Delivery of a heterologous antigen by a registered *Salmonella* vaccine (STM1)

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STM1 is an *aro A* attenuated mutant of *Salmonella enterica* serovar Typhimurium, and is a well-characterised vaccine strain available to the livestock industry for the prevention of salmonellosis in chickens. This strain has potential for heterologous antigen delivery, and here we show that the strain can be used to deliver a model antigen, ovalbumin, to immune cells *in vitro* and *in vivo*. Two plasmid constructs expressing the ovalbumin gene were utilized, one of which uses a prokaryotic promoter and the other the CMV promoter (DNA vaccine). *In vitro*, STM1 carrying ovalbumin-encoding plasmids was able to invade dendritic cells and stimulate a CD8+ cell line specific for the dominant ovalbumin epitope, SIINFEKL. *In vivo*, spleen cells were responsive to SIINFEKL after vaccination of mice with ovalbumin-encoding plasmids in STM1, and finally, humoral responses, including IgA, were induced after vaccination.

**INTRODUCTION**

Immunisation with a live, attenuated bacterial vaccine results in the induction of potent immune responses against the bacterial strain, due to the mimicking of natural infection by the attenuated pathogen. It has been known for some time that attenuated bacterial vaccines also have the potential to deliver heterologous antigens, either expressed from within the bacteria or delivered as a DNA vaccine that is subsequently transferred to host cells for expression (reviewed in [1]). Several bacterial species have been evaluated for this purpose, and the use of attenuated *Salmonella* strains as carriers of foreign antigens is a well-studied strategy (reviewed in [2,3]). *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is a facultative intracellular pathogen that invades enterocytes and M cells in the intestine. Salmonellosis is an important disease in livestock, causing significant disease in a variety of food animals. It is also an important contributor to gastrointestinal disease in humans, with *Salmonella* spp. being second only to *Campylobacter* spp. as the cause of gastroenteritis in people, generally due to the consumption of undercooked food. We have previously reported on the use of an *aro A S. typhimurium* vaccine, STM1, for use in poultry [4]. This vaccine can successfully protect...
chickens from subsequent challenge with virulent *Salmonella* [5], and is a registered vaccine that is commercially available to the livestock industry. STM1 has been rigorously evaluated for efficacy and safety in clinical trials and has been shown to be safe in food animals. Consequently it is an excellent candidate for development as a vehicle for the delivery of heterologous sequences for use in the field of veterinary vaccines or for future use for inducing mucosal immunity in humans. In this communication we describe the evaluation of this potential using a model antigen, ovalbumin. It has been demonstrated by others that *Salmonella* can deliver antigens to the MHC Class I pathway *in vitro* [6]. We have extended the analysis by correlating *in vitro* antigen presentation by an enriched population of murine dendritic cells with the observed immune responses obtained after vaccination of mice.

In mice, *S. typhimurium* crosses the epithelial lining of the gut by invading enterocytes and M cells, subsequently invading cells resident in Peyer’s patches which can drain to the mesenteric lymph nodes and disseminate the infection throughout the body [7]. It is well known that macrophages are a major cell type invaded by *S. typhimurium*, and are capable of the induction of inflammatory responses and antigen presentation after infection. It has become clear, however, that dendritic cells play a central role in the development of immune responses after *S. typhimurium* infection [8]. Both macrophages and dendritic cells can acquire *S. typhimurium* (although probably via a different mechanism, see [9]). However, it is dendritic cells that first take up *S. typhimurium* after oral administration [10]. Dendritic cells are present below the gut epithelium, underlying M cells, although it is possible that *S. typhimurium* do not need to penetrate the epithelium to be taken up by dendritic cells. It has been recently shown that *S. typhimurium* that are deficient in the invasion genes required to enter M cells can be found in the spleen after oral administration of mice [11]. It was found that dendritic cells could penetrate the epithelial layer and directly sample bacteria within the gut lumen. Dendritic cells populating peripheral tissues are in an immature state, and therefore have potent antigen capture capabilities [8]. Cheminay et al [12] have shown that dendritic cells that are activated by salmonellae up-regulate expression of surface markers such as MHC II and CCR7. Once antigen is captured the
dendritic cells mature, migrate to local lymph nodes and present antigen to naïve T cells. Dendritic cells are the only antigen presenting cells capable of priming naïve T cells [13].

