Optimization of *Salmonella enterica* as a carrier for vaccination

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This work is dedicated to martyrs of Egyptian Revolution, young generation committed to development of Egypt and my beloved Mohamed, Judi and Kareem'

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Introduction

1.1. Introduction

Infectious diseases, especially lower respiratory tract infections and diarrheal diseases are among the five leading causes of death worldwide with an even greater incidence in developing countries (WHO 2008). These diseases are caused by pathogens like bacteria, viruses, parasites or fungi, which are transmitted to human or animal hosts by different routes of infection. Vaccination is the most cost efficient strategy to prevent infectious diseases. However, for many important bacterial, viral and parasitic infections efficient vaccines are still missing. One important strategy in vaccine development is the use of attenuated live bacteria for the expression and presentation of heterologous antigens. As such carrier, *Salmonella* is specifically interesting since i) virulence can be attenuated by mutation of known virulence genes, ii) mucosal delivery of heterologous antigens is possible, iii) production of vaccines is easy and cost efficient, and iv) *Salmonella* is accepted as live vaccine. Therefore, it is of great importance to develop new carrier vaccines not only against Bacterial, Viral and Parasitic infections but also against Cancer.

1.2. Intracellular bacteria as a live vaccine carrier

Intracellular bacteria have evolved lifestyles in close interaction with eukaryotic host cells. Obligate intracellular bacteria including pathogenic bacteria such as *Chlamydia* spp. or *Rickettsia* spp cannot survive outside the host cell. In contrast, facultative intracellular bacteria can grow and replicate within the infected host cells as well as extracellular and comprise, among many others, species of the genera *Mycobacteria*, *Listeria*, *Legionella*, *Brucella* or *Salmonella*. Many of these facultative intracellular

pathogens have been utilized live carriers for new vaccination strategies. The applications include the use of live attenuated pathogens to display heterologous antigens to the immune system of the vaccinee, to translocate foreign protein antigens in specific populations of immune cells, or the use as carrier strains for DNA vaccination.

Because of the invasive properties and their capability to access the host cell cytosol intracellular bacteria have been employed in specific targeting of DNA vaccine vectors to professional antigen-presenting cells (APCs) [1]. Several earlier studies have shown that the delivery of DNA vaccines by several attenuated intracellular bacteria is a promising way of vaccination [2-3]. Towards this direction, a wide range of intracellular bacteria such as *Shigella flexneri* [4], *Salmonella enterica* [5-6], invasive *E. coli* [7], *Listeria monocytogenes* [2, 8] and attenuated strains of *Mycobacterium bovis* (BCG) [9] have been used as carriers of eukaryotic antigen expression vectors. Also, Attenuated *Yersinia enterocolitica* strains have been used as carriers for heterologous antigens [10] and more recently the type III secretion system of *Yersinia* spp. has been utilized to deliver fusion proteins into antigen-presenting cells (APC)[reviewed in 11]. The translocation approach appears also very effective and versatile in combination with *Salmonella* as live attenuated carrier and will be discussed in detail in this review.

1.3. Pathogenesis of infectious diseases by *Salmonella*

Salmonellae are Gram-negative, facultative anaerobic, motile, non-lactose fermenting rods belonging to the family of *Enterobacteriaceae.* The genus comprises the two species *Salmonella enterica* and *Salmonella bongori*, the latter being considered as the

phylogenetical older species [12]. *Salmonella enterica* is classified into seven subspecies and further characterization was done by typing of their O (somatic), K (capsular) and H (flagellar) antigens, with more than 2,500 serovars being identified.

Salmonella infects humans and animals generally by the oro-faecal route. While some *Salmonella* species are restricted to one or few host species, others have a wider host spectrum. Very diverse disease outcomes following *Salmonella enterica* infection can be observed with human diseases ranging from a general mild, self-limiting gastroenteritis, caused mainly by *S. enterica* serovars Typhimurium (*S.* Typhimurium) and Enteritidis (*S.* Enteritidis), to the severe, systemic infection of typhoid fever caused by *S. enterica* serovar Typhi (*S.* Typhi) or Paratyphi (*S.* Paratyphi). Salmonellosis is more prevalent in developing parts of the world [13]. Typhoid fever is an acute, life threatening febrile illness with 16 million to 33 million cases and 500,000 to 600,000 deaths every year according to World Health Organization (WHO) reports. Another studies conservatively estimated 22 million cases and 216,000 related deaths in 2000 [13-14].

