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(54) Title: RECOMBINANT BACTERIUM AND USES THEREOF

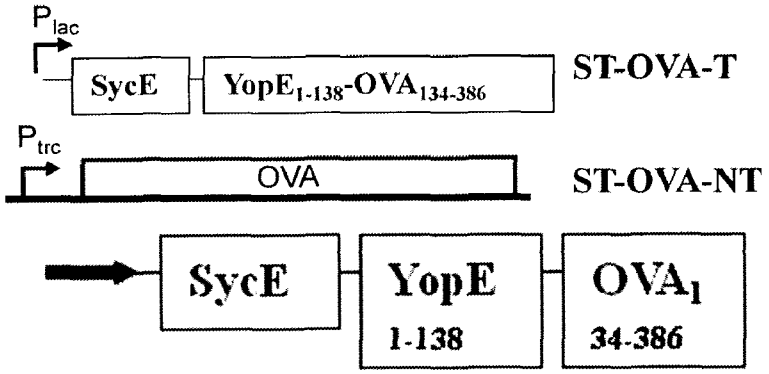

FIG. 1A

(57) Abstract: The present invention relates to a recombinant bacterium expressing an antigen that is translocated to the cytosol of a host organism, and uses thereof. To this end, the present invention provides a recombinant bacterium comprising a nucleic acid encoding an antigen that is translocated to the cytosol of a host cell utilizing Type III secretion system. The recombinant bacterium is generally chosen from intracellular pathogens that reside in the phagosome and fail to induce rapid T cell activation. The translocated antigen may be a viral antigen, a bacterial antigen, or a tumour antigen. Methods of imparting immunity using the recombinant bacterium are also provided.

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RECOMBINANT BACTERIUM AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to recombinant bacterium and uses thereof. More specifically, the invention relates to recombinant bacterium expressing an antigen that is translocated to the cytosol of a host organism, and uses thereof.

BACKGROUND OF THE INVENTION

Various vaccine vectors or adjuvants that induce potent T cell responses are known in the art (Kaufmann and Hess, 1997). However, very few vaccine vectors exist that induce rapid and potent memory CD8+ cytolytic T cell responses, and that are safe and cost-effective (Raupach and Kaufmann, 2001). Unlike other T cells, CD8+ T cells uniquely provide immune-surveillance to the entire body because they recognize targets in the context of MHC class I molecules, which are present in every cell (Bevan, 1995). Furthermore, CD8+ T cells can eliminate infected cells or tumour cells rapidly. Thus, the induction of specific, potent CD8+ T cells is highly desirable for diseases that are caused by intracellular pathogens and tumours.

Intracellular pathogens induce CD8+ T cell responses; however, the responses are either highly attenuated or the organism itself is highly toxic. Generally, rapid proliferation of pathogens is countered by rapid presentation of antigen to $CD8^+$ T cells within the first few days of infection and activated $CD8^+$ T cells undergo profound expansion (>1000-fold) within the first week of infection, which results in resolution of infection (Kaech and Ahmed, 2001). Similarly, CD8+ T cells play a key role in mediating immune-surveillance against tumours (Smyth et al., 2000). While antibodies and helper T cells mainly promote clearance of extracelluar pathogens (Kaech et al., 2002), CD8+ T cells play a principal role in controlling intracellular pathogens and tumours. Thus, rapid induction of memory CD8+ T cells is essential for developing vaccines against tumours or intracellular pathogens.

While the CD8+ T cells play a key role against various diseases, their induction is highly tedious. Antigenic proteins injected into hosts in the absence or presence of adjuvants does not lead to the induction of CD8+ T cells (Moore et al., 1988). This is mainly because extracelluar proteins do not gain access to the cytoplasm (cytosol) of antigen-presenting cells (APC) (Rock, 1996). Rather, these extracellular proteins or vaccines are trafficked through specialized intracellular vesicles called phagosomes, which leads to the activation of helper T cells to aid antibody production. For induction of CD8+ T cell responses, the pathogen or the vaccine has to reside within the cytosol of an antigen-presenting cell (Bahjat et al., 2006).

Alternative routes of cross-presentation of non-cytosolic antigens to T cells have been suggested (Schaible et al., 2003; Houde et al., 2003; Yrlid and Wick, 2000), however the efficiency of these pathways in controlling pathogens isn't clear (Freigang et al., 2003). Dendritic cells may pick up antigen from dying APCs and present it to $CD8^+$ T cells (Albert et al., 1998). *Salmonella enterica* serovar Typhimurium (ST) nduces rapid death of macrophages and dendritic cells (Hersh et al., 1999; van der Velden et al., 2000) and it has been shown that cross-presentation of ST antigens occurs through dendritic cells (Yrlid and Wick, 2000). Phagosomes have themselves been considered to be competent at promoting cross-presentation (Houde et al., 2003). However, these mechanisms are of little protective value since rapid pathogen elimination is not observed. Cells that are cross-presenting ST antigens don't appear to serve as good targets for $CD8^+$ T cells to mediate their function. Thus, target cell accessibility seems to be the critical difference between direct and cross-presentation.

Subunit vaccines that consist of purified proteins admixed with adjuvants typically do not induce CD8+ T cell response due to residence of these entities within phagosomes of cells (Bahjat et al., 2006). However, some adjuvants induce CD8+ T cell responses most likely by the cross-presentation pathway (Krishnan et al., 2000). Subunit vaccines are difficult to mass-produce and are faced with numerous technical difficulties including batch to batch variability, quantitation of the antigen-adjuvant ratio, and extensively laborious procedures. To avoid this problem, live vaccines are preferred. However, live vaccines can be either over- or under-attenuated and it is difficult to find the right balance (Raupach and Kaufmann, 2001).

Typically, viral infections (such as *Lyphochoriomeningitis virus, LCMV*) lead to potent activation of CD8+ T cell responses due to their replication within the cytosol of infected cells (Kaech et al., 2002; Murali-Krishna et al., 1998). However, it is difficult to justify the use of viral vectors as a live vaccine due to the lack of availability of reagents to control the virus, particularly in immunocompromised hosts. Live bacteria can be considered as an alternative option for vaccine development since antibiotics can be used in case they are not controlled by the host. However, extracellular bacteria do not gain access to the cytosol of infected cells, hence fail to induce CD8+ T cell response (Bevan, 1995). On the other hand, intracellular bacteria induce CD8+ T cell response, albeit poor, despite residing within the phagosomes of infected cells, perhaps by cross-presentation (Kaufmann, 1993) - the caveat being that intracellular bacteria (e.g., *Salmonella*, *Mycobacteria*, *Leishmania*) that reside within the phagosomes of infected cells induce a chronic infection, implying that CD8+ T cells fail to eradicate them from the host (Kaufmann, 1993; Hess and Kaufmann, 1993).

There remains a need in the art for a safe, cost-effective method to induce rapid and potent memory CD8+ cytolytic T cell responses.

SUMMARY OF THE INVENTION

The present invention relates to recombinant bacterium and uses thereof. More specifically, the invention relates to recombinant bacterium expressing an antigen that is translocated to the cytosol of a host organism, and uses thereof.

The present invention provides a recombinant bacterium, comprising a nucleic acid encoding an antigen that is translocated to the cytosol of a host cell. The bacterium may be *Salmonella*, *Mycobacteria*, *Brucella*, or *Leishmania.* In one example, the recombinant bacterium may be *Salmonella.*

The antigen expressed by the recombinant bacteria as just described may be a viral antigen, a bacterial antigen, or a tumour antigen. The antigen may be the nucleoprotein of LCMV, tyrosinase related protein 2 (TRP-2), MART-1, melanoma associated antigen 1 (MAGE1), gp100, or Her-2/neu or other viral or bacterial antigens.

The nucleic acid encoding the antigen may encode a fusion protein comprising the antigen and a translocation domain from a type III secretion system. For example, the translocation domain may be YopE, SopE, SptP, or a fragment thereof.; in one specific example, the chaperone may be SycE or a fragment thereof (such as, but not limited to MKISSFISTSLPLPTSVS, SEQ ID NO:2). The fusion protein may optionally further comprise a chaperone. The chaperone may be derived from a type III secretion system. For example, the chaperone may be SycE or HSP70.

The nucleic acid may be comprised in a vector. The vector may be a pHR vector; in a specific example, the vector may be a modified pHR-241 vector. In the modified pHR-241 vector, the vector may be modified to remove the sequence of p60/M45, may be optionally further modified to remove the sequence of SycE.

Specific, non-limiting examples of fusion proteins encompassed by the present invention are those of SEQ ID NO:7 to SEQ ID NO:12.

The present invention also provides a method of imparting immunity against naturally-occurring bacterium in a subject, the method comprising administering the recombinant bacterium described above to said subject.

The present invention further provides a method of imparting immunity against tumours in a subject, the method comprising administering the recombinant bacterium described above to said subject. The recombinant bacterium may be administered by intravenous, oral, or subcutaneous routes of immunization.

The present invention also encompasses a use of the recombinant bacterium described herein as a vaccine.

Previously, it was known that pathogen-specific CD8+ T cells remain ineffective as long as the pathogen remained in the phagosome. For example, when conventional memory $CD8^+$ T cells against a given antigen were adoptively transferred to naïve hosts, they failed to respond rapidly in response to the same antigen expressed by ST infection (Luu et al., 2006). Presently, a recombinant ST that injects an antigen directly into the host cytosol has been developed. This results in profound $CD8^+$ T cell activation and consequent elimination of ST. It is also shown that when $CD8^+$ T cells are engaged in this manner, they undergo profound expansion which results in massive pathogen and tumour control as well as abridgment of pathogen chronicity. For example, as is evident in present Figure 3E, the numbers of OVA-specific $CD8^+$ T cells were similar at day 60 in ST-OVA-T versus ST-OVA-NT groups, but the burden was controlled only in the ST-OVA-T infected group, reiterating the notion that antigenic accessibility is the key to $CD8^+$ T cell functionality. This strategy works even with attenuated strains of *Salmonella*.

Notwithstanding the numerous genes that pathogens such as ST employ for virulence and chronicity (Jones and Falkow, 1996; Kaufmann et al., 2001), the present data provide novel insights into the incapacity of the immune system to efficiently control the bacterium, as well as reveal the power of the acquired immune system, wherein engagement of potent antigen-presentation early on can be sufficient to control an otherwise uncontrollable bacterium. The present results provide compelling evidence that modulation of the cell biology of antigen trafficking is a key avenue that is employed by various pathogens for immune evasion. Thus, a novel vaccine vector (*Salmonella*) is presently provided, wherein a key modification makes the bacterium generate rapid, potent CD8+ T cell response, resulting in self-destruction of the vaccine *in vivo*, making it highly efficacious, safe and cost-effective at the same time.

The use of OVA as an antigen is described herein as a proof of principle. Using a similar approach, other putative antigens from other pathogens (bacteria, virus) or tumours can be cloned into ST and these antigens can be translocated into the host cell cytosol for rapid and potent antigen-presentation using the *YopE/SycE* system. When a tumour-antigen is cloned into ST using the *YopE/SycE* system, potent and rapid anti-tumour CD8+ T cell response is generated which consequently results in rapid destruction of the bacterium.

Additional aspects and advantages of the present invention will be apparent in view of the following description. The detailed description and examples, while indicating preferred embodiments of the invention, are given by way of illustration only, as various changes and

modifications within the scope of the invention will become apparent to those skilled in the art in light of the teachings of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will now be described by way of example, with reference to the appended drawings, wherein:

FIGURE 1A shows a schematic of the fusion protein constructed, where an antigen (OVA) is fused to YopE, which is then incorporated into the plasmid pHR241 containing the SycE chaperone. FIGURE 1B shows a schematic of the antigen (OVA) translocation into the cytosol for ST-OVA-T, and the lack thereof for ST-OVA-NT. Ag: antigen; OVA: ovalbumin; ST: *Salmonella* Typhimurium. FIGURE 1C shows expression of OVA (by western blot) in the bacterial pellet, supernatant, and the cytosol of spleen cells of mice infected for 24 h with ST-OVA-NT and ST-OVA-T.

FIGURE 2A shows a graph representing the doubling times of the ST-OVA-NT (closed circles) and ST-OVA-T (open circles) bacteria in liquid culture, based on the measurement of OD at 600nm. Based on these values, the bacteria were found to be similar. FIGURE 2B is a graph showing the ST burden in IC-21 macrophages (H-2^b) infected with ST-OVA-NT or ST-OVA-T (multiplicity of infection, MOI=10). No statistically significant difference was detected in the ability of ST-OVA-NT or ST-OVA-T to infect and replicate within macrophages ($p>0.05$). Results are representative of three independent experiments.

FIGURE 3A shows flow cytometry results of *in vitro* infection of IC-21 macrophages (H-2^b) with recombinant bacteria (ST-OVA-NT, ST-OVA-T, or ST). The reduction in CFSE intensity of OT-1 CD8+ T cells indicated that infection of macrophages with ST or ST-OVA-NT did not result in any detectable proliferation of OT-1 cells, and thus, a lack of antigen-presentation. Infection with ST-OVA-T resulted in strong dilution of CFSE expression, which is indicative of rapid and potent antigen-presentation. FIGURE 3B shows flow cytometry results of *in vivo* infection of B6.129F1 mice infected with ST-OVA-NT or ST-OVA-T (Day 5). In ST-OVA-T-infected mice, the majority of transferred OT-1 cells displayed reduced expression of CFSE while OT-1 cells in ST-OVA-NT-infected mice maintained high levels of CFSE expression. Results represent the mean of three mice ± SD per group, and are representative of 2-3 independent experiments. FIGURE 3C is a graphical representation of the kinetic evaluation of *in vivo* antigen-presentation. ST-OVA-NT infected mice displayed muted and delayed activation of CFSE-labelled OT-1 cells. ST-OVA-NT (closed circles); ST-OVA-T (open circles).

FIGURE 4 shows the numbers of spleen cells (A), spleen size at Day 14 (B) and bacterial burden (C) in resistant (B6.129F1) mice infected with ST-OVA-T or ST-OVA-NT, as well as the percentage (D) and numbers (E) of OVA-specific $CD8^+$ T cells in the spleen. Results represent the mean of three to five mice ± SD per group and are representative of three independent experiments. ST-OVA-NT (closed circles); ST-OVA-T (open circles).

FIGURE 5A shows the OVA-tetramer profile in the spleens of ST-OVA-T- or ST-OVA-NT-infected resistant (B6.129F1) mice at Day 7. The expression of CD62L (FIGURE 5B, 5D) and CD127 (FIGURE 5C, 5D) on OVA-tetramer+CD8+ T cells is also shown. Results are representative of three independent experiments. These results indicate early generation of memory CD8+ T cells in mice infected with ST-OVA-T. ST-OVA-NT (closed circles); ST-OVA-T (open circles).