The recognition of dendritic cells as potent stimulators of T cells has led to considerable effort into the targeting of such cells to initiate the induction of immune responses [14]. The recent observation that immature dendritic cells possess high phagocytic capacity, and can take up *S. typhimurium*, may explain the potent immune responses that infection by attenuated *Salmonella* spp. induces. Given that successful vaccination with encoded heterologous antigen requires the presentation of expressed antigen by antigen-presenting cells (APCs) [15], the observed utility of *S. typhimurium* to deliver heterologous antigens to a front-line APC of the host immune system is apparent. The purpose of this investigation was to determine whether STM1 could deliver a heterologous antigen, expressed either from a prokaryotic or eukaryotic promoter, to dendritic cells *in vitro* and whether *in vivo* T cell and humoral responses to ovalbumin could be elicited after oral vaccination. Correlations were made between the observed *in vitro* and *in vivo* responses.
MATERIALS AND METHODS

Bacterial strains and plasmids.

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Transformation of bacteria

Plasmid DNA was maintained in and purified from *E. coli* DH5α using standard procedures. *S. typhimurium* strains were transformed with plasmid DNA according to the following procedure. *S. typhimurium* (LT2-9121 or STM1) were cultured in LB broth at 37°C for 3-4 hours. The culture was then placed on ice for 30 min, the bacteria collected by centrifugation and washed in 20ml of sterile water. The cell pellet was suspended in 2 ml of 10% glycerol/water and again collected by centrifugation. Bacteria were finally resuspended in 500 μl of 10% glycerol/water. One hundred ng of plasmid was transformed into 40 μl of competent cells by electroporation at 25 μF, 2.48 Kv, and 200 ohms, using a Biorad gene pulser. Outgrowth and selection of transformed cells was by standard
procedures. Each of the plasmids was first transformed into LT2-9121, and plasmid DNA was subsequently isolated from this strain prior to electroporation into STM1. Passaging of plasmid DNA via LT2-9121 ensures stability of plasmid DNA in STM1.

Mice

Mice were obtained from the Animal Resource Centre, Perth, Australia and were housed at the animal facility of RMIT University. All mice were female C57BL/6, 6-10 weeks of age.

Cell Culture

Enrichment of dendritic cells (DCs) from bone marrow was as described elsewhere [18]. Briefly, marrow was flushed from femur and tibia bones and cells were depleted of erythrocytes by incubation with ammonium chloride buffer (8% NH₄Cl, 0.1% KHCO₃, 0.04% Na₂EDTA, pH 7.2), for 5 minutes. Cells were then washed with RPMI, counted and cultured at 1 x 10⁶ cells/ml in Petri dishes of 100 mm diameter with RPMI complete medium supplemented with recombinant murine GM-CSF and IL-4 (PharMingen, USA). Culturing continued at 37°C in a humid CO₂ incubator for 5 days before use.

B3Z86/90.14 (B3Z) cells are a murine T cell hybridoma specific for the dominant H₂Kb MHC class I / ovalbumin epitope 257-264, of sequence SIINFEKL [19]. These cells were routinely cultured in DMEM.

Antigen Presentation assay

The ability of mammalian cells infected with STM1 carrying ovalbumin-expressing plasmids to stimulate B3Z cells was assessed. Presentation of SIINFEKL via MHC class I to B3Z cells results in the artificial generation of a colorimetric response, allowing a quantitative comparison of antigen presentation to be made [19]. Prior to infection, bacteria were grown to stationary phase, diluted in
PBS and opsonized for 30 min in PBS containing 20% normal mouse serum. *In vitro* infection was performed as previously described by Cheminay et al [12], at a multiplicity of infection of 100:1.

Cultured DCs were infected with STM1 carrying plasmid (pKK, pKK-OVA or sOVA-C1) as follows. Immature DCs were resuspended in complete RPMI (without antibiotics) and seeded at 10⁵ cells per well in 24-well plates. 1 x 10⁷ STM1 containing the appropriate plasmid were added to each well. The 24 well plates were centrifuged at 270 x g for 5 min. Dendritic cells were incubated for 2 h at 37°C. After infection, cultures were treated with gentamicin (100 μg/ml) for one hour and washed twice with PBS. DCs were resuspended in RPMI and cultured overnight. To measure uptake of STM1 by DCs, an aliquot of infected DCs was lysed with Triton X100, and the lysate plated to calculate bacterial counts. Between 1 and 10% of the original bacterial culture were recovered (10⁵-10⁶ cells), demonstrating significant uptake of STM1 by DCs.

