]. This can explain, at least in part, the observed improvement in the T cell-activation capacity of DC infected with the double mutants.

DC play a key role during the induction of adaptive immune responses, since they constitute the bridge between innate and adaptive immunity. After encountering invading microbes in peripheral tissues, immature DC capture antigens and migrate into the T cell-dependent areas of the secondary lymphoid tissues. During migration, they undergo a maturation process, which is characterized by morphological and functional changes. Only mature DC have high surface expression of MHC and co-stimulatory molecules, which in turn are essential for efficient T cell activation. An up-regulated expression of activation markers was already observed on DC after infection with *Salmonella* grown to the stationary phase [28,29]. In this study, we demonstrated an enhanced expression of these molecules on DC infected with exponentially growing wild type bacteria. These conditions seem to mimic more closely the situation of rapid replication occurring during systemic dissemination. Similar results were obtained using attenuated *Salmonella* carrying a mutation in the gene *aroA*, which is involved in the biosynthesis of aromatic compounds, as well as bacteria defective in the production of the SPI-2-encoded effector protein SseC or the PhoP/Q two-component regulatory system. When DC were infected with *aroA*-, *sseC*- and *phoP*-derivatives carrying an additional mutation on *sopB*, we observed a significant increment in the expression levels of adhesion, (i.e. CD54), co-stimulatory (i.e. CD80, CD86) and MHC class I molecules.

These results suggest that the presence of a *sopB* mutation in attenuated bacteria results not only in an improved viability of infected DC, but also in their increased expression of surface molecules that are critical for antigen presentation. Thus, we evaluated the influence of the *sopB* mutation on the antigen presenting capacity of infected DC. To this end, antigen presentation studies were performed using *Salmonella* expressing a polypeptide encompassing epitopes recognized by T cells of HA-transgenic mice. DC infected with *Salmonella* carrying a mutation on the *sseC* or *phoP* genes were able to stimulate an antigen-

specific proliferation of T cells. However, when cells were infected with *Salmonella* derivatives carrying an additional mutation in the *sopB* locus, a significantly enhanced activation of T cells was observed. Thus, the incorporation of a mutation in the *sopB* locus improves the capacity of infected DC to process a heterologous antigen and present it to T cells. Studies from Cheminay et al. suggested that DC infected with *Salmonella* can suppress antigen-dependent T-cell proliferation by a SPI-2-dependent mechanism [30]. However, in these *in vitro* studies exogenous antigen was added to infected DC. This scenario is clearly different from our studies in which the antigen expression was performed *in vivo* by *Salmonella* itself, thereby mimicking the conditions occurring during infection or vaccination. However, it seems clear that several bacterial factors might affect DC viability and function.

The improved activation, maturation and antigen presentation capacities of DC infected with the *sopB* derivatives prompted us to evaluate if the *sopB* mutation can be incorporated into attenuated carrier strains to increase their performance. Previous studies showed that a *Salmonella* vaccine carrier strain with a mutation in the *sseC* gene promotes strong humoral and cellular immune responses against the model antigen β-galactosidase after oral immunization [4]. Thus, we compared the immune responses stimulated using a *Salmonella sseC* mutant as carrier, with those obtained using the double mutant *sseC sopB*. In agreement with the *in vitro* antigen presentation studies, the incorporation of the *sopB* mutation led to the elicitation of significantly stronger humoral and cellular immune responses (Fig. 3). Similar results were also obtained when the *sopB* mutation was combined with mutations in the *phoP* and *aroA* genes, respectively. This suggests that the observed effect is not restricted to a single type of attenuated mutant.

$CD4^+$ T cells can differentiate into two distinct types of effector cells, Th1 cells that regulate cell-mediated immunity and Th2 cells, which regulate humoral immune responses

[19]. While Th2-type responses are mainly protective against certain parasites, Th1-type responses enhance the microbicidal activity of macrophages, thereby facilitating the clearance of intracellular pathogens. Interestingly, the analysis of the IgG subclasses showed that mainly IgG1 was stimulated by the double mutant *sseC sopB* (i.e. Th2 dominant pattern), whereas the *sseC* deficient *Salmonella* promoted a Th1-like response. Thus, it seems that the incorporation of additional mutations can also be exploited to modulate the quality of the immune responses elicited using live carriers. Although additional work will be required to elucidate the underlying mechanisms.

In conclusion, the obtained results suggest that *Salmonella* can also escape the host immune system by a SopB-mediated mechanism, in which the antigen presentation capacities of DC are impaired. These results not only define a new potential immune escape mechanism of this important pathogen, but also demonstrate that this mechanism can be subverted to optimize the immune responses elicited using live attenuated *Salmonella* as vaccine carriers.