FIGURE 6 shows the bacterial burdens (A) in spleen cells of susceptible (C57BL/6J) mice infected with ST-OVA-T or ST-OVA-NT, along with the percentage (B) and numbers (C) of OVA-specific $CD8^+$ T cells, as well as the frequency of OVA-specific $CD8^+$ T cells evaluated by ELISPOT assay (D). The specific killing of OVA-pulsed targets in naïve mice exposed to OVA-pulsed and control spleen cells is shown if FIGURE 6E and F, indicating that ST-OVA-T infection results in rapid induction of antigen-specific CD8+ T cells that can efficiently kill antigen-bearing target cells. Results represent the mean of three to four mice ± SD per group, and two independent experiments. ST-OVA-NT (closed circles); ST-OVA-T (open circles).

FIGURE 7A shows the OVA-tetramer profile in the spleens of susceptible (C57BL/6J) mice infected with ST-OVA-T or ST-OVA-NT at Day 7. FIGURE 7B shows the expression of CD62L versus CD127 on splenic OVA-tetramer$^+$$CD8^+$ T cells in the ST-OVA-T versus ST-OVA-NT infected mice. CD8+ T cells generated with ST-OVA-T infection express high levels of CD127 and CD62L (memory markers). Results are representative of three independent experiments.

FIGURE 8A shows the bacterial burden in spleens of C57BL/6J mice treated with anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43) or Rat IgG isotype antibodies following infection with ST-OVA-T. Results represent the mean of three to four mice ± SD per group. Anti-CD4 and anti-CD8 antibody treatment resulted in near complete elimination of CD4 and CD8+ T cells respectively. FIGURE 8B shows the bacterial burden in spleens of WT, MHC-I- or MHC-II-deficient mice following infection with ST-OVA-T. These results indicate that the control of bacterial burden in ST-OVA-T infected mice is mediate exclusively by CD8+ T cells. Results represent the mean of five mice ± SD per group.

FIGURE 9 shows the relative numbers of OVA-specific $CD8^+$ T cells in the spleen (FIGURE 9A) and peripheral blood (FIGURE 9B) of B6.129F1 mice infected with wild type (WT) or attenuated (ΔaroA) ST-OVA expressing non-translocated (NT) or translocated (T) OVA. Results represent the mean of five mice ± SD per group. Results indicate that even attenuated strain of ST can induce potent and rapid CD8 T cell response when antigen is translocated to the cytosol of infected cells. WT-OVA-NT (closed circles); WT-OVA T (open circles); AroA-OVA-NT (closed inverted triangles); AroA-OVA-T (open inverted triangles).

FIGURE 10A is a graphical representation of the results of prophylactic vaccination with ST-OVA-T in C57BL/6J mice followed by subcutaneous challenge with B16-OVA tumor cells. This protocol resulted in potent protection against tumor challenge. Non-infected (closed circles); ST-OVA-T (open squares). FIGURE 10B shows a graph of results of therapeutic vaccination with ST-OVA-T in C57BL/6J mice after subcutaneous challenge with B16-OVA tumor cells. Mice receiving ST-OVA-T displayed the best protection against B16 melanoma cells. Protection induced by ST-OVA-T was far greater than that induced by ST-OVA-NT and the another recombinant bacterium, *Listeria* expressing OVA (LM-OVA). Results represent the mean of five mice ± SD per group. Non-infected (full circles); ST-OVA-T (open squares); ST-OVA-NT (closed triangles); LM-OVA (open diamonds).

FIGURE 11A shows the frequency of CD8+ T cells against a tumour antigen (Trp-2) in the spleens of mice infected with wild-type (WT) or attenuated (*aroA*) ST-Trp2-T on Day 7. FIGURE 11B shows the bacterial burden in the spleens of mice at various time intervals post-infection with WT ST-Trp2-T (open squares) or ST-Trp2-NT (closed circles). FIGURE 11C shows the bacterial burden in the spleens of mice infected with *aroA* mutant of ST-Trp2-T (open squares) or NT (closed circles).

FIGURE 12A shows the bacterial burden in the spleens of mice infected with translocated or non-translocated aroA-ST expressing another tumour antigen (gp100). *aroA*-gp100-T (open squares) or *aroA*-gp100-NT (closed circles). FIGURE 12 B shows the numbers of gp100-tetramer+ CD8+ T cells in the spleens of infected mice at various time intervals. *aroA*-ST-gp100-T (open squares); *aroA*-ST-gp100-NT (closed circles).

FIGURE 13A shows the schematic of the fusion constructs. FIGURE 13B shows the frequency of NP-specific CD8+ T cells in mice infected with ST-NP-T or ST-NP-NT at day 7 post-infection. FIGURE 13C shows the *in vivo* cytolytic activity of NP-specific CD8+ T cells on NP-pulsed target cells at day 7 post-infection. Cytolytic activity was evaluated after transferring naïve spleen cells (pulsed with media or NP peptide) into infected mice at day 7 and evaluated the killing of peptide-pulsed targets at 24 h post-transfer. FIGURE 13D shows

the frequency of NP-specific CD8+ T cells in mice infected with *aroA*-NP-T (black bars) or *aroA*-NP-NT (white bars). FIGURE 13E shows the bacterial burden in the spleens at various time intervals. *aroA*-NP-T (open squares) or *aroA*-NP-NT (closed circles)FIGURE 13F shows the influence of antigenic translocation on the induction of inflammation in the spleen. *aroA*-NP-T (open squares); *aroA*-NP-NT (closed circles).

FIGURE 14 shows that truncated YopE is equally effective at inducing CD8+ T cell response. FIGURE 14A shows the schematic representation of the full length (upper panel) and the truncated YopE (lower panel). FIGURE 14B shows the OVA-specific CD8+ T cell response in the spleens of mice infected with full YopE or truncated YopE. FIGURE 14C shows that both the full length and truncated YopE induce the rapid generation of OVA-specific CD8+ T cells expressing memory marker (CD127). FIGURE 14D shows the inflammation induced (numbers of spleen cells) in mice infected with full length or truncated YopE. FIGURE 14E shows the bacterial burden in the spleens of mice infected with full length or truncated YopE. ST-OVA-NT (closed circles); ST-OVA-T (open circles); ST-OVA-tYopE (closed triangles).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to recombinant bacterium and uses thereof. More specifically, the invention relates to recombinant bacterium expressing an antigen that is translocated to the cytosol of a host organism, and uses thereof.

The present invention provides a recombinant bacterium, comprising a nucleic acid encoding an antigen that is translocated to the cytosol of the host organism.

The bacterium may be any virulent or attenuated bacterium that resides in the phagosome of macrophages and/or dendritic cells and induces poor T cell activation. Such a bacterium may be, but is not limited to *Salmonella*, *Mycobacteria*, *Brucella*, *Leishmania,* and the like, which are all intracellular pathogens that reside in the phagosome and fail to induce rapid T cell activation, hence causing diseases that are not controlled by the immune system.

In one example, the virulent or attenuated bacterium may be *Salmonella.* Any suitable strain of *Salmonella* known in the art may be used; for example, and without wishing to be limiting in any manner, the virulent or attenuated bacterium may be *Salmonella enterica*, serovar Typhimurium (ST). ST is a highly virulent pathogen that induces gastroenteritis in humans, and typhoid-like disease in mice (Jones and Falkow, 1996). In susceptible C57BL/6J mice, which lack natural resistance-associated macrophage proteins (NRAMP), ST (strain SL1344) induces a systemic lethal infection even at doses as low as (10^2) (iv), and all mice die within 7

days of infection (Albaghdadi et al., 2009). In contrast, ST induces a chronic but non-lethal infection in resistant 129SvJ mice (which express NRAMP). F1 hybrids between susceptible and resistant mice (B6.129F1) also harbour a chronic, non fatal, infection (Luu et al., 2006). Genes that are involved in *Salmonella* invasion of epithelial cells are clustered at the *Salmonella* pathogenicity island-1 loci (SPI-1) (Bliska et al., 1993; Zhou and Galan, 2001; Galan and Curtiss, III, 1989; Hardt et al., 1998). They encode several factors, including a type III secretion system (TTSS) apparatus that exports specific proteins (effectors) into the host cell. Two major virulence loci allow *Salmonella* to survive inside cells (Jones and Falkow, 1996). The two-component regulatory system *phoP/phoQ*, which controls >40 genes (Groisman et al., 1989; Miller et al., 1989), is involved in intracellular survival (Garvis et al., 2001). Another pathogenicity island (SPI-2) encodes a second TTSS, mediates resistance to intracellular killing, and is key to virulence (Hensel et al., 1995; Shea et al., 1996).

The CD8+ T cell response against ST is delayed, which fails to control the bacterium leading to a chronic infection (Albaghdadi et al., 2009). *aroA* mutant of ST was developed as a vaccine against Salmonella (Hoiseth and Stocker, 1981), which induces minimal inflammation and poor immunogenicity (Albaghdadi et al., 2009; Dudani et al., 2008). The virulent or attenuated bacterium of the present invention may be the *aroA* mutant of ST, comprising a vaccine vector modified such that the bacterium resides in the phagosome of infected cells, but translocates antigen to the cytosol. This modification allows rapid induction of CD8+ T cells; without wishing to be bound by theory, this may lead to the self-destruction of the vaccine. Phagosomal localization is considered a major impediment to T cell activation, and the antigenic translocation strategy described herein can be used for other intracellular bacterial vaccine vectors, including *Mycobacteria*, *Brucella* or *Leishmania*.

By the term "recombinant" it is meant that the bacterium has been genetically altered or engineered; such genetic engineering may be the inclusion of a recombinant (or artificial) nucleic acid or vector (comprising a nucleic acid) encoding a foreign protein that is an antigen.

The antigen may be any suitable protein or fragment thereof that is processed and presented efficiently by dendritic cells and/or macrophages resulting in efficient T cell activation. Without wishing to be limiting in any manner, the antigen or fragment thereof may be a nascent protein, a bacterial antigen, viral antigen, or a tumour antigen. For example, the antigen may be, but is not limited to tyrosinase related protein 2 (TRP-2), MART-1, melanoma associated antigen 1 (MAGE1), gp100, Her-2/neu or other proteins or fragments thereof known in the art. Other proteins may include, but are not limited to ovalbumin, hen egg lysozyme, and myelin basic protein, nuclear protein of LCMV. In a specific, non-limiting example, the antigens may be ovalbumin, TRP-2, gp-100, LCMV-NP, or fragments thereof.

Upon infection, the antigen is translocated into the cytosol of the host cell (for example macrophages and/or dendritic cells). The antigen may naturally translocate to the cytosol, or may be a recombinant protein engineered to do so. Thus, the antigen may be comprised in a fusion protein that further comprises a translocation domain from a type III secretion system; optionally, the fusion protein may further comprise a chaperone. As would be known to those of skill in the art, the fusion protein, also referred to herein as "fused proteins", comprising the antigen may be generated via recombinant methods well-known to those of skill in the art. The antigen and translocation domain, and the optional chaperone, may be joined directly or by a linker; appropriate linkers would be well-known to those of skill in the art.

By the term "translocation domain", it is meant a protein domain or fragment thereof that directs translocation of a protein from the phagosome to the cytosol of the host cell. The translocation domain may be any suitable translocation domain from known type III secretion systems of bacteria, which are well-known to those of skill in the art. For example, and without wishing to be limiting in any manner, the translocation domain may be YopE or a fragment thereof. YopE is a 23kDa protein comprising a N-terminal secretion domain of approximately 11 amino acids and a translocation domain of at least 50 aa. In one specific, non-limiting example, the YopE translocation domain may comprise the sequence:

MKISSFISTSLPLPTSVSGSSSVGEMSGRSVSQQKSEQYANNLAGRTESPQGSSLASRI
TEKLSSMARSAIEFIKRMFSEGSHKPVVTPAPTPAQMPSPTSFSDSIKQLAAETLPKYIQ
QLSSLDAETLQKNHDQFAT (SEQ ID NO:1),

a fragment thereof (such as, but not limited to MKISSFISTSLPLPTSVS, SEQ ID NO:2), or a sequence substantially identical thereto. Another suitable translocation domain may be the SptP protein of ST (Russmann et al., 1998); again, the SptP translocation domain could be the full length protein or a truncated version thereof. In one specific example, the SptP translocation domain may comprise the sequence:

MLKYEERKLNNLTLSSFSKVGVSNDARLYIAKENTDKAYVAPEKFSSKVLTWLGKMPLF
KNTEVVQKHTENIRVQDQKILQTFLHALTEKYGETAVNDALLMSRINMNKPLTQRLAVQI
TECVKAADEGFINLIKSKDNVGVRNAALVIKGGDTKVAEKNNDVGAESKQPLLDIALKGL
KRTLPQLEQMDGNSLRENFQEMASGNGPLRSLMTNLQNLNKIPEAKQLNDYVTTLTNI
QVGVARFSQWGTCGGEVERWVDKASTHELTQAVKKIHVIAKELKNVTAELEKIEAGAP
MPQTMSGPTLGLARFAVSSIPINQQTQVKLSDGMPVPVNTLTFDGKPVALAGSYPKNTP
DALEAHMKMLLEKECSCLVVLTSEDQMQAKQLPPYFRGSYTFGEVHTNSQKVSSASQ
GEAIDQYNMQLSCGEKRYTIPVLHVKNWPDHQPLPSTDQLEYLADRVKNSNQNGAPG

RSSSDKHLPMIHCLGGVGRTGTMAAALVLKDNPHSNLEQVRADFRDSRNNRMLEDAS QFVQLKAMQAQLLMTTAS (SEQ ID NO:3),

a fragment thereof, or a sequence substantially identical thereto. Yet another example of a suitable translocation domain is SopE, a type III secretion protein in *Salmonella* ST (Zhu et al., 2010). In a specific example, the SopE translocation domain may comprise the sequence:MTKITLSPQNFRIQKQETTLLKEKSTEKNSLAKSILAVKNHFIELRSKLSERFIS HKNTESSATHFHRGSASEGRAVLTNKVVKDFMLQTLNDIDIRGSASKDPAYASQTREAI LSAVYSKNKDQCCNLLISKGINIAPFLQEIGEAAKNAGLPGTTKNDVFTPSGAGANPFITP LISSANSKYPRMFINQHQQASFKIYAEKIIMTEVAPLFNECAMPTPQQFQLILENIANKYIQ NTP (SEQ ID NO:4),

a fragment thereof, or a sequence substantially identical thereto.