Several clusters of virulence genes are important for the adhesion to specific cell types of the mucosa, the invasion of enterocytes and for the triggering of fluid secretion leading to the diarrheal symptoms. Pathogenesis of *Salmonella* depends on its ability to survive and replicate inside host cells. This virulence trait is linked to the ability to cause systemic infections [15]. A large number of genes are required to enable *Salmonella* to cope with nutritional limitations (*aro*, *pur,* etc.) and to avoid clearance by the host immune system. Further virulence factors enable *Salmonella* to avoid or survive damage by antimicrobial peptides [16-17]. For experimental studies, *S.* Typhimurium

has proven to be a useful model organism as it is a genetically amenable pathogen that provides a mouse model with systemic infections reminiscent of typhoid fever in humans [for recent reviews on Salmonella pathogenesis, see 18].

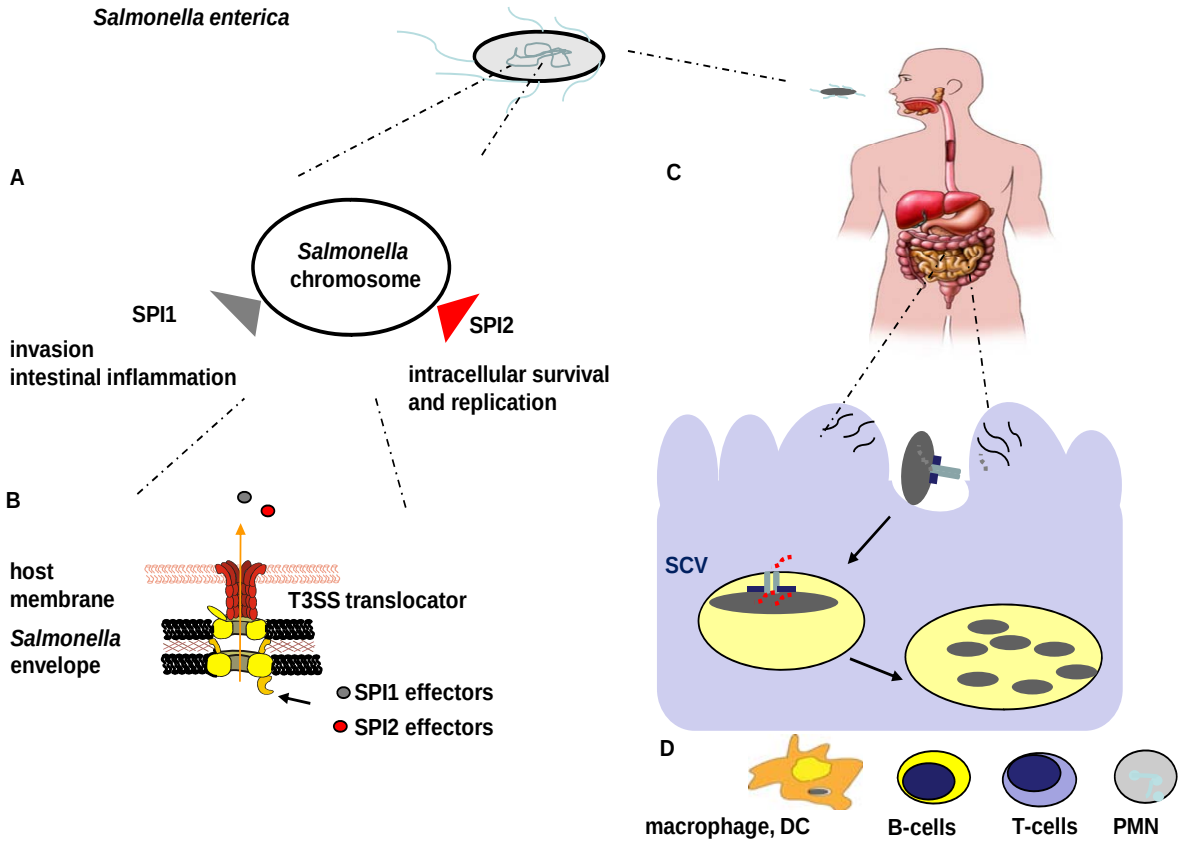


Fig 1.1. Key events in *Salmonella* pathogenesis. A) Within the chromosome of *Salmonella enterica*, several virulence clusters known as *Salmonella* Pathogenicity Islands (SPI) are present. In particular, SPI1 and SPI2 which play an important roles in invasion and intracellular survival, respectively. **B**) *Salmonella* encodes two distinct virulence-associated T3SS by genes within SPI1 and SPI2 that are important for different phases of pathogenesis. C) Orally ingested *Salmonella* survive at the low pH of the stomach, enter the small intestine and invade epithelial cells. Upon invasion, *Salmonella* SPI1-T3SS

delivers its effector proteins across the host cell plasma membrane which leads to temporal reorganization of the host cell actin cytoskeleton and induces uptake of the bacteria by means of macropinocytosis and plays an important role in *Salmonella*-induced inflammatory responses. *Salmonella* remains in a specialized phagosome, *Salmonella*-containing vacuole (SCV), where multifunctional virulence system SPI2-T3SS plays its role in *Salmonella* survival inside SCV and inducing systematic responses. D) *Salmonella* preferentially enters M cells, which transport them to the lymphoid cells (T and B) in the underlying Peyer's patches. Non-typhoidal *Salmonella* strains induce an early local inflammatory response, which results in the infiltration of PMNs (polymorphonuclear leukocytes) into the intestinal lumen and diarrhea. On the other hand, *Salmonella* serotypes that are associated with systemic illness enter intestinal macrophages and disseminate throughout the reticuloendothelial system.

1.4. Type III secretion systems