On the next day 10⁵ B3Z cells per well were seeded in 96-well tissue culture plates (Greiner, Germany). Infected DCs were irradiated at 5000 rad for 13 min then distributed at 10⁵ cells/well in 100 μl. For a positive control, B3Z cells were pulsed with 25 μg/ml SIINFEKL peptide for 2-3 h, followed by washing 3 times. All conditions were analysed in triplicate. Cells were co-cultured overnight at 37°C, then collected by centrifugation and washed once with PBS. The colourimetric assay was developed as described [20] with some modifications; briefly, the B3Z cells were lysed in 100 μl PBS containing 100 mM 2-mercaptoethanol, 9 mM MgCl₂, 0.125% NP-40 and 0.15 mM CPRG (chlorophenol red β–galactoside, Calbiochem), and absorbance was measured at 550/650 nm.

**Immunization of mice.**

Two immunisation experiments were conducted. In the first, groups of 3 mice were vaccinated three times at three weekly intervals with STM1 with and without DNA plasmid (oral vaccination) or naked plasmid DNA (intramuscular vaccination). Vaccination groups are detailed in the results. Oral
vaccination was performed by inoculation of $5 \times 10^7$ CFU and IP vaccination with $1 \times 10^5$ CFU. For
naked DNA vaccination, 50 $\mu$g of plasmid DNA in saline was injected into each quadriceps muscle.
Serum samples were taken prior to the third vaccination and three weeks after the third vaccination, at
which time mice were killed and splenocytes isolated for ELISPOT.

In the second experiment groups of 5 mice were vaccinated either once, or twice at a three-
week interval. Blood was sampled three weeks after the first and (where appropriate) three weeks after
the second vaccination. Mice were killed and splenocytes isolated at each of these time points, hence
duplicate groups of mice received one or two vaccinations.

**Antibody assay**

Humoral responses to ovalbumin were analysed by ELISA, using microtitre plates coated with
10 $\mu$g/ml grade V chicken ovalbumin (Sigma, USA). Briefly, diluted sera were incubated for 4 hours at
room temperature. For the detection of IgG, peroxidase-conjugated goat anti-mouse IgG (Biorad) was
added at 1:1000 dilution and incubated at 37$^\circ$C for 1 hour, followed by washing and addition of
substrate. For isotype detection, rabbit anti mouse IgG1 was diluted to 1:2000, anti mouse IgG2a was
diluted to 1:3000 and anti mouse IgA was diluted to 1:1000. Assays were developed by addition of
substrate 3,3’, 5,5’-tetramethylbenzidin (Sigma, USA).

**Cytokine assay**

Cytokine secretion by stimulated lymphocytes was detected by using the ELISPOT assay as
described [21]. Mice were killed three weeks after vaccinations, spleens removed and cell suspensions
prepared. Anti-IFN$\gamma$ capture antibody was coated to 96 well PVDF plates (Millipore) by incubating
overnight at 4$^\circ$C. After washing with PBS and blocking with 10% FCS in PBS, splenocytes were added
at $1 \times 10^6$ per well in 100 $\mu$l RPMI. The antigen SIINFEKL was added at 10 $\mu$g per ml before incubating
for 24h at 37°C. After washing, biotinylated IFNγ secondary antibody was added, and cytokine-secreting cells detected by the addition of BCIP/NBT substrate.

RESULTS AND DISCUSSION

STM1 can deliver ovalbumin epitopes to the MHC Class I processing pathway

STM1 harboring plasmid DNA encoding ovalbumin was used to infect dendritic cells \textit{in vitro}, and the ability of the infected dendritic cells to trigger B3Z cells was measured. Hence processing of ovalbumin by dendritic cells and presentation via the MHC class I pathway is measured. Figure 1 shows the results from one typical experiment (a second experiment gave similar qualitative, statistically significant results). This shows that the positive control, the peptide SIINFEKL, was able to trigger T cells, and also shows that dendritic cells infected by STM1 carrying ovalbumin-expressing plasmids were also able to do so. These results indicate that dendritic cells can process ovalbumin either expressed within STM1 (pKK-OVA/STM1) or expressed by a DNA vaccine carried by STM1 that is presumably transferred to the nucleus of the host cell and transcribed (sOVA-C1/STM1). In both cases, OD readings are significantly higher than that induced after the infection of dendritic cells with STM1 harboring the empty plasmid (pKK/STM1). Normally peptides presented by MHC class I molecules are generated from the degradation of proteins synthesized within the cell. Exogenous and foreign antigens endocytosed by the cell are degraded in the lysosomes and presented by MHC class II molecules. Dendritic cells are unique among the cells of the immune system in their capacity to take up exogenous antigen, process and present it on the MHC class I by an alternative pathway termed “cross priming” [13]. Our \textit{in vitro} results indicate that cultured dendritic cells infected with STM1 are capable of presenting a foreign antigen by this pathway.