The fusion protein may optionally comprise a chaperone. By the term "chaperone', it is meant a protein that assists in translocation of the immunodominant antigen. The chaperone protein may be any suitable protein known in the art, and must be compatible with translocation domain chosen. The chaperone may also be from a type III secretion system. For example, and without wishing to be limiting, the chaperone may be SycE. SycE is a YopE-specific chaperone that is required for YopE-mediated translocation of fused proteins to the cytosol (Russmann et al., 2001). SycE assists in translocation of the fused protein into the cytosol of infected cells through the type III secretion system of ST. In a specific, non-limiting example, the SycE chaperone may comprise the sequence:

MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE TLLSHNIFSQDILKPILSWDEVGGHPVLWNRQPLNNLDNNSLYTQLEMLVQGAERLQTS SLISPPRSFS (SEQ ID NO:5),

or a sequence substantially identical thereto. In another example, the SopE translocation domain has been used in combination with the chaperone protein heat shock protein 70 (Hsp70) to deliver an antigen to the cytosol (Zhu et al., 2010). In a specific, non-limiting example, the chaperone may comprise the sequence:

MGKIIGIDLGTTNSCVAIMDGTQARVLENAEGDRTTPSIIAYTQDGETLVGQPAKRQAVT NPQNTLFAIKRLIGRRFQDEEVQRDVSIMPYKIIGADNGDAWLDVKGQKMAPPQISAEVL KKMKKTAEDYLGEPVTEAVITVPAYFNDAQRQATKDAGRIAGLEVKRIINEPTAAALAYG LDKEVGNRTIAVYDLGGGTFDISIIEIDEVDGEKTFEVLATNGDTHLGGEDFDTRLINYLV DEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADATGPKHMNIKVT

RAKLESLVEDLVNRSIEPLKVALQDAGLSVSDINDVILVGGQTRMPMVQKKVAEFFGKE
PRKDVNPDEAVAIGAAVQGGVLTGDVKDVLLLDVTPLSLGIETMGGVMTPLITKNTTIPT
KHSQVFSTAEDNQSAVTIHVLQGERKRASDNKSLGQFNLDGINPAPRGMPQIEVTFDID
ADGILHVSAKDKNSGKEQKITIKASSGLNEEEIQKMVRDAEANAESDRKFEELVQTRNQ
GDHLLHSTRKQVEEAGDKLPADDKTAIESALNALETALKGEDKAAIEAKMQELAQVSQK
LMEIAQQQHAQQQAGSADASANNAKDDDVVDAEFEEVKDKK (SEQ ID NO:6),

or a sequence substantially identical thereto. The inclusion of the chaperone is optional, as the translocation domain, or a fragment thereof, alone may be sufficient to cause translocation of the antigen to the cytosol; for example, and without wishing to be limiting, YopE alone, or an 18-amino acid fragment thereof (MKISSFISTSLPLPTSVS, SEQ ID NO:2) are presently shown to produce the desired effect. Similarly, expression of the endogenous *Salmonella* chaperone protein InvB is sufficient to mediate the translocation function of SopE (Lee and Galan, 2003).

In one specific example of the present invention, the recombinant bacterium comprises a nucleic acid encoding an antigen comprising a fusion protein comprising the sequence of SycE, YopE, and ovalbumin:

MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE
TLLSHNIFSQDILKPILSWDEVGGHPVLWNRQPLNNLDNNSLYTQLEMLVQGAERLQTS
SLISPPRSFSMKISSFISTSLPLPTSVSGSSSVGEMSGRSVSQQKSEQYANNLAGRTES
PQGSSLASRITEKLSSMARSAIEFIKRMFSEGSHKPVVTPAPTPAQMPSPTSFSDSIKQL
AAETLPKYIQQLSSLDAETLQKNHDQFATGSNFQTAADQARELINSRVESQTNGIIRNVL
QPSSVDSQTAMVLVNAIVFKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRV
ASMASEKMKILELPFASGTMSMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIK
VYLPRMKMEEKYNLTSVLMAMGITDVFSSSANLSGISSAESLKISQAVHAAHAEINEAGR
EVVGSAEAGVDAASVSEEFRADHPFLFCIKHIATNAVLFFGRCVSP (SEQ ID NO:7),

a fusion protein comprising the sequence of SycE, a fragment of YopE, and ovalbumin:

MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE
TLLSHNIFSQDILKPILSWDEVGGHPVLWNRQPLNNLDNNSLYTQLEMLVQGAERLQTS
SLISPPRSFSMKISSFISTSLPLPTSVSGSNFQTAADQARELINSRVESQTNGIIRNVLQPS
SVDSQTAMVLVNAIVFKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASM
ASEKMKILELPFASGTMSMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLP

RMKMEEKYNLTSVLMAMGITDVFSSSANLSGISSAESLKISQAVHAAHAEINEAGREVV
GSAEAGVDAASVSEEFRADHPFLFCIKHIATNAVLFFGRCVSP (SEQ ID NO:8),

a fusion protein comprising the sequence of a fragment of YopE and ovalbumin:

MKISSFISTSLPLPTSVSGSNFQTAADQARELINSRVESQTNGIIRNVLQPSSVDSQTAM
VLVNAIVFKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKIL
ELPFASGTMSMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEK
YNLTSVLMAMGITDVFSSSANLSGISSAESLKISQAVHAAHAEINEAGREVVGSAEAGVD
AASVSEEFRADHPFLFCIKHIATNAVLFFGRCVSP (SEQ ID NO:9),

a fusion protein comprising the sequence of SycE, YopE, and TRP-2:

MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE
TLLSHNIFSQDILKPILSWDEVGGHPVLWNRQPLNNLDNNSLYTQLEMLVQGAERLQTS
SLISPPRSFSMKISSFISTSLPLPTSVSGSSSVGEMSGRSVSQQKSEQYANNLAGRTES
PQGSSLASRITEKLSSMARSAIEFIKRMFSEGSHKPVVTPAPTPAQMPSPTSFSDSIKQL
AAETLPKYIQQLSSLDAETLQKNHDQFATMKISSFISTSLPLPTSVSGSSSVGEMSGRSV
SQQKSEQYANNLAGRTESPQGSSLASRITEKLSSMARSAIEFIKRMFSEGSHKPVVTPA
PTPAQMPSPTSFSDSIKQLAAETLPKYIQQLSSLDAETLQKNHDQFATGSGILLRARAQF
PRVCMTLDGVLNKECCPPLGPEATNICGFLEGRGQCAEVQTDTRPWSGPYILRNQDD
REQWPRKFFNRTCKCTGNFAGYNCGGCKFGWTGPDCNRKKPAILRRNIHSLTAQERE
QFLGALDLAKKSIHPDYVITTQHWLGLLGPNGTQPQIANFSVYDFFVWLHYYSVRDTLL
GPGRPYKAIDFSHQGPAFVTWH (SEQ ID NO:10),

a fusion protein comprising the sequence of SycE, YopE, and gp100:

MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE
TLLSNIFSQDILKPILSWDEVGGHPVLWNRQPLNSLDNNSLYTQLEMLVQGAERLQTSS
LISPPRSFSMKISSFISTSLPLPASVSGSSSVGEMSGRSVSQQKSDQYANNLAGRTESP
QGSSLASRIIERLSSMAHSVIGFIQRMFSEGSHKPVVTPALTPAQMPSPTSFSDSIKQLA
AETLPKYMQQLSSLDAETLQKNHDQFATGSGKNTMDLVLKRCLLHLAVIGALLAVGATK
VPRNQDWLGVSRQLRTKAWNRQLYPEWTEAQRLDCWRGGQVSLKVSNDGPTLIGAN
ASFSIALNFPGSQKVLPDGQVIWVNNTIINGSQVWGGQPVYPQETDDACIFPDGGPCPS
GSWSQKRSFVYVWKTWGQYWQVLGGPVSGLSIGTGRAMLGTHTMEVTVYHRRGSR
SYVPLAHSSSAFTITDQVPFSVSVSQLRALDGGNKHFLRNQPLTFALQLHDPSGYLAEA
DLSYTWDFGDSSGTLISRALVVTHTYLEPGPVTAQVVLQAAI PLT (SEQ ID NO:11),

a fusion protein comprising the nuclear protein ofSycE, YopE, and LCMV-NP :

MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE
TLLSHNIFSQDILKPILSWDEVGGHPVLWNRQPLNNLDNNSLYTQLEMLVQGAERLQTS
SLISPPRSFSMKISSFISTSLPLPTSVSGSSSVGEMSGRSVSQQKSEQYANNLAGRTES
PQGSSLASRITEKLSSMAHSAIEFIKRMFSEGSHKPVVTPAPTPAQMPSPTSFSDSIKQL
AAETLPKYMQQLSSLDAETLQKNHDQFATGSFVSDQVGDRNPYENILYKVCLSGEGWP
YIACRTSIVGRAWENTTIDLTSEKPAVNSPRPAPGAAGPPQVGLSYSQTMLLKDLMGGI
DPNAPTWIDIEGRFNDPVEIAIFQPQNGQFIHFYREPVDQKQFKQDSKYSHGMDLADLF
NAQPGLTSSVIGALPQGMVLSCQGSDDIRKLLDSQNRKDIKLIDVEMTREASREYEDKV
WDKYGWLCKMHTGIVRD (SEQ ID NO:12),

or a sequence substantially identical thereto. The fusion protein further comprises the sequence of the antigen of interest.

A substantially identical sequence may comprise one or more conservative amino acid mutations. It is known in the art that one or more conservative amino acid mutations to a reference sequence may yield a mutant peptide with no substantial change in physiological, chemical, or functional properties compared to the reference sequence; in such a case, the reference and mutant sequences would be considered "substantially identical" polypeptides. Conservative amino acid mutation may include addition, deletion, or substitution of an amino acid; a conservative amino acid substitution is defined herein as the substitution of an amino acid residue for another amino acid residue with similar chemical properties (e.g. size, charge, or polarity).

In a non-limiting example, a conservative mutation may be an amino acid substitution. Such a conservative amino acid substitution may substitute a basic, neutral, hydrophobic, or acidic amino acid for another of the same group. By the term "basic amino acid" it is meant hydrophilic amino acids having a side chain pK value of greater than 7, which are typically positively charged at physiological pH. Basic amino acids include histidine (His or H), arginine (Arg or R), and lysine (Lys or K). By the term "neutral amino acid" (also "polar amino acid"), it is meant hydrophilic amino acids having a side chain that is uncharged at physiological pH, but which has at least one bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Polar amino acids include serine (Ser or S), threonine (Thr or T), cysteine (Cys or C), tyrosine (Tyr or Y), asparagine (Asn or N), and glutamine (Gln or Q). The term "hydrophobic amino acid" (also "non-polar amino acid") is meant to include amino acids exhibiting a hydrophobicity of greater than zero according to the normalized consensus hydrophobicity scale of Eisenberg (1984). Hydrophobic amino acids include proline (Pro or P), isoleucine (Ile or I), phenylalanine (Phe or F), valine (Val or V), leucine (Leu or L), tryptophan (Trp or W), methionine (Met or M), alanine (Ala or A), and glycine (Gly or G).

"Acidic amino acid" refers to hydrophilic amino acids having a side chain pK value of less than 7, which are typically negatively charged at physiological pH. Acidic amino acids include glutamate (Glu or E), and aspartate (Asp or D).

Sequence identity is used to evaluate the similarity of two sequences; it is determined by calculating the percent of residues that are the same when the two sequences are aligned for maximum correspondence between residue positions. Any known method may be used to calculate sequence identity; for example, computer software is available to calculate sequence identity. Without wishing to be limiting, sequence identity can be calculated by software such as NCBI BLAST2 service maintained by the Swiss Institute of Bioinformatics (and as found at http://ca.expasy.org/tools/blast/), BLAST-P, Blast-N, or FASTA-N, or any other appropriate software that is known in the art.

The substantially identical sequences of the present invention may be at least 70%, 80%, 90%, or 95% identical; in another example, the substantially identical sequences may be at least 70, 71, 72, 73, 74, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% identical at the amino acid level to sequences described herein. Importantly, the substantially identical sequences retain the activity and specificity of the reference sequence.

The present invention also encompasses nucleic acids encoding the antigen as described above, as well as vectors comprising the nucleic acid.

Thus, the recombinant bacterium of the present invention may comprise a nucleic acid encoding the antigen as described above, or may comprise a vector (also referred to herein as"plasmid") comprising such nucleic acid that is fused with the nucleic acid of a translocation domain and optionally the nucleic acid of a chaperone; for example, and without wishing to be limiting in any manner, the vector in which antigen is translocated to the cytosol of infected cells may be a modified pHR plasmid. The modified pHR construct uses the type III secretion protein to generate fusion proteins that are transported out of the phagosome and through the host bacterial type III secretion system for direct cytosolic antigen presentation. The pHR constructs may comprise sequences encoding a translocation domain and optionally a chaperone protein to aid in proper trafficking of the downstream fusion protein. In a specific, non-limiting example, the vector may be a modified pHR-241 plasmid (Russmann et al., 2001); more specifically, the pHR-241 comprising the sequence of SycE-YopE-p60/M45 fusion protein (Russmann et al., 2000) modified to remove the sequence of p60/M45. In another example, the pHR241 vector is modified to comprise YopE or a fragment of YopE (for example, but not limited to MKISSFISTSLPLPTSVS, SEQ ID NO:2) with the sequence for the SycE and p60/M45 proteins removed. Replacement of the antigenic sequence by appropriate

restriction enzymes and subsequent ligation of other antigens would result in development of the desired CD8+ T cell response against said antigens upon vaccination. Furthermore, the recombinant bacterium that harbors this plasmid need not be a highly virulent bacterium; for example, attenuated *Salmonella* is presently shown to be effective at inducing the desired response. The response can be accentuated further by vaccination with higher doses of the attenuated strain.

The recombinant bacterium as described above may be utilized to impart immunity against other naturally-occurring and virulent bacteria. This may be accomplished by administering an effective amount of the recombinant bacterium of the present invention to a subject, and allowing a $CD8^+$ T cell response to be mounted. Similarly, the recombinant bacterium may be utilized to impart immunity against tumors in a subject, by administering an effective amount of the recombinant bacterium of the present invention to said subject. In both methods, the recombinant bacterium may be administered through intravenous, oral or subcutaneous routes of immunization. This approach avoids the unwanted side-effects of persisting bacteria and undesirable toxicity/inflammation associated with live vaccines. Thus, higher doses of the vaccine can be used for improved efficacy. Because the recombinant bacterium of the present invention is eliminated after a few weeks, there is little concern regarding toxicity. Furthermore, *Salmonella* when given orally induces a mucosal CD8+ T cell response (Jones and Falkow, 1996). Thus, the modified bacterium can be administered through the oral route for induction of the desired CD8+ T cell response.

