**Salmonella Typhimurium interferes with the humoral immune response in pigs**


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**Abstract**

Foodborne salmonellosis is one of the most important bacterial zoonotic diseases worldwide. *Salmonella Typhimurium* is the serovar most frequently isolated from slaughter pigs in Europe. Circumvention of the host’s immune system by *Salmonella* might contribute to persistent infection of pigs. We found that *Salmonella Typhimurium* strain 112910a, which is able to persist in pigs, was capable of downregulating the expression of major histocompatibility class II (MHC II) molecules on porcine alveolar macrophages (PAM) in a *Salmonella* pathogenicity island 2 (SPI-2) dependent way and that MHC II downregulation was *Salmonella* strain dependent. The MHC II downregulation capacity was abolished when bacteria were opsonized with *Salmonella*-specific antibodies. Furthermore, intracellular proliferation of *Salmonella Typhimurium* opsonized with *Salmonella* positive pig serum was significantly impaired compared to that of the bacteria opsonized with negative pig serum. In a subsequent *in vivo* experiment, *Salmonella Typhimurium* strain MB2216 that did not induce MHC II downregulation *in vitro*, was shed less and persisted less but induced earlier seroconversion in pigs than strain 112910a. From the *in vitro* data, it is proposed that *Salmonella Typhimurium* downregulates the humoral immune response to promote intracellular survival inside porcine macrophages, contributing to long-term *Salmonella* persistence in pigs. The fact that the less persistent strain MB2216 induced earlier seroconversion than strain 112910a is of major interest for *Salmonella*-monitoring programs primarily based on serology, since this indicates that more persisting strains are more likely to escape serological detection.

**Introduction**

In European countries, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella Typhimurium*) is the serovar most frequently isolated from slaughter pigs (Anon., 2008). In most cases, the bacterium will asymptomatically colonize pigs, resulting in a so called ‘carrier status’ (Wood et al., 1989). In the past, *Salmonella* infections in pig herds have traditionally been diagnosed by culturing intestinal or faecal samples. LPS-based serological surveillance is perceived as a practical and cost-effective alternative for monitoring *Salmonella* infection in pig herds (Proux et al., 2000) and is currently widely applied. The success of many persisting pathogens relies on their ability to interfere with the host’s immune responses, for example by interfering with major histocompatibility complex (MHC) molecule expression and antigen presentation. SPI-2 plays a role in *Salmonella*-mediated downregulation of MHC II in human Mel JuSo cells (Mitchell et al., 2004). No data are available on this phenomenon in porcine cells.

In the present study, we hypothesized that *Salmonella Typhimurium* strain 112910a induced *Salmonella* pathogenicity island 2 (SPI-2) mediated downregulation of MHC II expression on porcine macrophages as a possible mechanism to circumvent antibody production by the pig’s immune system. We then examined the role of antibodies in intracellular survival and proliferation of *Salmonella* in macrophages and in the bacterium’s ability to interfere with the MHC II presentation pathway, and if this MHC II downregulating capacity was *Salmonella* strain dependent. Finally, we examined whether the *Salmonella* induced MHC II downregulation in porcine macrophages coincides with *Salmonella* persistence in pigs.
Material and Methods
Bacterial strains and manipulations
Salmonella Typhimurium strain 112910a, a pig stool isolate (Boyen et al., 2009), and its isogenic deletion mutants ΔsseA and ΔssrA/B, constructed as described by Datsenko and Wanner (2000), were used. Other strains used in this study are Salmonella Typhimurium pig isolates MB2150, MB2216, MB2222, MB2223, MB2233 and MB2498 and the pigeon isolate DAB69, Salmonella serovars Brandenburg, Derby and Infantis, all isolated from pigs and a chicken isolate Salmonella serovar Enteritidis 76Sa88. For flow cytometric analysis, strains were transformed with a plasmid expressing green fluorescent protein (GFP; Valvidia and Falkow, 1996).

Pig antisera against Salmonella Typhimurium
Porcine serum containing Salmonella-specific antibodies was raised by injecting pigs twice intramuscularly with a bacterin consisting of formalin-inactivated Salmonella Typhimurium strain 112910a suspended in phosphate buffered saline (PBS) and Freund’s incomplete adjuvant (positive serum). Negative serum was collected from pigs, injected twice with an emulsion of Freund’s incomplete adjuvant and PBS.

The effect of Salmonella Typhimurium on MHC expression on the surface of porcine macrophages
Porcine alveolar macrophages (PAM) were isolated and incubated with GFP-transformed Salmonella strains and serovars, as described by Boyen et al. (2006). Macrophages were incubated with a primary MHC class I or class II antibody to detect MHC I or II expression, respectively, and then incubated with a secondary Alexa Fluor 633 antibody. MHC expression of uninfected and infected PAM was measured using a FACScanto™ II cytometer and analysed with FACS Diva software.

Impact of opsonization with antibodies on intracellular survival of Salmonella Typhimurium in porcine macrophages
Intracellular survival of bacteria that were either non-opsonized or opsonized with positive or negative pig serum, was assessed a gentamicin protection assay (Boyen et al., 2006).

Comparison of the in vivo behaviour of Salmonella Typhimurium strains 112910a and MB2216 after experimental inoculation of piglets
Salmonella-free piglets were orally inoculated with Salmonella Typhimurium strain 112910a (n=19), inducing MHC II downregulation in vitro, or strain MB2216 (n=9), not inducing MHC II downregulation in vitro, respectively. Faeces were collected at different days post inoculation (pi) and bacteriologically analyzed. Six days before inoculation and 11, 18, 26, 33 and 40 days pi, blood was collected and serum was isolated. Serum was analysed using an LPS-based Elisa (IDEXX Labs), and an in-house whole-cell ELISA (Leyman et al., 2011). Forty days pi, all pigs were euthanized and tonsils, ileocecal lymph nodes, ileum, ileum contents, caecum, caecum contents, colon and colon contents were collected and bacteriologically analysed (Van Parys et al., 2010).