\textit{In vivo} evaluation of T cell activation by STM1 delivery

Having demonstrated that the invasion of dendritic cells by recombinant STM1 results in the processing and presentation of ovalbumin epitopes to T cells \textit{in vitro}, mice were immunised with the
same vaccines to determine if immunological responses to ovalbumin could be detected *in vivo*. Two vaccination experiments were performed. In the first, spleen cells from mice vaccinated three times at three-weekly intervals were isolated and examined for the ability to respond to a challenge with the dominant CD8⁺ epitope, SIINFEKL. Plasmids were delivered within STM1 as in the *in vitro* assays, or as naked DNA injected into skeletal muscle for control groups. Secretion of IFNγ by stimulated cells was measured by ELISPOT. The results are shown in Figure 2A. Results are expressed as number of positive cells per million splenocytes, with cells from each mouse assayed in triplicate. As expected, cells isolated from mice that were vaccinated with either STM1 alone or pKK alone did not respond. No response was evident from pKK-OVA injected intramuscularly, again as expected, as the ovalbumin gene will not be expressed from the prokaryotic promotor in mammalian cells. Cells from mice vaccinated intramuscularly with the mammalian expression vector, sOVA-C1, did induce a modest response after re-stimulation with SIINFEKL or ovalbumin.

STM1 harboring ovalbumin-expressing plasmids could induce a T cell response, as evident by the strong IFNγ responses to SIINFEKL. These results correlate with those observed *in vitro*, where both constructs delivered by STM1 enabled dendritic cells to trigger T cells. Therefore ovalbumin expressed by STM1 can induce T cell responses *in vitro* and *in vivo*, and plasmid DNA carried by STM1 can be transferred to host cells resulting in the production of ovalbumin and the induction of an immune response. It is noteworthy that the delivery of the DNA vaccine in STM1 results in a better cellular response than direct vaccination with the naked plasmid. Intramuscular vaccination with naked DNA is known to be very inefficient, with the majority of DNA degraded prior to uptake by host cells [22], and hence the delivery by STM1 to antigen presenting cells (macrophages or dendritic cells) may offer higher numbers of transfected APCs and hence induction of an increased immune response. In addition, microbial infections deliver “danger signals” to the immune system through recognition of pathogen-associated molecular patterns (PAMPs) such as LPS, CpG sequences or other components by
toll-like receptors (TLR) on cells of the immune system such as dendritic cells and macrophages [23]. A potent pro-inflammatory response results with the secretion of appropriate cytokines at the infection site and the activation and maturation of dendritic cells that then migrate to the draining lymph node for effective priming of an immune response. The nature of the immune response induced is dependent on the differentiation induced in the immune cells, which is in turn dependent on the PAMP recognized [24]. Vaccines such as soluble antigen delivered without adjuvant are inefficient in inducing an effective immune response because they do not deliver such signals and are ignored by the immune system.

**T cell responses are evident after a single vaccination**

In the above experiment mice were vaccinated three times prior to evaluation of responses. We also investigated the ability of spleen cells to secrete cytokines after re-stimulation following fewer doses of vaccine. In a second experiment, groups of mice were vaccinated once or twice and T cell responses measured three weeks later. Figure 2B shows that at each time point the responses to STM1 delivering ovalbumin sequences were significantly higher (P < 0.05) than STM1 delivering the empty vector.

**Delivery of ovalbumin by STM1 induces humoral responses**

It was established in the above experiments that the delivery of ovalbumin sequences by STM1 could induce T cell responses, and in particular, CD8⁺ responses. We next tested sera from vaccinated mice to evaluate humoral responses to ovalbumin. Figure 3 shows the results from both vaccination experiments. In the first trial, humoral responses were generated in mice vaccinated with either sOVA-C1 naked DNA, pKK-OVA in STM1 or sOVA-C1 in STM1 (Figure 3A). These results therefore correlate with the ability of the various vaccines to elicit T cell responses, as detected by ELISPOT. Only those vaccines that induced a T cell response were able to induce a humoral response. Both IgG1 and IgG2a responses were detected, with IgG2a titres consistently higher than IgG1 titres (Figure 3B).
Both conjugates were used well in excess of their titres (1:15,800 and 1:10,200 for IgG1 and IgG2a respectively, used at 1:3000 or less). It was therefore assumed the assays were effectively saturated and the titres can be compared between the two conjugates. This analysis shows that there was IgG2a dominance in the response to ovalbumin, indicating a Th1-type response. We also measured responses to STM1 lysate, and again found higher titres of IgG2a compared to IgG1 (data not shown). Finally, IgA levels were measured. As shown in Figure 3B, all ova-expressing constructs induced the IgA isotype. As expected, the ratio of IgA:IgG1 was higher when the DNA vaccine was delivered orally in STM1 (9:1), rather than as a naked DNA vaccine (1.2:1).