The recombinant bacterium as described above may also be utilized as a vaccine; the vaccine may protect against other naturally-occurring and virulent bacteria, other bacterial pathogens, viral pathogens, or tumors. When the antigen is a tumour-antigen, the tumour-antigen will be translocated to the host cell cytosol, resulting in rapid activation of tumor-specific $CD8^+$ T cells, which will translate to better tumour control by tumor-specific CD8+ T cells.

A recombinant ST that injects an antigen directly into the host cytosol has presently been developed. This results in profound $CD8^+$ T cell activation and consequent elimination of ST. It is also shown that when $CD8^+$ T cells are engaged in this manner, they undergo profound expansion which results in massive pathogen and tumour control as well as abridgment of pathogen chronicity. The present data provide novel insights into the incapacity of the immune system to efficiently control the bacterium, as well as reveal the power of the acquired immune system, wherein engagement of potent antigen-presentation early on may be sufficient to control an otherwise uncontrollable bacterium. The present results provide compelling evidence that modulation of the cell biology of antigen trafficking is a key avenue that is employed by various pathogens for immune evasion. The recombinant bacterium described

herein may be used as a novel vaccine, wherein a key modification makes the bacterium generate rapid, potent CD8+ T cell response, resulting in self-destruction of the vaccine *in vivo*, making it highly efficacious, safe and cost-effective.

The utility of the recombinant bacterium described herein is demonstrated using OVA, TRP-2, and gp-100 as antigens. Using a similar approach, other putative antigens from other pathogens (bacteria, virus) or tumours can be cloned into the recombinant bacterium; these antigens can then be translocated into the host cell cytosol for rapid and potent antigen-presentation using the a translocation domain/chaperone system.

The present invention will be further illustrated in the following examples. However, it is to be understood that these examples are for illustrative purposes only and should not be used to limit the scope of the present invention in any manner.

Example 1: Preparation of recombinant bacteria

Recombinant bacteria comprising *Salmonella enterica*, serovar Typhimurium (ST) expressing ovalbumin (OVA) were prepared. Construct ST-OVA-NT, which does not translocate antigen to the cytosol, was prepared as previously described (Luu et al., 2006). A recombinant construct, ST-OVA-T, that produces an OVA fusion protein that is translocated to the cytosol; Figure 1A shows a schematic of the fusion protein, where OVA is fused to YopE and SycE. YopE is a 23kDa protein comprising a N-terminal secretion domain (~11 aa) and a translocation domain (at least 50 aa); the latter domain provides the binding site for the YopE-specific chaperone (SycE) that is required for YopE-mediated translocation of fused proteins to the cytosol (R). SycE is a chaperone necessary for translocation of the fused protein into the cytosol of infected cells through the type III secretion system of ST. A schematic of both ST-OVA-NT and ST-OVA-T constructs and their proposed actions are shown in Figure 1B.

Plasmid pHR-OVA was constructed by the modification of the plasmid pHR-241 (Russmann et al., 2001), which contains the sequence of the fusion protein SycE-YopE-p60/M45 (Russmann et al., 2000). In a first step, the genes of p60/M45 were removed by cutting plasmid pHR-241 with BamHI and KpnI. Then, the pKK-OVA plasmid was purified from the recombinant ST-OVA-NT bacteria by mid prep kit (Invitrogen, US) according to the manufacturer's instructions. The OVA gene was PCR-amplified using the plasmid pKK-OVA as a template (forward primer BamHI 5'-CGGGATCCAACTTTCAAACAGCTG-3' (SEQ ID NO:13) and reverse primer KpnI 5'-GGGGTACCTTAAGGGGAAACACATC-3' (SEQ ID NO:14). Subsequently, the OVA gene was inserted between the BamHI-KpnI sites of the cut pHR-241 palsmid, creating new plasmid pHR-OVA. PCR amplification of the inserts was performed with Taq polymerase using the

following cycling parameters: 94°C, 5 min; 25 cycles of 94°C, 30 s to 58°C, 1 min to 72°C, 1 min; followed by a 7 min extension time at 72°C. The amplified insert was ligated into the intended vector then sequenced to verify the accuracy of the amplified cDNA. The pHR-OVA plasmid was then transfected into the highly virulent ST (strain SL1344). 50 µL of electrocompetent *Salmonella* (WT or *aroA*) were mixed with ~20 ng plasmid DNA and pulsed in a Bio-Rad micropulser using one pulse of 2.5 kV. Immediately afterwards, 1 mL of SOC recovery medium was added to the bacteria and they were allowed to recover with shaking at 37°C. The bacteria were then plated on LB agar plates with ampicillin for the selection of individual clones.

Example 2: Detection of antigen

ST-OVA-NT and ST-OVA-T constructs of Example 1 were grown and expression and translocation of ovalbumin was evaluated. Pellet and supernatant of ST-OVA-NT and ST-OVA-T growing in liquid cultures were tested for the presence of OVA.

C57BL/6J mice were injected intravenously with 10^6 ST-OVA-NT or ST-OVA-T reconstituted in 200 microlitres normal saline. Two days later, spleens were obtained from infected mice; spleen cells were isolated and lysed with Triton X-100 in the presence of protease inhibitor, phenylmethylsulfonyl fluoride. The soluble lysate containing cytosolic proteins was tested for OVA expression by western blotting. Samples were normalized for cell number and were loaded on SDS-10% polyacrylamide gels. SDS-PAGE was performed and proteins were transferred to membranes, which were then blocked with 5% skim milk powder in PBS-Tween. OVA expression was detected using a 1/10,000 dilution of polyclonal anti-OVA antibody (Sigma-Aldrich), followed by incubation with HRP-conjugated goat anti-rabbit Ab (1/5,000 dilution in PBS-Tween) from Roche Applied Science. Immuno-reactive bands were detected with enhanced chemiluminescence substrate (Roche Applied Bioscience). Results show that OVA-expression by ST-OVA-NT and ST-OVA-T (from ~$5x10^6$) in the bacterial pellets was similar (Fig. 1 C). However, OVA could only be detected in the supernatant of ST-OVA-T cultures. Expression of OVA was detectable in the cytosol of spleen cells from mice infected with ST-OVA-T- but not ST-OVA-NT (Fig. 1 C).

Example 3: Proliferation of ST-OVA-T and ST-OVA-NT

The ability of ST-OVA to proliferate extra- and intra-cellularly was also analyzed.

Liquid cultures of ST-OVA-NT and ST-OVA-T were set up in flasks to enumerate extracellular proliferation. At various time intervals (eg, 60 min., 120 min., 240 min., etc), aliquots were

removed for measurement of OD at 600nm. Both ST-OVA-NT and ST-OVA-T displayed similar proliferation and doubling time (Fig. 2A).

The influence of antigenic translocation on the ability of ST-OVA to proliferate within the intracellular compartment was evaluated. IC-21 macrophages (H-2^b) ($5x10^4$/well) were infected with ST-OVA-NT or ST-OVA-T (MOI=10). After 30 min, cells were washed and cultured in media containing gentamicin (50 µg/ml) to remove extracellular bacteria. After 2 h, cells were washed again and cultured in media containing reduced levels of gentamicin (10µg/ml). At various time intervals cells were lysed and bacterial burden in the cells determined. No statistically significant difference was detected in the ability of ST-OVA-NT or ST-OVA-T to infect and replicate within macrophages ($p>0.05$). Results are shown in Figure 2B and are representative of three independent experiments. Thus, the ability of ST-OVA to infect and survive within macrophages *in vitro* was not influenced by antigenic translocation.

Example 4: Translocation and antigen presentation

It was previously reported that ST-OVA-NT infection does not induce a detectable $CD8^+$ T cell response within the first week of infection (Luu et al., 2006), due to delayed presentation of antigen to $CD8^+$ T cells (Albaghdadi et al., 2009). Therefore, it was evaluated whether translocation of antigen to the cytosol would result in rapid antigen-presentation.

In vitro antigen-presentation was performed as previously described (Albaghdadi et al., 2009). IC-21 macrophages (H-2^b) cells (10^5/well) were infected with different MOI of ST (Albaghdadi et al., 2009), ST-OVA-NT (Example 1), or ST-OVA-T (Example 1) for 30 min. Extracellular bacteria were removed after incubation in medium containing gentamicin (50 µg/ml). At 2 h, cells were cultured in media containing lower levels of gentamicin (10 µg/ml) and incubated with CFSE-labelled OT-1 ($CD45.1^+45.2^-$) TCR transgenic cells (10^6/well). After 4 days of culture, cells were harvested, stained with anti-CD45.1 and anti-CD8 antibodies, and the reduction in CFSE intensity of OT-1 $CD8^+$ T cells was evaluated by flow cytometry.

Infection of macrophages with ST or ST-OVA-NT did not result in any detectable proliferation of OT-1 cells, indicating lack of antigen-presentation (Fig. 3A). Interestingly, infection with ST-OVA-T, even at reduced doses, resulted in strong dilution of CFSE expression, which is indicative of rapid and potent antigen-presentation in vitro (Fig. 3A).

In vivo antigen-presentation was done as previously described (Albaghdadi et al., 2009). B6129F1 mice were infected with the recombinant bacteria of Example 1, followed by adoptive transfer of CFSE labelled OT-1 cells. B6.129F1 mice were used because B6 parents are

highly susceptible and die within the first week of infection (Albaghdadi et al., 2009). Briefly, B6129F1 mice were generated in house by mating 129x1SvJ female mice with C57BL/6J male mice; mice were obtained from The Jackson Laboratory and were maintained at the Institute for Biological Sciences (National Research Council of Canada, Ottawa, Canada) in accordance with the guidelines of the Canadian Council on Animal Care. For immunization, frozen stocks of ST-OVA-NT or ST-OVA-T (Example 1) were thawed and diluted in 0.9% NaCl; mice were inoculated (iv) with 10^3 organisms suspended in 200 µl. At various time intervals, CFSE-labelled OT-1 cells were injected ($5x10^6$, iv). Four days after the transfer of OT-1 cells, spleens were isolated from recipient mice and spleen cells were stained with OVA-tetramer and anti-CD8 antibody. Reduction in the expression of CFSE intensity was evaluated by flow cytometry, as described above.

Results are shown in Figures 3B and 3C; results represent the mean of three mice ± SD per group, and are representative of two-three independent experiments. At day 5 of infection, the majority of transferred OT-1 cells displayed reduced expression of CFSE in mice infected with ST-OVA-T (Fig. 3B). In contrast, OT-1 cells in ST-OVA-NT-infected mice maintained high levels of CFSE expression. When *in vivo* antigen-presentation was evaluated kinetically, ST-OVA-NT infected mice displayed muted and delayed activation of CFSE-labelled OT-1 cells (Fig. 3C). Interestingly, the massive antigen-presentation that was induced early on in ST-OVA-T infected mice was subsequently reduced to baseline levels as the pathogen was cleared.

Example 5: Antigen translocation and $CD8^+$ T cell response

The question of whether the induction of rapid antigen-presentation *in vitro* and *in vivo* by antigenic translocation to the cytosol would result in the development of a rapid $CD8^+$ T cell response *in vivo* and whether this had any influence on pathogen control was examined.

B6.129F1 mice were infected (10^3, iv) with ST-OVA-T or ST-OVA-NT without any adoptive transfer of OT-1 cells. At various time intervals, the numbers of spleen cells, spleen size and bacterial burden were evaluated. OVA-specific CD8+ T cell response was enumerated by Flow cytometry. Briefly, aliquots of spleen cells (5×10^6) were incubated in 80µl of PBS plus 1% BSA (PBS-BSA) with anti-CD16/32 at 4°C. After 10 min., cells were stained with H-$2K^bOVA_{257-264}$ tetramer-PE (Beckman Coulter, US) and various antibodies (anti-CD8 PerCP-Cy5, anti-CD62L APC-Cy7, and anti-CD127 (PE-Cy7) for 30 min. All antibodies were obtained from BD Biosciences. Cells were washed with PBS, fixed in 0.5% formaldehyde and acquired on a BD Biosciences FACSCanto analyzer.

Results are shown in Figure 4; these results represent the mean of three to five mice ± SD per group and are representative of three independent experiments. Infection of mice with ST-OVA-T resulted in the development of a rapid and potent OVA-specific $CD8^+$ T cell response as evaluated by staining with OVA-tetramers (Fig. 4D, 4E; Fig. 5A); these mice displayed reduced spleen cell numbers and size (Fig. 4A, 4B). At day 3 of infection, similar bacterial burdens were noted in mice that received ST-OVA-T or ST-OVA-NT (Fig. 4C). However, at subsequent time intervals, the burden of ST-OVA-T were enormously controlled which was reduced to non-detectable levels by day 30. In contrast, ST-OVA-NT burden was maintained at high levels and the burden was detectable even at day 60 (Fig. 4C). Interestingly, at day 60, while both groups of mice had similar numbers of OVA-$tetramer^+$ cells (Fig. 4E), the ST-OVA-T group of mice had controlled the burden whereas the ST-OVA-NT group of mice failed to control it (Fig. 4C); this suggests that direct antigen-presentation in case of ST-OVA-T makes the targets susceptible.

Phenotypic analysis of OVA-specific $CD8^+$ T cells induced against ST-OVA-T versus ST-OVA-NT was also performed. Figure 5A shows the OVA-tetramer profile in the spleens of infected mice, and the expression (MFI) of CD62L (Fig. 5B, 5D) and CD127 (Fig. 5C, 5D) on OVA-$tetramer^+CD8^+$ T cells. In contrast to ST-OVA-NT, OVA-specific $CD8^+$ T cells induced against ST-OVA-T displayed rapid activation (CD62L down-regulation) and rapid progression to the memory state (CD127 up-regulation) (Fig. 5B-D). Taken together, these results clearly indicate that antigenic translocation to the cytosol in the context of ST infection accelerates the kinetics and increases the potency of antigen-presentation, $CD8^+$ T cell differentiation, and memory development. Thus, the differentiation of $CD8^+$ T cells that is noted with ST-OVA-T infection mirrors the one that is induced against the potent pathogen, LM.

Example 6: Rapid $CD8^+$ T cells response and survival of susceptible mice.

Given the results noted with antigenic translocation in resistant mice (Example 5), determination of whether the rapid induction of $CD8^+$ T cells would influence the survival of susceptible C57BL/6J mice was undertaken.