Results
Infection with Salmonella Typhimurium strain 112910a did not result in a decreased MHC I expression level on PAM. In contrast, the MHC II expression level on strain 112910a infected PAM was significantly decreased (Figure 1A). MHC II expression levels between PAM inoculated with strain 112910a, ΔsseA or ΔssrA/B did not significantly differ from each other, nor from the respective ratios directly after inoculation, suggesting that MHC II expression was partly restored when SPI-2 was abolished (Figure 1A). Among the 7 different Salmonella Typhimurium isolates tested, strain MB2216 did not induce MHC II downregulation on PAM, while the other strains exhibited downregulation of MHC II expression similar to strain 112910a. Furthermore, Salmonella Typhimurium strain DAB69 and the Salmonella Derby and Infantis isolates showed no MHC II downregulation, in contrast to serovar Brandenburg and Enteritidis strains (Figure 1B). PAM inoculated with Salmonella Typhimurium opsonized with negative pig serum showed a decrease in MHC II expression level, while the MHC II expression on PAM inoculated with Salmonella Typhimurium opsonized with positive pig serum remained unaffected. Furthermore, Salmonella was able to significantly proliferate intracellularly in PAM when they were not opsonized or when they
were opsonized with negative pig serum, while bacteria that were opsonized with positive pig serum proliferated less.

![Diagram](image_url)

**Figure 1. The capacity of Salmonella enterica to downregulate MHC expression on the surface of PAM.** The average median FL-5 signal ratios between uninfected and infected porcine alveolar macrophages (PAM\textsuperscript{uninf} and PAM\textsuperscript{inf}), respectively, are presented with standard deviation, immediately (0 h) and 24 h post inoculation (pi) with *Salmonella* Typhimurium strain 112910a. The values were calculated from the ratios obtained from 3 independent experiments. **A.** Average median PAM\textsuperscript{uninf}/PAM\textsuperscript{inf} ratios 0 and 24 h post inoculation with *Salmonella* Typhimurium strain 112910a or its isogenic deletion mutants ΔsseA or ΔssrA. An asterisk refers to a significantly different average median fluorescence ratio between 0 and 24 h pi (p ≤ 0.05). 112910aNEG: *Salmonella* Typhimurium strain 112910 opsonized with negative pig serum; 112910aPOS: *Salmonella* Typhimurium strain 112910 opsonized with pig serum containing *Salmonella* Typhimurium-specific antibodies. **B.** Average median PAM\textsuperscript{uninf}/PAM\textsuperscript{inf} ratios 0 and 24 h post inoculation with different *Salmonella* Typhimurium strains and different *Salmonella* serovars. An asterisk (*) refers to a statistically significant higher ratio at 24h pi than 0h pi (p ≤ 0.05).

In a subsequent *in vivo* experiment, seroconversion and *Salmonella* faecal shedding and persistence were compared between pigs inoculated with either strain 112910a or MB2216, respectively inducing and not inducing MHC II downregulation, as assessed earlier in our study. The number of times that pigs tested positive for *Salmonella* in the faeces was significantly lower for strain MB2216 than for strain 112910a. Using the LPS-based ELISA, the proportion of positive piglets at 33 days pi was significantly higher in the MB2216 group. Using the whole-cell ELISA, the average antibody titre in the MB2216 group was higher than in the 112910a group at 33 and 40 days pi. At euthanasia, strain MB2216 was isolated in lower numbers from the tonsils than strain 112910a. The proportion of *Salmonella* positive lymph nodes was higher in the 112910a than in the MB2216 group.

**Discussion**

In this study, we found that *Salmonella* Typhimurium strain 112910a specifically interferes with the MHC II presentation pathway in porcine macrophages, leaving the MHC I pathway undisturbed. The fact that MHC II downregulation in ΔsseA and ΔssrA/B infected PAM was partly restored, suggests a role for SPI-2 in downregulation of MHC II expression (Mitchell et al., 2004). The absence of MHC II downregulation and the decrease in survival in macrophages after opsonization of *Salmonella* might emphasize the importance for the bacterium to postpone seroconversion in pigs for successful persistence. The extent of MHC II downregulation differed considerably among various *Salmonella* Typhimurium strains and *Salmonella* serovars, suggesting that the MHC II downregulation capacity might have evolved independently.

Oral inoculation of pigs demonstrated that *Salmonella* strain MB2216, which did not downregulate MHC II expression *in vitro*, induced earlier seroconversion than strain 112910a which did suppress MHC II expression. Tonsils and lymph nodes play a role in the host’s immune response towards bacterial infections (Horter et al., 2003) and both organs are predominantly colonized by *Salmonella* Typhimurium (Wood et al., 1989). Since MB2216 was less able to persist in these organs in pigs, the earlier onset of *Salmonella*-specific antibody production may have led to a more efficient reduction of *Salmonella*, resulting in decreased persistence compared to the 112910a infected pigs. However, extrapolation of *in vitro* MHC II downregulation capacity to the observed *in vivo* results must be done with care.
Conclusion
We found that Salmonella Typhimurium strain 112910a was able to downregulate MHC II expression on PAM in a SPI-2 dependent way and that the MHC II downregulation capacity was strain dependent. We furthermore showed a correlation between early onset of seroconversion, reduced faecal shedding and reduced persistence capacity and vice versa, in an infection experiment with 2 different Salmonella Typhimurium strains. Circumvention of the pig’s antibody response might therefore attribute to long-term Salmonella persistence in pigs.

References