Humoral responses were also measured during the dose response experiment, and the results are shown in Figure 3C. Again, delivery of either ova-expressing plasmid by STM1 has induced humoral responses to ovalbumin.

Conclusions

This study demonstrates that the Salmonellosis vaccine STM1 has promise as a future vehicle for delivering heterologous antigens. The advantage of conducting such experiments on well characterised, registered vaccines is that future registration will be streamlined- hence it is important to validate basic principles of heterologous antigen delivery in such vehicles. STM1 can deliver the model antigen ovalbumin via the oral route to mice, and immune responses (T cell and humoral) were induced. In particular, we have found that the passenger antigen can be processed and presented via MHC class I, as has been found previously for bacteria that target an intracellular location. The results obtained in vitro show that dendritic cells can take up STM1, and this results in the presentation of passenger-antigen derived peptides to CD8+ T cells; in vivo macrophages presumably also have a major role in antigen presentation and/or dissemination of the infection [8].
STM1 can elicit an immune response to sequences delivered either from a plasmid with a prokaryotic or eukaryotic promoter. In vitro, presentation by dendritic cells after infection by either vaccine is equivalent, although in vivo the results varied, with the DNA vaccine in STM1 yielding more IFNγ-secreting cells in experiment 1, but slightly less than the prokaryotic vector in the second experiment. Humoral responses induced by either were similar. In comparing the DNA vaccine delivered as either a naked vaccine, injected intramuscularly, or delivered in STM1, higher immune responses were obtained after delivery by the bacterial vector. This demonstrates the promise of STM1 as a vehicle for the delivery of plasmids encoding heterologous antigens and “danger signals” for effective immune responses. The potential of STM1 to deliver identified disease antigens is now being evaluated.

Acknowledgements

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Figure Legends

Figure 1. *In vitro* signaling of T cells by STM1-infected dendritic cells. STM1 was transformed with either empty plasmid (pKK/STM1) or plasmid expressing ovalbumin from a prokaryotic or eukaryotic promoter, and was then used to infect cultured dendritic cells. The signaling of 3BZ cells (TCR recognizing MHC ClassI/SIINFEKL) was measured in a colourimetric assay. 3BZ cells incubated with peptide were used as a positive control. Values obtained from OVA-expressing plasmids were compared to empty vector using the *t*-test. * P < 0.0001.

Figure 2. Secretion of cytokines by splenocytes. (A). Vaccination experiment #1. Splenocytes were isolated from mice vaccinated with the various constructs shown, and the number of IFNγ-secreting cells measured after re-stimulation with SIINFEKL. Reactivity was evaluated against controls using the *t*-test. * P < 0.05, ** P < 0.0001. ***Vaccination with sOVA-C1/STM1 enumerated higher numbers of IFNγ-secreting cells than vaccination with naked DNA, sOVA-C1 (P < 0.005). B. Vaccination experiment #2. Splenocytes were isolated from mice vaccinated 1 or 2 times with the indicated constructs, and the proportion of measured after re-stimulation with SIINFEKL. In each case, the number of IFNγ-secreting cells enumerated by either of the ova-expressing plasmids in STM1 was higher than that of the empty plasmid in STM1 (P < 0.005).

Figure 3. Humoral responses elicited by vaccination. Sera taken from mice vaccinated with the indicated constructs was pooled and reactivity to ovalbumin measured. Panel A shows the total IgG response determined in vaccination experiment 1. Week 6 and week 9 refer to weeks after the first vaccination. Panel B shows the isotype response at week 9 in the same experiment. C. IgG responses elicited in vaccination experiment 2, determined after one or two vaccine doses.
Figure 1

![Graph showing OD 550/650 for different samples: SIINFEKL, pKK/STM1, pKK-OVA/STM1, sOVA-C1/STM1. The graph indicates that SIINFEKL has a significantly higher OD than the other samples, with pKK-OVA/STM1 and sOVA-C1/STM1 showing similar but lower OD values.](image-url)
Figure 2

A

![Bar chart showing spots per million cells for different samples.]

B

![Bar chart showing spots per million cells at different doses.]

**Spots per million cells**

**Dose**

- pKK
- pKK-OVA
- sOVA-C1
- STM1
- pKK-STM1
- pKK-OVA/STM1
- sOVA-C1/STM1

**Statistical significance:**
- *p < 0.05
- **p < 0.01
- ***p < 0.001
Figure 3

A

![Graph A](image1)

B

![Graph B](image2)

C

![Graph C](image3)