C57BL/6J mice were infected (10^3, iv) with ST-OVA-T or ST-OVA-NT. At different time points (day 1, 3, 5, 7 and 14) after infection, spleens were removed and the bacterial burdens were enumerated. Spleen cells were stained with OVA-tetramers and antibodies against CD8, CD62L and CD127. The percentage and numbers of OVA-specific $CD8^+$ T cells were determined, as was the expression of CD62L versus CD127 on OVA-$tetramer^+CD8^+$ T cells.

Results are shown in Figure 6 and represent the mean of three to four mice ± SD per group; results are representative of two independent experiments. At days 1 and 3, similar bacterial burdens were noted in ST-OVA-NT- and ST-OIVA-T-infected groups (Fig. 6A). At later time periods, while the bacterial burden in ST-OVA-NT-infected mice continued to increase exponentially to lethal levels, the burden in ST-OVA-T-infected mice was rapidly controlled and became undetectable after day 14. Abridgment of bacterial burden in ST-OVA-T-infected mice correlated to the early emergence of potent OVA-specific $CD8^+$ T cell response, as detected by OVA-tetramer staining (Fig. 6B-C) that peaked at day 7.

Example 7: Antigen translocation induces functional $CD8^+$ T cells

Two functional assays were carried out to determine whether the CD8+ T cells that were induced by antigenic translocation would result in induction of CD8+ T cells that mediate appropriate functions.

Enumeration of IFN-γ secreting cells was performed by ELISPOT assay as reported previously at day 7 of infection (Dudani et al., 2002). ST-OVA-T-infected mice mounted a profound $CD8^+$ T cell response (Fig. 6D), indicative of IFN-γ production. In contrast, infection of susceptible mice with ST-OVA-NT did not result in any detectable IFN-γ-secreting $CD8^+$ T cells.

The ability of stimulated $CD8^+$ T cells to kill target cells specifically was enumerated as another functional readout. To do this, CFSE-labelled, OVA-pulsed and control spleen cells from naïve mice were transferred to ST-OVA-T- and ST-OVA-NT-infected mice on day 7, and the specific killing of OVA-pulsed targets was evaluated. In vivo cytolytic activity of $CD8^+$ T cells was performed according to previously published reports (Luu et al., 2006; Barber et al., 2003). OVA-specific CD8+ T cells that were induced at day 7 in ST-OVA-T-infected mice displayed rapid, potent and specific cytolytic activity towards OVA-pulsed target cells (Fig. 6E-F). In contrast, ST-OVA-NT-infected mice displayed little cytolytic activity, as expected (Luu et al., 2006). Thus, the kinetics of CD8+ T cells response in ST-OVA-T infected susceptible C57BL/6J was similar to that observed in resistant B6.129 F1 mice, as was their phenotype.

Figure 7 shows results of phenotypic analysis of OVA-specific $CD8^+$ T cells induced against ST-OVA-T versus ST-OVA-NT. Similar to the profile in resistant mice, OVA-specific $CD8^+$ T cells induced against ST-OVA-T in susceptible mice displayed rapid down-regulation of CD62L and rapid transition to the memory phenotype (increased CD127 up-regulation; Figure 7).

Example 8: Control of bacterial growth

While the data of Example 6 indicated that rapid emergence of functional $CD8^+$ T cells by antigenic translocation can control ST burden rapidly, it was still correlative. In order to test if the rapid emergence of $CD8^+$ T cells are responsible for elimination of bacteria during ST-OVA-T infection, C57BL/6J mice were treated with anti-CD8 or anti-CD4 antibody or isotype control then infected with ST-OVA-T to eliminate those cells specifically. C57BL/6J mice were treated with (100 μg/injection) anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43), or Rat IgG isotype antibodies on days -3, 0 and 3 after infection with 10^3 ST-OVA-T. At day 7 after infection, spleens were harvested and the bacterial burden evaluated. At day 7 after infection, anti-CD8 antibody treated mice had a >100-fold higher ST-OVA-T burden (Fig. 8A), suggesting that when $CD8^+$ T cells are depleted, ST-OVA-T cannot be controlled by the host. Depletion of $CD4^+$ T cells had no effect on the bacterial burden.

The importance of $CD8^+$ T cells in controlling bacterial burden was further confirmed by infecting WT, MHC-I or MHC-II-deficient C57BL/6J mice with ST-OVA-T. Since MHC class I deficient mice lack $CD8^+$ T cells they should be susceptible to infection. Twenty days after infection, MHC-I deficient mice were moribund, displaying very high bacterial loads (Fig. 8B) whereas control mice had undetectable burden, and MHC class II-deficient hosts (lacking $CD4^+$ T cells) showed only a minor effect. MHC-I deficient mice were sick due to high bacterial loads, while MHC-II-deficient and WT mice were healthy. Taken together, these results indicate that antigenic translocation to cytosol in the context of ST infection results in a rapid emergence of a potent $CD8^+$ T cell response which is sufficient to control the burden.

Example 9: Translocation of antigen in attenuated strain of Salmonella

In order to design vaccines, attenuated strains of bacteria are often used to avoid undesirable toxicity that occurs with highly virulent bacteria. It was therefore determined whether translocation of OVA in a highly attenuated strain of ST (ΔaroA) would induce rapid activation of $CD8^+$ T cells.

B6.129F1 mice were infected with 10^3 (virulent) wild type (WT; SL1344)) or 10^5 attenuated (avirulent; Δ*aroA*) ST-OVA expressing non-translocated or non-translocated OVA. At various time intervals (day 7, 14, 21 and 30), spleens and peripheral blood were collected and the relative change in the numbers of OVA-specific $CD8^+$ T cells enumerated after staining with OVA-tetramers and anti-CD8 antibodies as described in Example 5.

Results in Figure 9 represent the mean of five mice ± SD per group. Translocation of OVA by avirulent ST also resulted in rapid and profound induction of OVA-specific $CD8^+$ T cell response in the spleen (Fig. 9A) and peripheral blood (Fig. 9B). Thus, these results indicate that antigenic translocation works equally well for virulent and avirulent bacteria.

Example 10: Translocation of antigen and tumour control.

It was also investigated whether antigenic translocation would result in effective protection upon tumour challenge.

C57BL/6J mice were infected with 10^3 ST-OVA-T; non-infected (naïve) mice served as controls. On day 60, mice were challenged subcutaneously in the lower dorsal region with 10^6 B16 melanoma cells carrying the OVA gene (B16-OVA). Survival of mice was measured subsequently. As shown in Figure 10A, prophylactic vaccination with ST-OVA-T resulted in potent protection against tumour challenge. Protection in a therapeutic model, where mice were first challenged with tumours and then vaccinated with immunogens, was also tested. B6.129F1 mice were challenged first with 10^6 B16-OVA tumour cells subcutaneously in the lower dorsal region. Three days later, mice were vaccinated with ST-OVA-NT or ST-OVA-T. Non-infected mice served as negative controls and LM-OVA infected mice served as positive controls. At various time intervals subsequently, survival of mice was monitored. Mice receiving ST-OVA-T displayed the best protection against B16 melanoma cells (Figure 10B). Protection induced by ST-OVA-T was far greater than that induced by ST-OVA-NT and LM-OVA. Results represent the mean of five mice ± SD per group.

Example 11: CD8+ T cell response against tumor-antigens.

The use of OVA as an immunodominant antigen is described herein as a proof of principle. Using a similar approach, other putative antigens from other pathogens (bacteria, virus) or tumours can be cloned into ST and these antigens can be translocated into the host cell cytosol for rapid and potent antigen-presentation using the YopE/SycE system.

The gene for the tumour-antigen (Trp-2) (Schumacher and Restifo, 2009) was cloned into the WT or *aroA* mutant of ST, which translocates antigen to the cytosol. PCR was done using pCDNA3-Trp2 as template using the following primers:

Forward primer: TAGGATCCGGAATTCTGCTCAGAG (SEQ ID NO:15), and

Reverse primer: AGATGGTACCTTTAGTGCCACGTG (SEQ ID NO:16).

The PCR product and pHR-OVA were digested with BamHI and KpnI and ligated. PCR amplification of the inserts was performed with Taq polymerase using the following cycling parameters: 94°C, 5 min; 25 cycles of 94°C, 30 s to 58°C, 1 min to 72°C, 1 min; followed by a 7 min extension time at 72°C. The amplified insert was ligated into the intended vector, then sequenced to verify the accuracy of the amplified cDNA. The PCR product was digested with BglII and KpnI; pHR-241 was digested with BamHI and KpnI and the digested products were ligated. pHR-Trp2 plasmid was then transfected into the highly virulent ST (SL1344) or *aroA* mutant of ST. 50 µL of electrocompetent *Salmonella* (WT or aroA) were mixed with ~20 ng plasmid DNA and pulsed in a Bio-Rad micropulser using one pulse of 2.5 kV. Immediately afterwards, 1 mL of SOC recovery medium was added to the bacteria and they were allowed to recover shaking at 37°C. The bacteria were then plated on LB agar plates with ampicillin for the selection of individual clones.

The gene for gp100 tumour-antigen (Rosenberg et al., 2008) was cloned into a pHR or pKK plasmid. PCR was done using pCDNA3-gp100 as template with the following primers:

Forward primer: GAAGATCTGGGAAGAACACAATGG (SEQ ID NO:17), and

Reverse primer: GGGGTACCTTAGGTGAGAGGAATGG (SEQ ID NO:18).

The PCR product was digested with BglII and KpnI; pHR-241 was digested with BamHI and KpnI and the digested products were ligated. Infection of B6.129F1 mice with these recombinant nucleic acids resulted in the induction of CD8+ T cell response against Trp-2 (Fig. 11A). This was associated with accelerated control of the bacterium (Fig. 11B,C). Similarly, infection of mice with the gp100 expressing aroA-ST resulted in accelerated control of the bacterium (FIGURE 12A) and induction of a better CD8+ T cell response against gp100 (FIGURE 12B).

Example 12: CD8+ T cell response to a viral antigen.

The immunodominant epitope recognized to stimulate a $CD8^+$ T cell response from LCMV nucleoprotein (NP) in C57Bl/6 mice was also used as an antigen and cloned into ST, and its effect on T cell response in mice was evaluated.

LCMV-NP was encoded over amino acids 396-404 (FQPQNGQFI) of the protein (Basler et al., 2004). cDNA encoding amino acids 288 – 463 of the NP protein was cloned into plasmid pKK to generate pKK-NP (Fig. 13), using PCT methods as described in Example 1 and 11. Again, DH5α clones were selected using ampicillin. In this case, NcoI and HindIII restriction sites

were added to the oligonucleotides used for amplification of the insert sequence. The oligonucleotide sequences used for the cDNA amplification were:

5' TACCATGGCATTTGTTTCAGACCAAGT 3' (SEQ ID NO:19) and

5' TAAAGCTTCTAGTCCCTTACTATTCCAG 3' (SEQ ID NO:20).

The final insert in the pKK plasmid was truncated prior to the end of the amplified insert due to the presence of an internal HindIII restriction site, ending at codon 461. After confirmation of the sequence, this plasmid was also transferred into ST wild type and STΔAro using a standard electroporation protocol (as described below and in Examples 1 and 11). cDNA encoding amino acids 288 – 461 of the NP protein was similarly cloned into the plasmid, pKK, to generate pKK-NP (Fig. 13). Again, DH5α clones were selected using ampicillin. In this case, NcoI and HindIII restriction sites were added to the oligonucleotides used for amplification of the insert sequence. The oligonucleotide sequences used for the cDNA amplification are:

5' TACCATGGCATttgtttcagaccaaGT 3' (SEQ ID NO:21) and

5' TAAAGCTTCTAGTCCCTTACTATTCCAG 3' (SEQ ID NO:22).

The final insert in the pKK plasmid was truncated prior to the end of the amplified insert due to the presence of an internal HindIII restriction site, ending at codon 461. After confirmation of the sequence, this plasmid was also transferred into ST wild type and STΔAro using a standard electroporation protocol. Briefly, 50 µL of electrocompetent *Salmonella* (WT or *aroA*) were mixed with ~20 ng plasmid DNA and pulsed in a Bio-Rad micropulser using one pulse of 2.5 kV. Immediately afterwards, 1 mL of SOC recovery medium was added to the bacteria and they were allowed to recover shaking at 37°C. The bacteria were then plated on LB agar plates with ampicillin for the selection of individual clones. PCR amplification of the inserts was performed with Taq polymerase using the following cycling parameters: 94°C, 5 min; 25 cycles of 94°C, 30 s to 58°C, 1 min to 72°C, 1 min; followed by a 7 min extension time at 72°C. The amplified insert was ligated into the intended vector then sequenced to verify the accuracy of the amplified cDNA.

B6.129F1 mice were infected intravenously with 10^3 recombinant ST expressing NP. Both virulent (Fig. 13B,C) and avirulent (Fig. 13D) ST induced profound NP-specific CD8+ T cell response when NP was translocated to the cytosol. Furthermore, antigenic translocation

resulted in decreased bacterial burden (Fig. 13E) and control of vaccine induced inflammation (Fig. 13F).

Example 13: Use of truncated YopE as a means to induce potent CD8+ T cell response.

To determine whether the full length YopE was needed for induction of a better CD8+ T cell response, or whether a truncated version of this protein would be sufficient, the gene for OVA was fused with truncated YopE (first eighteen amino acids only), which does not carry the C-terminal domain for binding to the SycE chaperon (Fig. 14A). PCR was done using pHR-OVA as template with the following primers:

Forward primer: GTGTCAAAGTTGGGGAATTCGC (SEQ ID NO:23), and

Reverse primer: CTGCTGGATCCTGACACTGATG (SEQ ID NO:24).

The PCR product and pHR-OVA were digested with EcoRI and BamHI and ligated. PCR amplification of the inserts was performed with Taq polymerase using the following cycling parameters: 94°C, 5 min; 25 cycles of 94°C, 30 s to 58°C, 1 min to 72°C, 1 min; followed by a 7 min extension time at 72°C. The amplified insert was ligated into the intended vector, then sequenced to verify the accuracy of the amplified cDNA.

B6.129F1 mice were infected with ST-OVA-NT, ST-OVA-T (carrying full length YopE), and ST-OVA-t*YopE* (carrying truncated *YopE*). As is clear from results shown in Figure 14B, the fusion of the desired antigen with the first eighteen amino acids of *YopE* is sufficient to induce rapid CD8+ T cell response. CD8+ T cells induced by the truncated *YopE* differentiated rapidly into memory cells (Fig. 14C), which lead to curtailment of inflammation (Fig. 14D) and bacterial burden (Fig. 14E).