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Figure legends

Fig. 1. Flow cytometric analysis of DC after infection with *S. enterica* Serovar Typhimurium. Bone marrow-derived DC were infected with either the wild type *Salmonella* strain ATCC 14028 or its isogenic *sseC* and *sseC sopB* derivatives at a bacteria:cell ratio of 10:1 for 2 h. After 22 h of incubation, DC were analyzed by two-color flow cytometry. Results represent the expression of different surface markers on 50,000 viable $CD11c^+$-gated cells treated with medium alone (shaded area) or after infection with the wild type strain (dotted line) or its *sseC* (thin solid line) and *sseC sopB* (solid thick line) derivatives. Results correspond to one representative experiment out of five independent tests.

Fig. 2. Stimulation of antigen-specific T cells co-cultivated with DC after infection with *Salmonella* carrying a plasmid coding for a polyepitope of the influenza virus HA. DC were infected with the *sseC*, *sseC sopB*, *phoP*, and *phoP sopB* derivatives of the *S. enterica* ATCC 14028 strain. Cells were then co-incubated for 3 days at different ratios with T cells obtained from HA TCR transgenic mice (see Material and methods section). Proliferation was determined by measuring the up-take of radioactive [^{3}H]-thymidine. Results represent average of quadruplicates and they are expressed as cpm from T cells co-cultured with DC with subtracted background (T cells cultured alone). One representative experiment out of two independent tests is shown. The results obtained using DC infected with the *sseC sopB* or *phoP sopB* mutant were significantly different from those generated using cells infected with the ss*eC* or *phoP* derivative at $P < 0.05$ (*).

Fig. 3. Immune responses elicited in vaccinated animals. (A) β-galactosidase specific sIgA responses were measured in intestinal lavages of BALB/c mice (n=5) immunized with *S.*

enterica ATCC 14028 derivatives carrying mutations on either the *sseC* or the *sseC sopB* loci by ELISA. The values obtained in animals receiving the single mutant were arbitrarily considered as 100%. (B) The presence of anti-β-galactosidase IgG isotypes was determined in sera of immunized animals by ELISA. Results are expressed as IgG1:IgG2a ratios. (C) $CD8^+$-enriched splenocytes ($1x10^5$/well) of mice immunized with the *Salmonella* derivatives carrying mutations on either the *sseC* or the *sseC sopB* loci were incubated for 16 h in the presence or absence of a peptide encompassing a MHC-I-restricted epitope from β-galactosidase. The number of INFγ-secreting cells was determined by ELISPOT. Values obtained in animals receiving the single mutant were arbitrarily considered as 100%. (D) β-galactosidase-specific IgG responses in sera of BALB/c mice (n=5) immunized with *S. enterica* ATCC 14028 derivatives carrying mutations on either the *aroA* and *aroA sopB* or the *phoP and phoP sopB* loci were determined by ELISA. The values obtained in animals receiving the single mutant were arbitrarily considered as 100%. Results correspond to one representative experiment out of two independent tests.

Table 1. ***Oligonucleotides used for PCR amplification***

Name	Sequence (5'-3')	Application/characteristics
1406	GCTGTCGACTTGTAGTGAGTGAGCAAG	Cloning of the *sseC* 5' flanking region (5' primer), contains a *Sal*I site
1405	GGATCTAGATTTTAGCTCCTGTCAGAAAG	Cloning of the *sseC* 5' flanking region (3'primer), contains a *Xba*I site
0096	GGATCTAGATCTGAGGATAAAAATATGG	Cloning of the *sseC* 3' flanking region (5' primer), contains a *Xba*I site
1407	GCTGAGCTCTGCCGCTGACGGAATATG	Cloning of the *sseC* 3' flanking region 3' primer), contains a *Sac*I site
0787	GCTGTCGACATGCAGCGTGGTGAGGGG	Cloning of the *aroA* 5' flanking region (5' primer), contains a *Sal*I site
0788	GGATCTAGAAACCCCACAGACTGGCCGTGG	Cloning of the *aroA* 5' flanking region (3' primer), contains a *Xba*I site
0789	GGATCTAGAGCCAGTTACGATCCTGG	Cloning of the *aroA* 3' flanking region (5' primer), contains a *Xba*I site
1021	GCTGAGCTCCATGGCCCATTTCATGGC	Cloning of the *aroA* 3' flanking region (3' primer), contains a *Sac*I site
1427	GGGGTACCTACAGAGCTTCTATCACTCAGC	Amplification of a s*opB* fragment (5' primer), contains a *Kpn*I site
1428	GGGGTACCTTCTGATGCTGTAGATAATTCC	Amplification of a s*opB* fragment, (3' primer), contains a *Kpn*I site