Type III secretion systems (T3SS) are specialized molecular machines of Gram-negative bacterial pathogens that deliver effector proteins to host cell membranes and cytosol [19]. T3SS fulfill distinct functions that include antiphagocytic and cytotoxic effects on host cells (Ysc/Yop system of *Yersinia* spp.), invasion of host cells (*S. enterica* SPI1 system, *Shigella* spp. Mxi/Spa system) and intracellular pathogenesis (*S. enterica* SPI2 system, *Chlamydia* spp. T3SS) [20]. T3SS consists of at least 20 different subunits which enable these bacteria to translocate specific effectors directly into the host cell cytoplasm in order to exert a broad range of virulence functions. The T3SS assemble needle-like appendages, share similar with the flagellar basal body and some of its proteins, including those which form the core of the central channel, are highly conserved between the two systems [21].

The T3SS apparatus, also referred to as injectisome, spans the inner and outer membranes of the bacterial envelope and secretes translocon and effector proteins. Translocon proteins allow access of effector proteins to the eukaryotic cells by forming pores in the host cell membrane and forming a connecting channel-like complex between the bacterium and the eukaryotic membrane. The effector proteins further subvert different aspects of host cell physiology and immunity thereby promoting bacterial virulence [22]. *S. enterica* encodes two distinct virulence-associated T3SS with roles in different phases of pathogenesis.

1.5. *Salmonella* Pathogenicity Islands

The genes for virulence factors of most bacterial pathogens cluster in pathogenicity islands (PAI) owing to integration of foreign genome fragments into the bacterial chromosome. PAI are large chromosomal regions that are present in pathogenic bacteria and confer virulence properties. PAI can be characterized by their large size, lower GC content, association with 'mobile DNA elements' such as integrases, insertion sequence (IS) elements, bacteriophage genomes, genetic instability and presence of one or more virulence genes [17]. Important virulence characters of *S. enterica* are encoded by genes within *Salmonella* Pathogenicity Islands (SPI), such as the interaction with enterocytes resulting in diarrhea, the invasion of non-phagocytic cells, and the ability to survive phagocytosis and to proliferate within eukaryotic host cells. A remarkable feature of *S. enterica* is the presence of a large number of SPI. Two major SPI, SPI1 and SPI2, encode T3SS that translocates bacterial effectors [23]. The contribution of SPI to *Salmonella* pathogenesis is depicted in **Fig. 1.1**.