The embodiments and examples described herein are illustrative and are not meant to limit the scope of the invention as claimed. Variations of the foregoing embodiments, including alternatives, modifications and equivalents, are intended by the inventors to be encompassed by the claims. Furthermore, the discussed combination of features might not be necessary for the inventive solution.

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CLAIMS:

1. A recombinant bacterium, comprising a nucleic acid encoding an antigen that is translocated to the cytosol of a host cell.

2. The recombinant bacterium of claim 1, wherein the bacterium is an intracellular bacterium such as *Salmonella*, *Mycobacteria*, *Brucella*, or *Leishmania.*

3. The recombinant bacterium of claim 1, wherein the bacterium is *Salmonella*.

4. The recombinant bacterium of any one of claims 1 to 3, wherein the protein is a viral antigen, a bacterial antigen, or a tumour antigen.

5. The recombinant bacterium of any one of claims 1 to 4, wherein the antigen is a fusion protein comprising an antigen and a translocation domain from a type III secretion system.

6. The recombinant bacterium of claim 5, wherein the translocation protein is YopE, SopE, SptP, or a fragment thereof.

7. The recombinant bacterium of claim5 or 6, wherein the fusion protein further comprises a chaperone.

8. The recombinant bacterium of claim 7, wherein the chaperone is derived from type III secretion systems.

9. The recombinant bacterium of claims 8, wherein the chaperone is SycE or HSP70.

10. The recombinant bacterium of any one of claims 1 to 9, wherein the nucleic acid is comprised in a vector.

11. The recombinant bacterium of claim 10, wherein the vector is a pHR vector.

12. The recombinant bacterium of claim 10, wherein the vector is pHR-241.

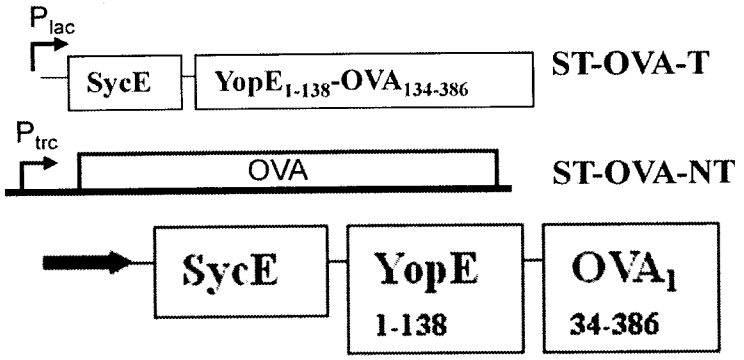
13. The recombinant bacterium of any one of claims 1 to 12, wherein the antigen is tyrosinase related protein 2 (TRP-2), MART-1, melanoma associated antigen 1 (MAGE1), or Her-2/neu, gp100, or other viral or bacterial antigens.

14. A method of imparting immunity against naturally-occurring bacterium or virus in a subject, the method comprising administering the recombinant bacterium of any one of claims 1 to 13 to said subject.

15. A method of imparting immunity against tumors in a subject, the method comprising administering the recombinant bacterium of claim 13 to said subject.

16. The method of claim 14 or 15, wherein the recombinant bacterium is administered through intravenous, oral or subcutaneous routes of immunization.

17. The use of the recombinant bacterium of any one of claims 1 to 13 as a vaccine.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YopE 1-138 -OVA 134-386
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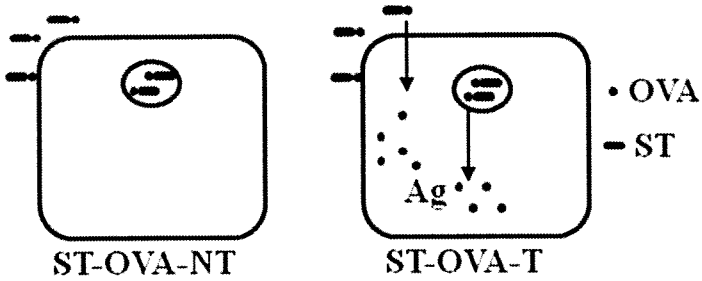
FIG. 1A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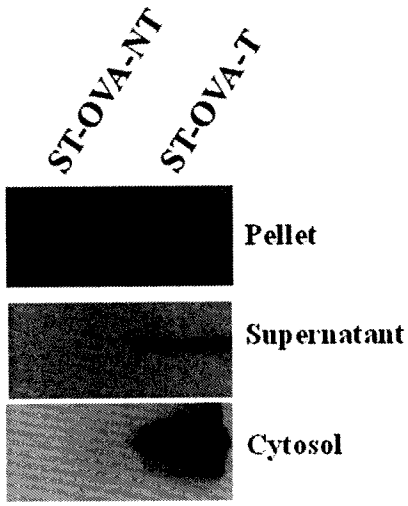
Ag
ST-OVA-NT
ST-OVA-T

FIG. 1B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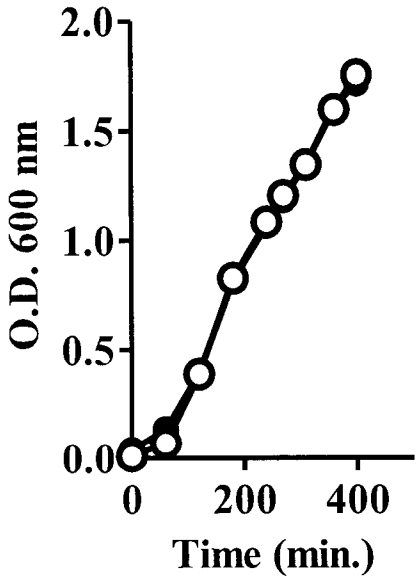
ST-OVA-T
Pellet
Supernatant
Cytosol

FIG. 1C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1.0
0.5
0.0
O.D. 600 nm
0
200
400
Time (min.)

FIG. 2A

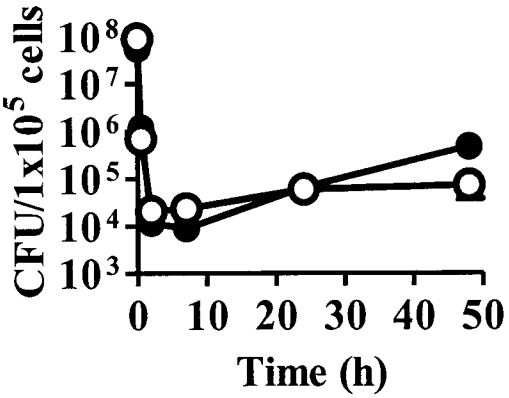
CFU/1x10^5 cells
10^8
10^7
10^6
10^5
10^4
10^3
0
10
20
30
40
50
Time (h)

FIG. 2B

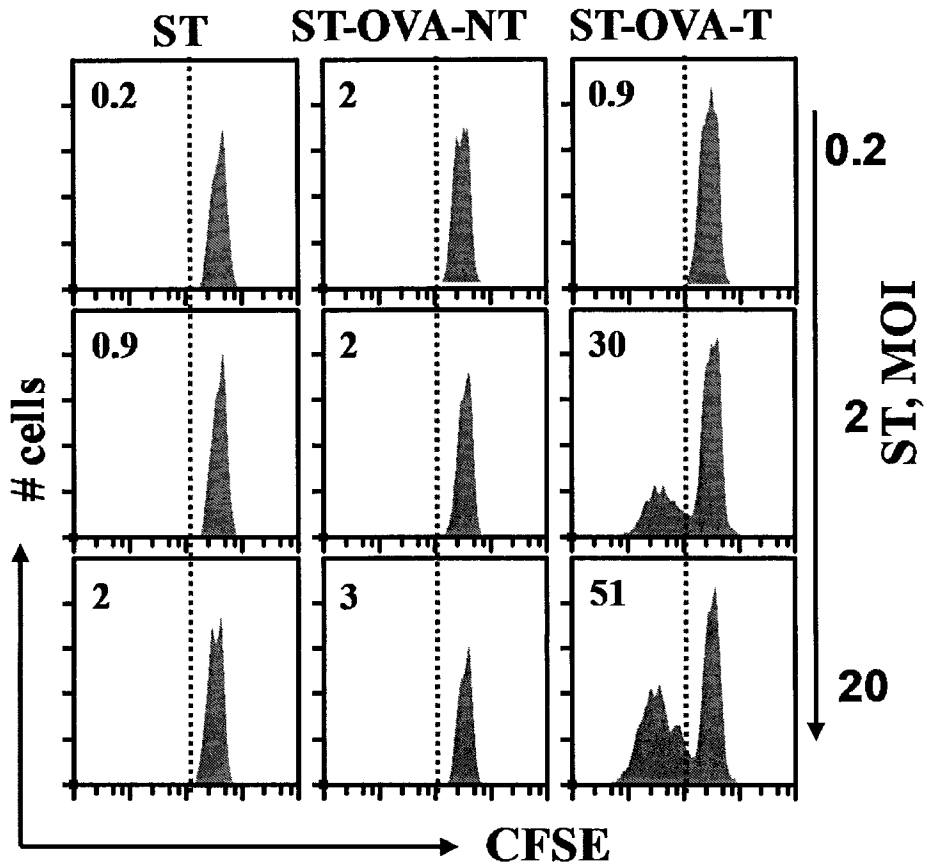
ST
ST-OVA-NT
ST-OVA-T
0.2
2
0.9
0.9
2
30
2
3
51
cells
0.2
2
20
ST, MOI
CFSE

FIG. 3A

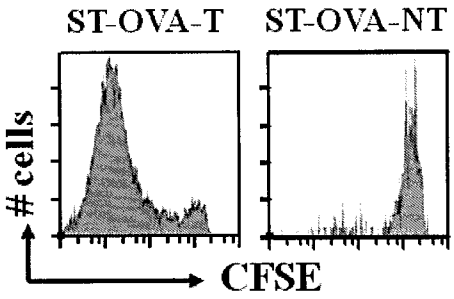

FIG. 3B

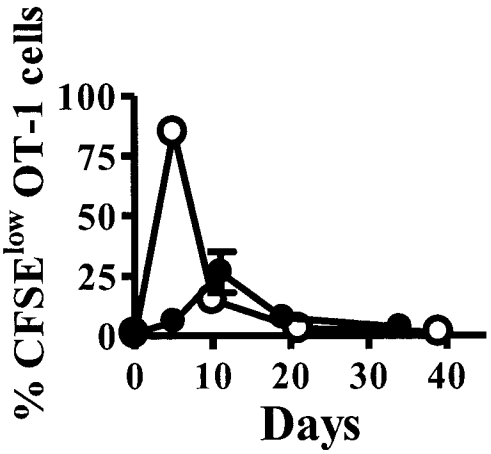

FIG. 3C

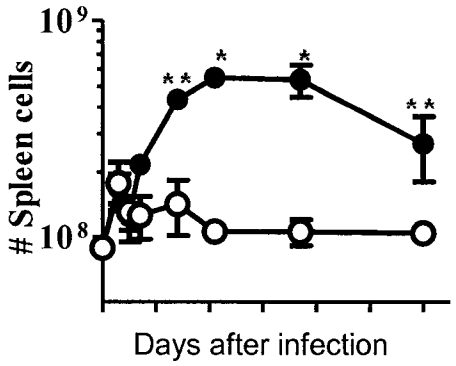

FIG. 4A

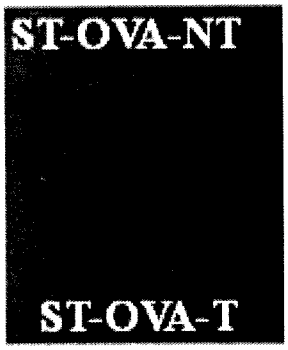

FIG. 4B

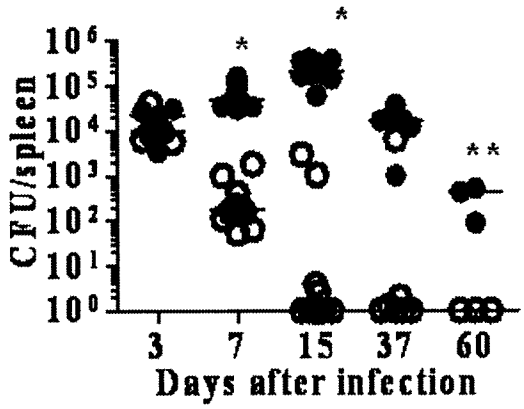

FIG. 4C

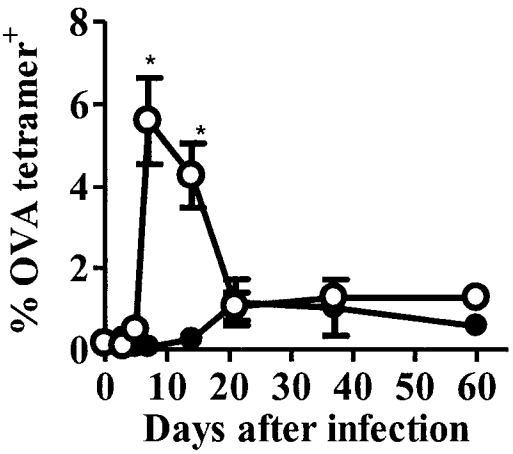

FIG. 4D

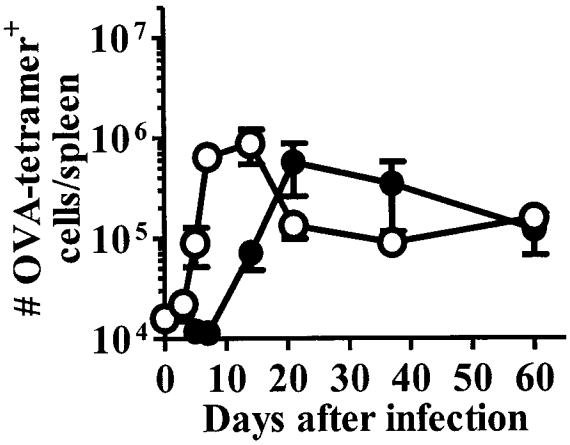
OVA-tetramer+ cells/spleen
10^7
10^6
10^5
10^4
0 10 20 30 40 50 60
Days after infection