Table 2. ***DC infection by wild type and attenuated*** **Salmonella**

Cell phenotype	Control*	Wild type	*sseC*	*sseC sopB*	*aroA*	*aroA sopB*
Viable	67.5	1.2	1.6	4.7	2.0	9.3
Apoptotic	23.7	23.9	22.9	57.6	23.6	53.6
Necrotic	8.8	74.9	75.5	37.7	74.4	37.1

(*) Results are expressed as percentages of the total number of gated $CD11c^+$cells (i.e., 50,000 events). Results correspond to one representative experiment out of two independent tests.

Table 3. ***Surface markers on DC infected with wild type and attenuated Salmonella***

Surface marker	Control		wt		*sseC*		*sseC sopB*		*phoP*		*phoP sopB*	
	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
CD40	20.2	9.9	53.9	22.6	38.1	16.6	47.2	19.9	45.2	14.9	59.5	20.5 **
CD54	32.4	62.3	31.6	64.6	37.7	67.9	48.7	82.1 *	36.6	64.0	50.6	84.5 *
CD80	15.9	55.4	45.6	112.7	38.0	101.4	62.6	197.0 *	41.8	106.8	52.5	131.7 *
CD86	19.5	23.3	45.4	46.4	47.2	38.1	65.6	52.4 *	33.9	38.4	46.8	53.2 *
MHCI	55.1	79.7	47.8	73.5	56.7	89.9	84.2	158.6 *	46.3	92.1	61.7	119.2 *
MHCII	55.3	147.3	69.3	249.8	68.5	250.1	68.7	299.1	70.7	265.1	68.9	300.41

Results are expressed as percentages of the total number (i.e., 50,000) of viable gated $CD11c^+$cells (%) and as geometric mean fluorescence intensity (MFI). The results obtained from $CD11c^+$ cells infected with the *Salmonella* mutant with an additional mutation in *sopB* were different from those of cells infected with the corresponding single mutant at $P<0.01$ (*) or $P<0.05$ (**).Results correspond to one representative experiment out of five independent tests.