1.5.1. SPI1

SPI1 is about 40 kb in size and encodes a T3SS, translocated effectors and their chaperones, regulatory components, as well as, an iron uptake system. The SPI1-encoded T3SS and its effectors are required for invasion of epithelial cells and is activated under conditions thought to be present in the intestinal lumen before host cell invasion [15-16, 23]. The functions of effectors in early infection can be summarized in cytoskeleton rearrangement, triggering cell entry, inflammation, loss of electrolytes and apoptosis [24].

The SPI1-T3SS delivers at least 13 effector proteins across the host cell plasma membrane. The coordinated action of a set of translocated effectors leads to temporal reorganization of the host cell actin cytoskeleton and induces uptake of the bacteria by means of macropinocytosis and plays an important role in *Salmonella*-induced inflammatory responses. A second subset is associated with the enteropathogenesis and inflammation of the intestinal epithelium and diarrheal symptoms [17, 25].

Salmonella mutants lacking a functional SPI1-T3SS are unable to invade epithelial cells or induce proinflammatory cytokines in *in-vitro* models [26]. These mutants are not only attenuated for virulence in the mouse typhoid fever model when inoculated orally, but also when inoculated intravenously, and they fail to produce significant secretory or inflammatory responses [27].

1.5.2. SPI2

Genes within SPI2 were initially identified by signature-tagged mutagenesis (STM) screening of a mutant bank for clones with reduced capacity to survive and replicate in the murine model of systemic *Salmonella* infections [23]. The function of SPI2 is

essential for the second hallmark of *Salmonella* pathogenesis, the ability to cause systemic infections and to proliferate within host organs. This virulence phenotype is linked to the ability of *Salmonella* to survive in phagocytic cells and to replicate within the *Salmonella*-containing vesicle (SCV) in a variety of eukaryotic cells. The SPI2-T3SS is a multifunctional virulence system: phenotypes linked to its function include avoidance of the oxidative burst, cytotoxicity, intracellular actin assembly, alteration of inducible nitric oxide synthase (INOS) localization in infected macrophages, cholesterol accumulation near the SCV and, most prominently, the redirection of vesicular traffic in infected cells and the maintenance of the SCV [28].

More than twenty SPI2 effectors are known to be translocated over the SCV membrane into the host cell cytoplasm, most of them encoded outside the SPI2 locus [23]. SPI2 genes are induced inside host cells and *in vitro* by conditions such as nutritional starvation, acidic pH, low concentrations of Mg^{2+} or Ca^{2+} as well as starvation of phosphate, factors which might reflect the situation inside the host cell phagosome [29].

Most of the SPI2-dependent phenotypes characterized so far are linked to the manipulation of host-cell vesicle trafficking, thus ensuring nutrient supply and evading bactericidal activities. SPI2 mutant strains are attenuated when administered by the oral, intravenous and intraperitoneal routes. In addition, SPI2 mutant strains were unable to proliferate within host organs and showed reduced survival and proliferation inside host cells [30-31]. However, SPI2 mutants show wild-type phenotypes in the interaction with the intestinal epithelium and cause diarrhea in different animal models [25].

1.6. *Salmonella* as carrier for delivering heterologous antigens

Live attenuated strains of *Salmonella* that synthesize and secrete foreign antigens are being developed as vaccines for a number of infectious diseases by bacterial, viral and parasitic pathogens and against cancer. Moreover, the use of live attenuated *Salmonellae* to deliver recombinant antigens to the immune system is an attractive strategy for the construction of multivalent vaccines. *Salmonella*-based vaccines provide a number of advantages over other antigen delivery strategies including low cost of production, oral delivery, the absence of animal products, genetic stability and safety. In addition, *Salmonella* vaccines delivering heterologous antigens stimulate innate immunity and also activate both the arms of the adaptive immune system by which they exert efficient immune response. The existence of the serovar Typhimurium mouse model for preclinical work and favorable prior human experience with the live attenuated vaccine *S*. Typhi Ty21a, are important benefits for the development of Salmonella vaccine carriers. Ty21a is a U.S. Food and Drug Administration approved vaccines for typhoid fever and serves as an important safety benchmark for researchers undertaking clinical trials[32].