FIG. 4E

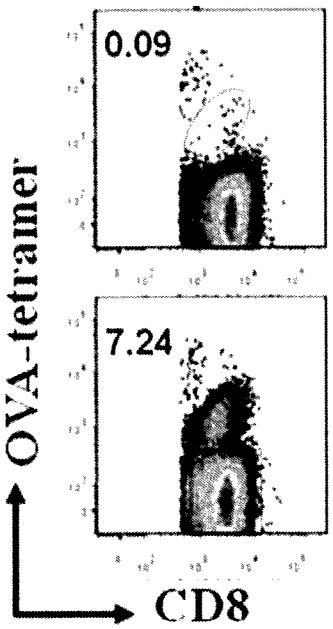
0.09
7.24
OVA-tetramer
CD8

FIG. 5A

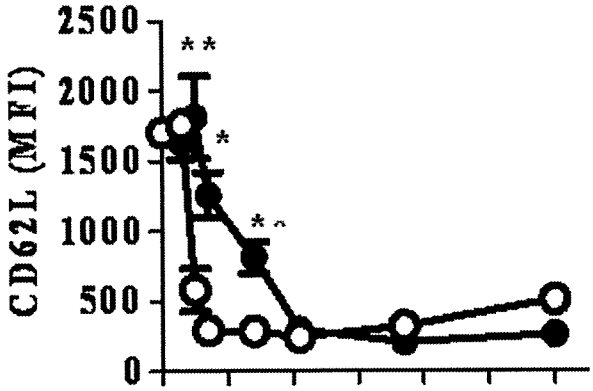
2500
2000
1500
1000
500
0
CD62L (MFI)
**
*
**

FIG. 5B

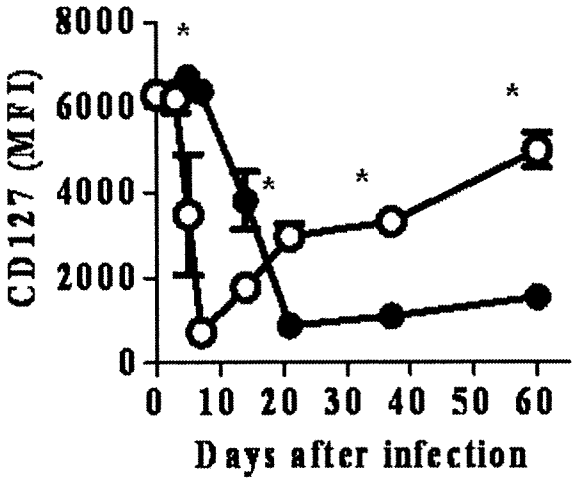
8000
6000
4000
2000
0
CD127 (MFI)
*
*
*
*
0 10 20 30 40 50 60
Days after infection

FIG. 5C

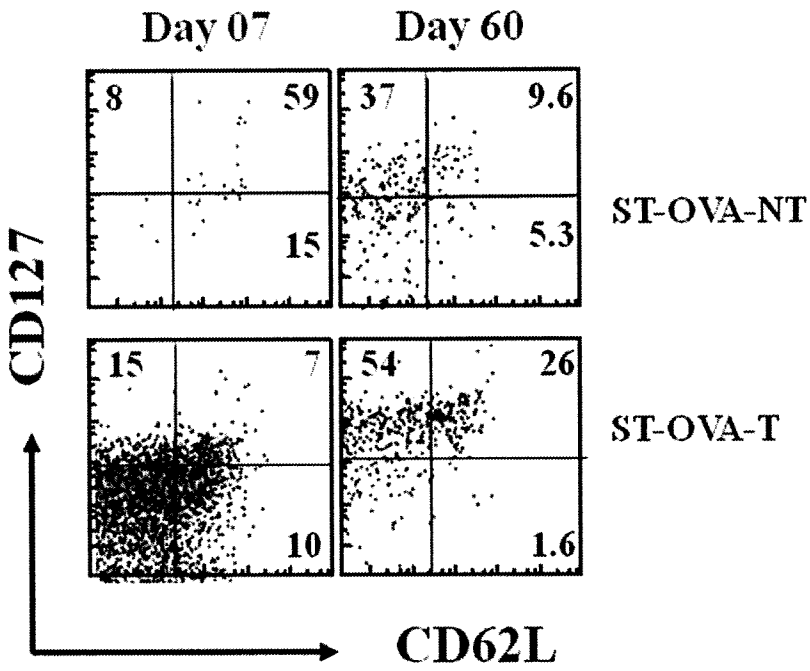
Day 07
Day 60
8
59
15
37
9.6
5.3
ST-OVA-NT
15
7
10
54
26
1.6
ST-OVA-T
CD127
CD62L

FIG. 5D

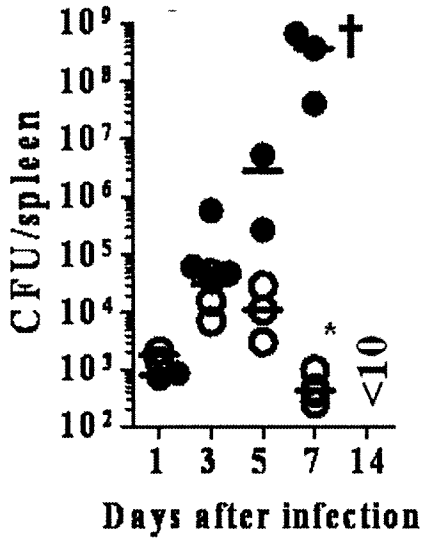
CFU/spleen
Days after infection
<10

FIG. 6A

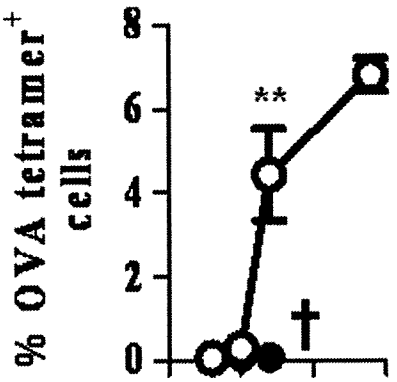
% OVA tetramer+ cells
8
6
4
2
0
**
†

FIG. 6B

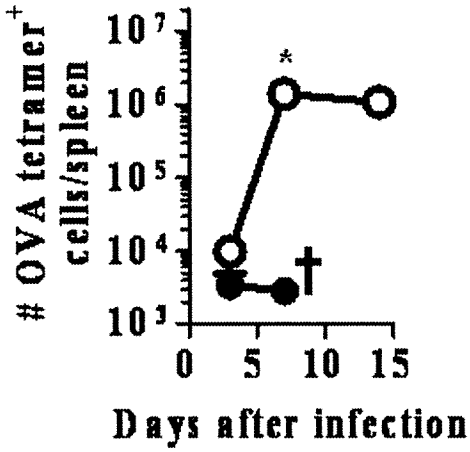
OVA tetramer+ cells/spleen
10 7
10 6
10 5
10 4
10 3
*
†
0 5 10 15
Days after infection

FIG. 6C

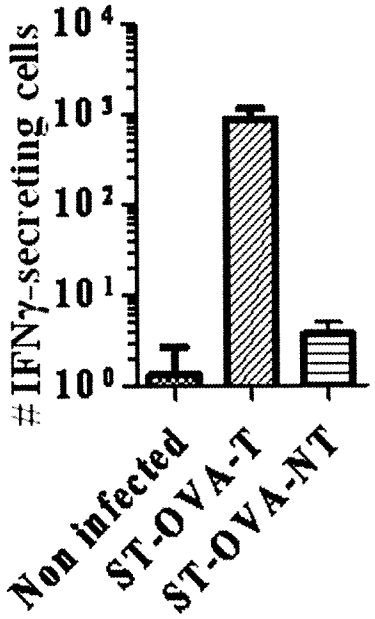

FIG. 6D

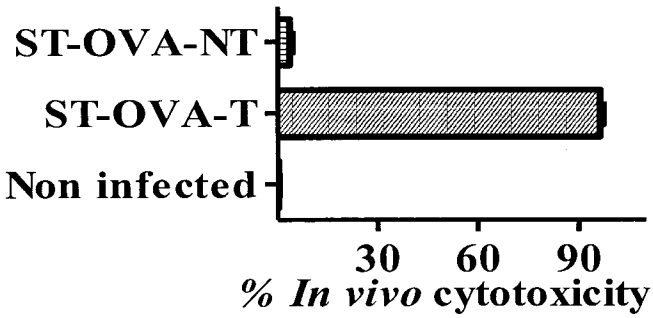

FIG. 6E

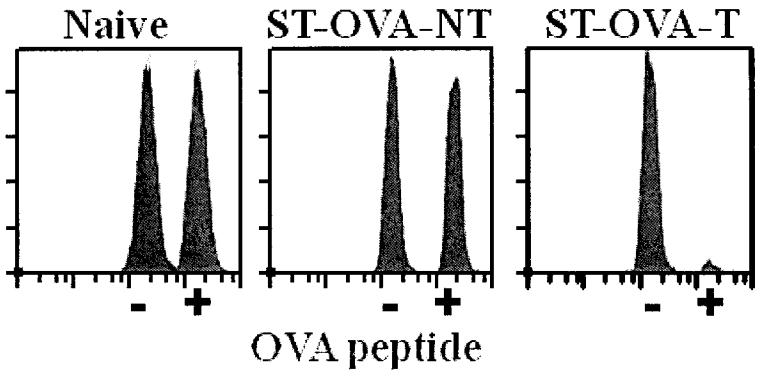

FIG. 6F

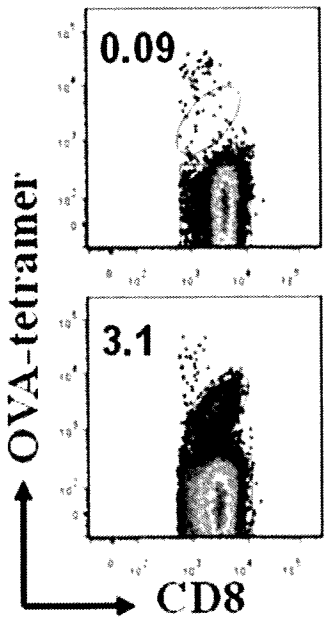
0.09
3.1
OVA-tetramer
CD8

FIG. 7A

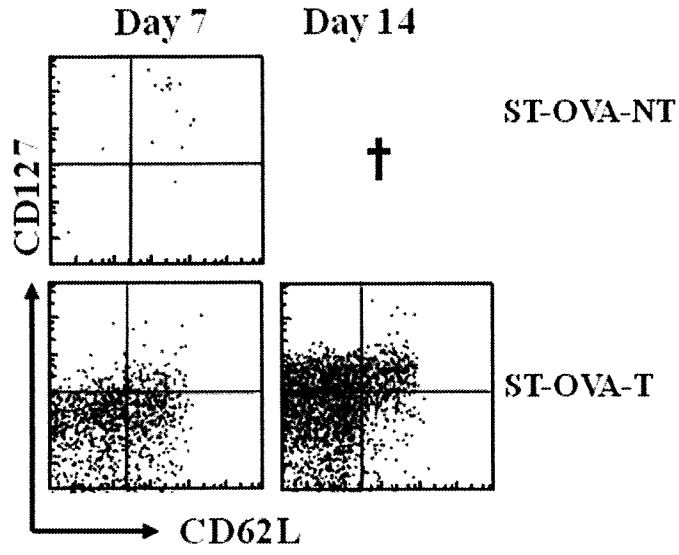
Day 7
Day 14
ST-OVA-NT
ST-OVA-T
CD127
CD62L