Fig.1

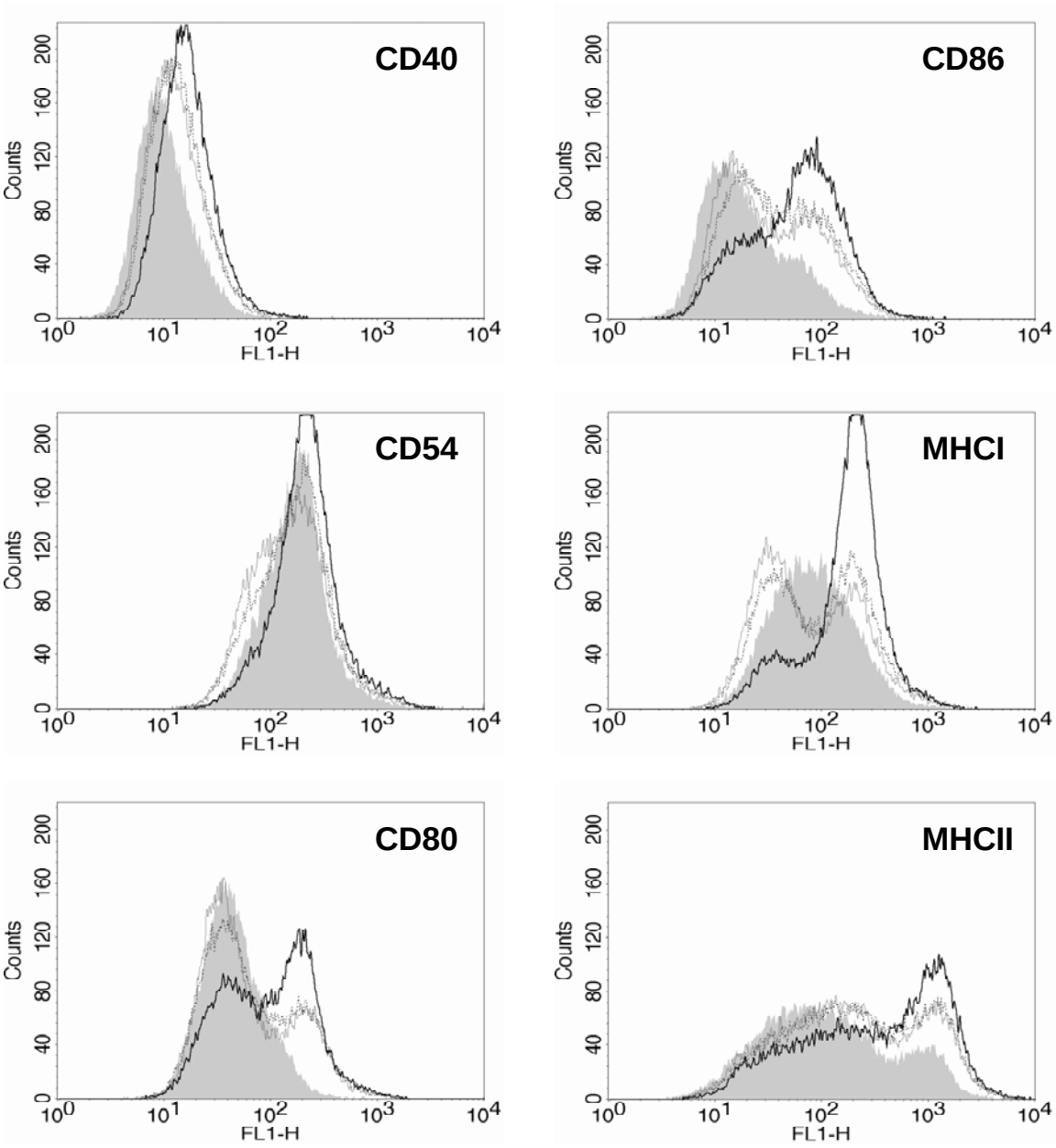
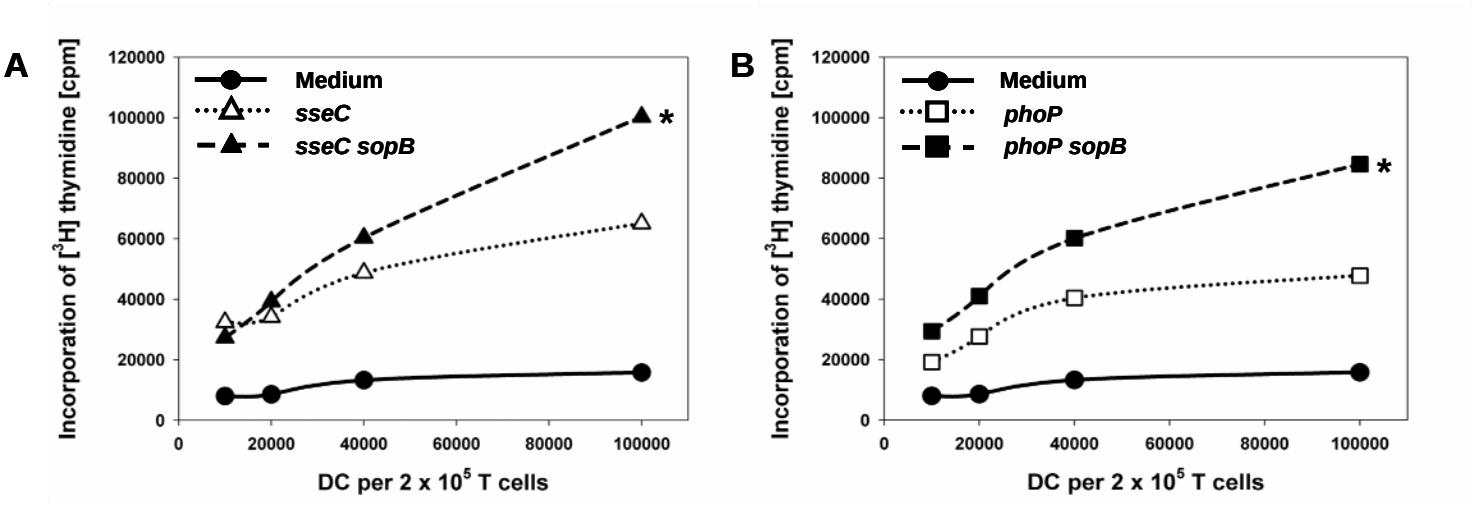