FIG. 7B

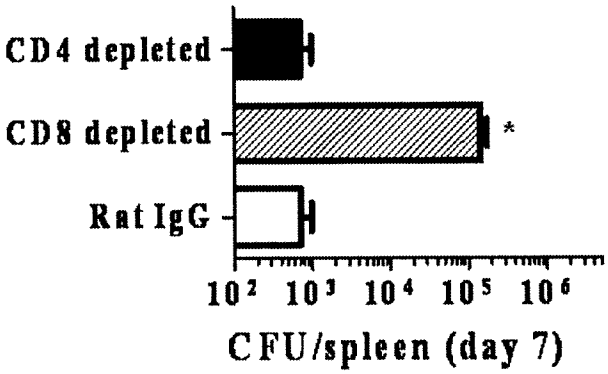

FIG. 8A

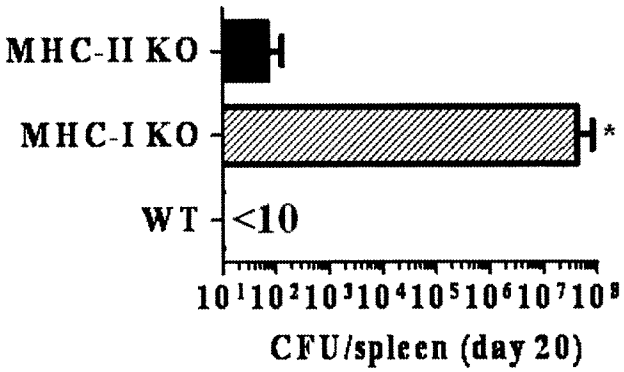

FIG. 8B

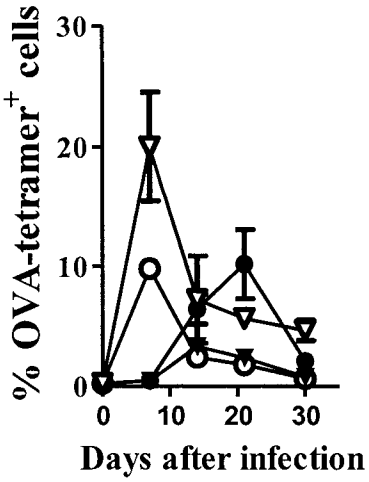

FIG. 9A

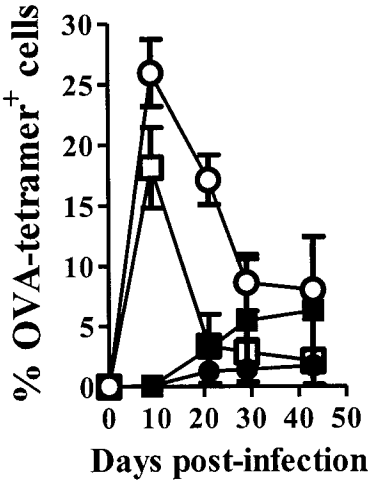

FIG. 9B

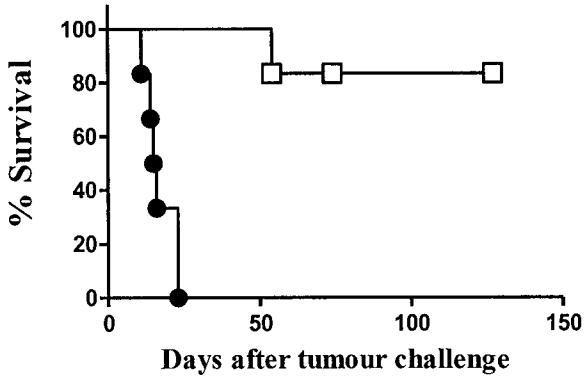

FIG. 10A

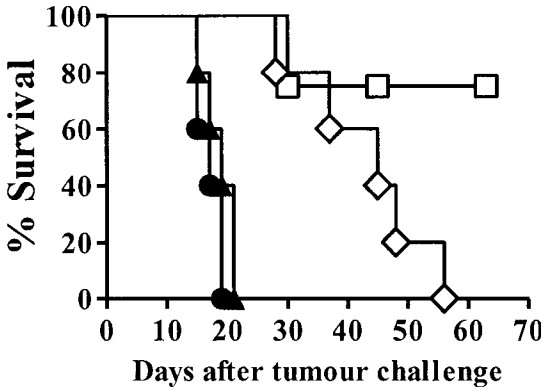

FIG. 10B

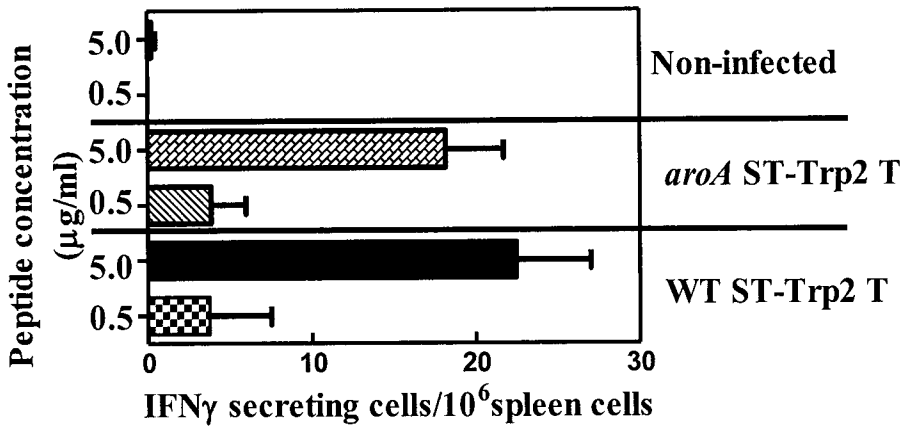

FIG. 11A

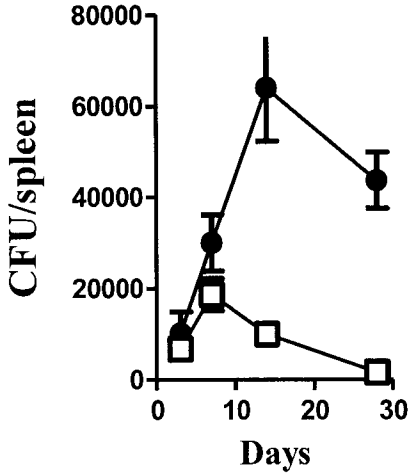

FIG. 11B

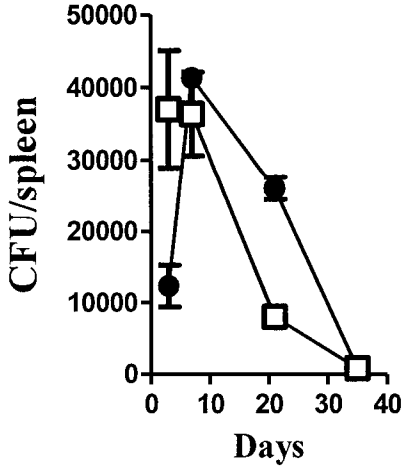

FIG. 11C

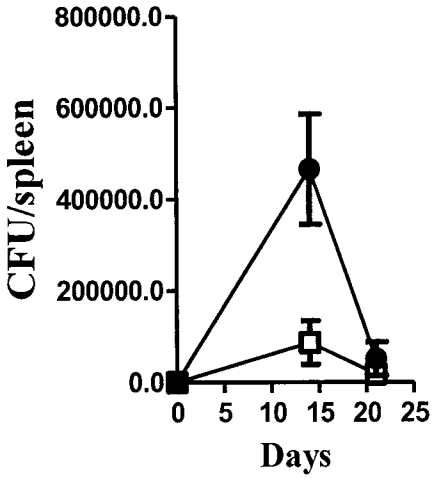

FIG. 12A

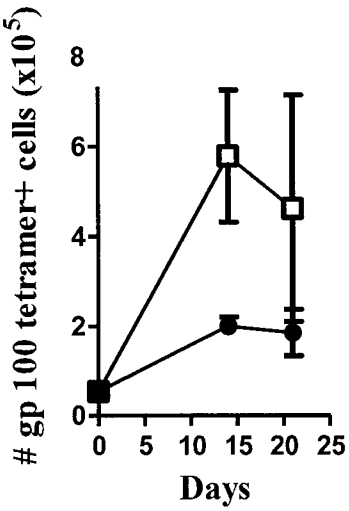

FIG. 12B

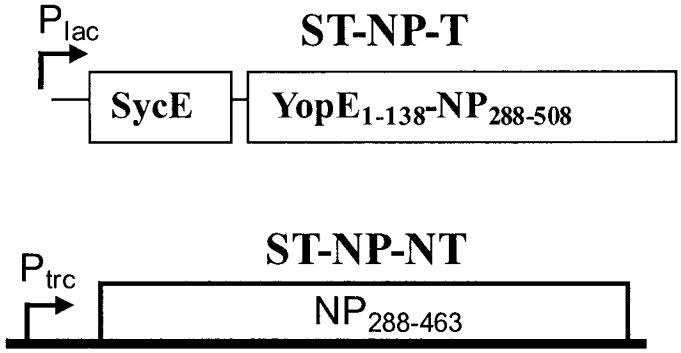

FIG. 13A

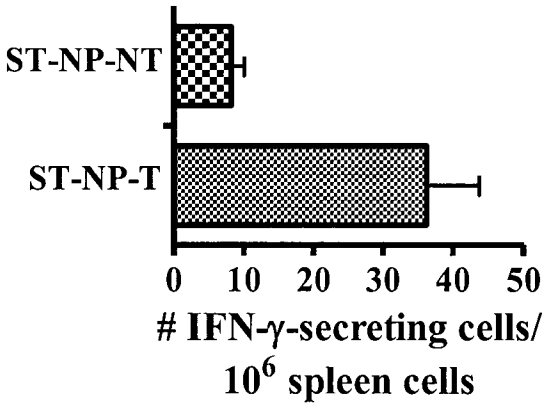

FIG. 13B

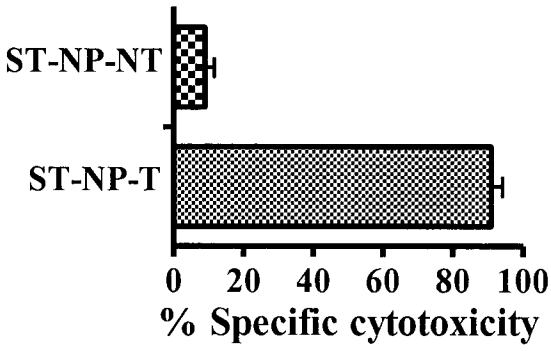

FIG. 13C

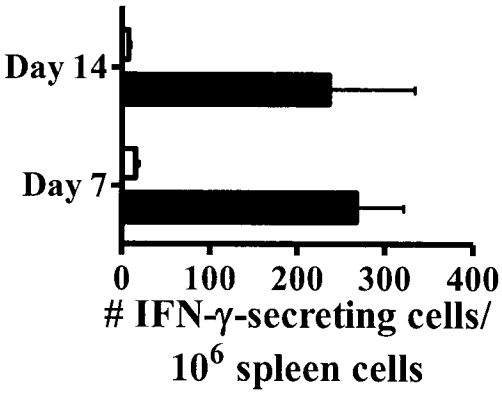

FIG. 13D

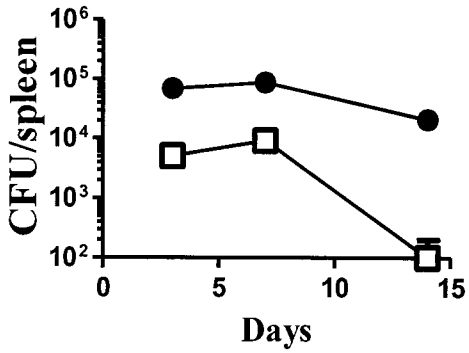

FIG. 13E

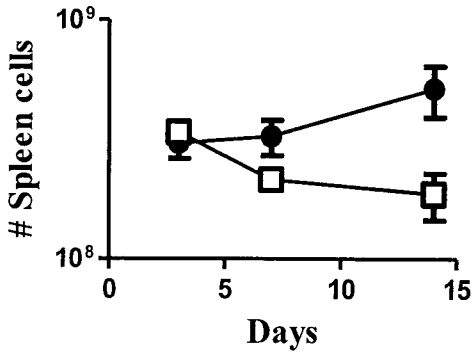

FIG. 13F

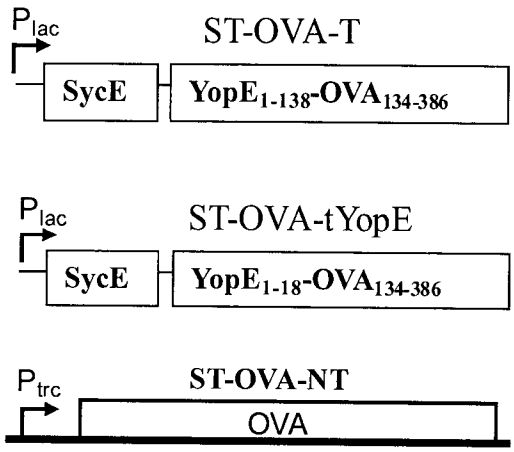

FIG. 14A

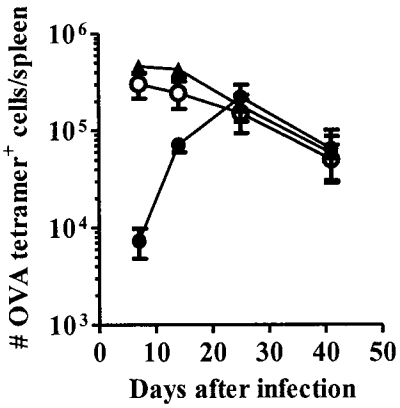

FIG. 14B

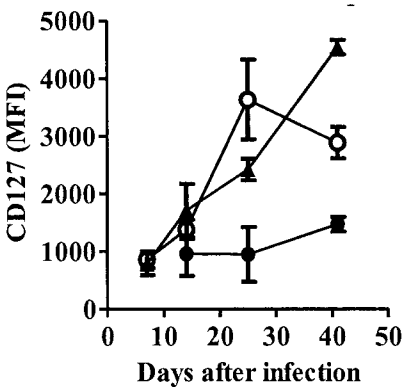

FIG. 14C

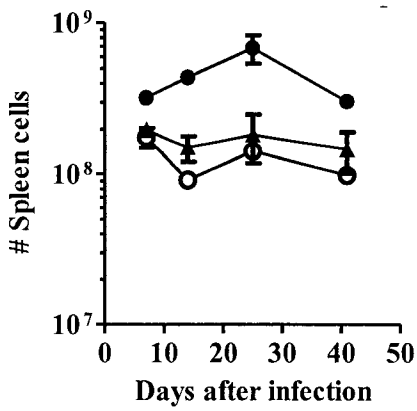

FIG. 14D

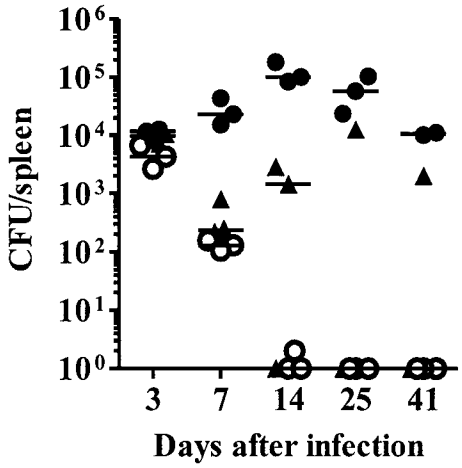
CFU/spleen
10^6
10^5
10^4
10^3
10^2
10^1
10^0
3
7
14
25
41
Days after infection

FIG. 14E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2011/000848

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C12N 1/21*** (2006.01) , ***A61K 39/295*** (2006.01) , ***A61P 31/04*** (2006.01) , ***A61P 31/12*** (2006.01) , ***A61P 37/04*** (2006.01) , ***C12N 15/00*** (2006.01), ***C12N 15/74*** (2006.01) , *C07K 14/255* (2006.01) , *C07K 19/00* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: ***C12N 1/21*** (2006.01) , ***A61K 39/295*** (2006.01) , ***A61P 31/04*** (2006.01) , ***A61P 31/12*** (2006.01) , ***A61P 37/04*** (2006.01) , ***C12N 15/00*** (2006.01), ***C12N 15/74*** (2006.01) , *C07K 14/255* (2006.01) , *C07K 19/00* (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Scopus, PubMed, Total Patent, Canadian Patent Database, Google, Keywords: recombinant bacteria, Salmonella, vaccine vector, translocation, cytosol, translocated antigen, translocation domain, type III secretion system, CD8+ T cell, Sad, Krishna, Tzelepis, Alcon, Young, National Research Council of Canada, NRC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IGW E. I. ET AL., Concomitant cytosolic delivery of two immunodominant Listerial antigens by *Salmonella enterica* Serovar Typhimurium confers superior protection against murine listeriosis INFECTION AND IMMUNITY December 2002 (12-2002) 70(12):7114-7119 (see whole document)	1-17
X	WO2007/044406 A2 (LUDWIG INSTITUTE FOR CANCER RESEARCH) 19 April 2007 (19-04-2007) (see whole document)	1-17

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 October 2011 (31-10-2011)	8 November 2011 (08-11-2011)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Sarita Chaudhary (819) 934-7926

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RÜSSMANN H. ET AL. Delivery of epitopes by Samonella type III secretion system for vaccine development SCIENCE 24 July 1998 (24-07-1998) 281:565-568	
A	WIEDIG C. A. ET AL. Induction of CD8+ T cell responses by Yersinia vaccine carrier strains VACCINE 2005 23:4984-4998	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2011/000848

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [X] Claim Nos. : 14-16

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 14-16 are directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search. However, this Authority has carried out a search based on the alleged effects or purposes/uses of the product defined in claims 1-13.

2. [ ] Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
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