Fig. 2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Incorporation of [3H] thymidine [cpm]
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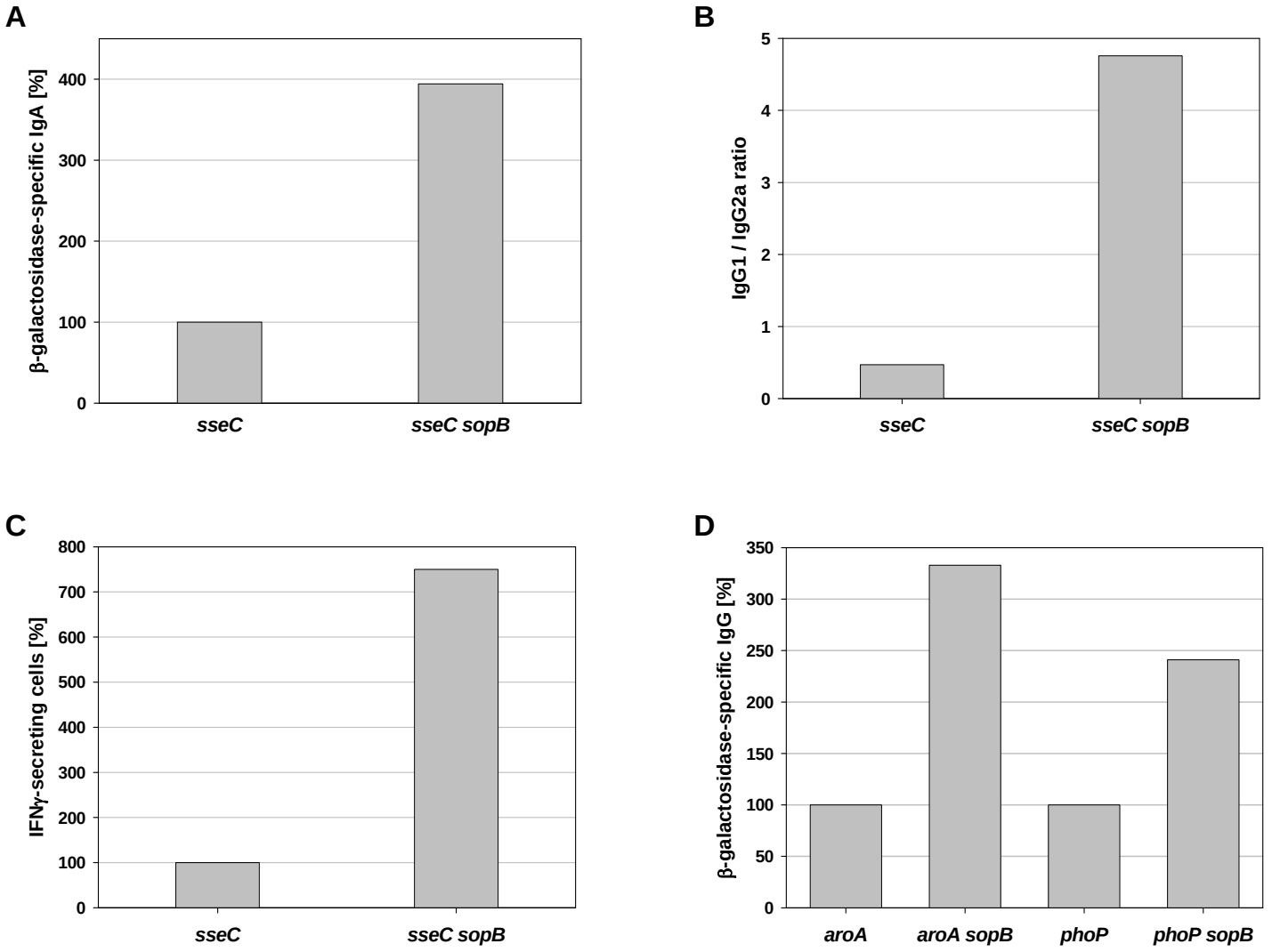
100000
80000
60000
40000
20000
0
0
20000
40000
60000
80000
100000
DC per 2 x 10^5 T cells
Medium
sseC
sseC sopB
*
B
Incorporation of [3H] thymidine [cpm]
120000
100000
80000
60000
40000
20000
0
0
20000
40000
60000
80000
100000
DC per 2 x 10^5 T cells
Medium
phoP
phoP sopB
*

Figure 3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β-galactosidase-specific IgA [%]
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100
200
300
400
sseC
sseC sopB
B
IgG1 / IgG2a ratio
0
1
2
3
4
5
sseC
sseC sopB
C
IFNγ-secreting cells [%]
0
100
200
300
400
500
600
700
800
sseC
sseC sopB
D
β-galactosidase-specific IgG [%]
0
50
100
150
200
250
300
350
aroA
aroA sopB
phoP
phoP sopB