As a consequence of the intracellular location of *Salmonella*, secreted proteins are trapped in the phagolysosome and presented to the immune system preferentially in the context of MHC class II molecules. There have been several approaches in the literature for bioengineering *Salmonella* strains which could deliver antigens directly to the intracellular compartment instead of the phagosome. In the next paragraphs two main strategies used by researchers for using *Salmonella* to deliver foreign antigens are discussed. The major strategies of *Salmonella*-based vaccines are shown in **Fig. 1.2**.

1.6.1. *Salmonella*-mediated DNA transfer strategies

The direct injection of naked DNA for vaccination has several advantages but also suffers from severe problems. Due to low number of APC in muscle tissues the efficiency of the DNA uptake seems to be quite low and dose-dependent and require injection of high amounts of DNA to elicit protective immune responses [33]. Further, injection of plasmid DNA does not seem to induce efficient immune responses at distant mucosal surfaces [34]. Classically, DNA vectors are classified into non-viral and viral vectors, but gene transfer can also occur from bacteria to a very broad range of recipients that include yeast, plants and mammalian cells [32].

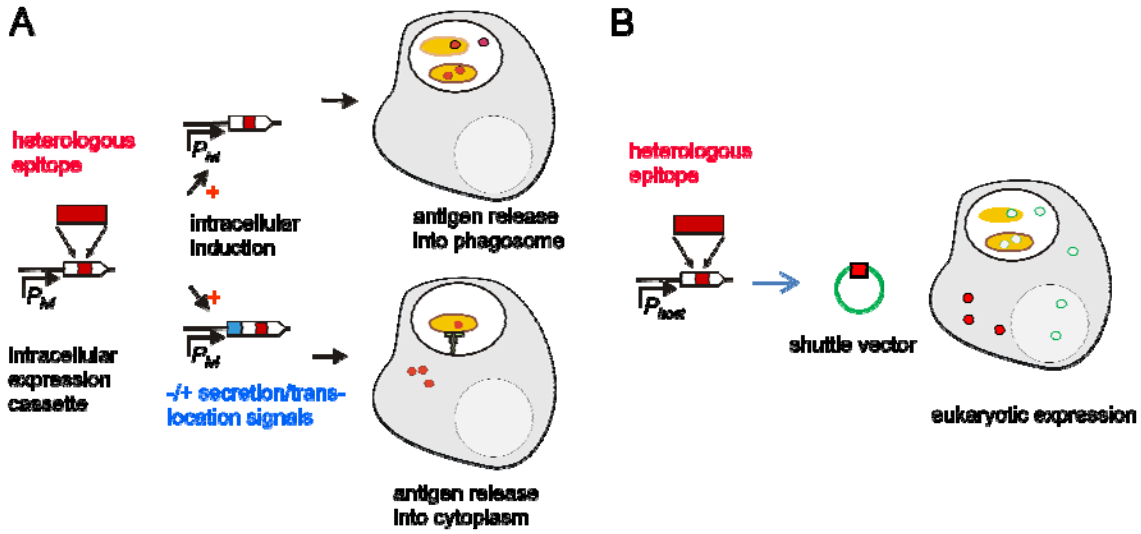


Fig. 1.2. Strategies for heterologous antigen delivery by *Salmonella*. A) Expression of heterologous antigens using in vivo activated promoters. The antigen may be release into the phagosome after lysis of internalized bacteria, The T3SS-mediated delivery of SPI2-effector antigen fusions expressed under control of *in-vivo* inducible promoter allows the controlled delivery into the target cell cytosol. **B)** In *Salmonella*-mediated DNA transfer strategy, antigen is expressed under control of a eukaryotic promoter. The plasmids expression cassettes are introduced into suitable attenuated *Salmonella* mutant carriers

which invade epithelial cells. In *Salmonella*-based DNA vaccines, *Salmonella* cannot escape the SCV, The plasmid DNA is released into the phagosome or subsequently enter the nucleus. The mechanism of plasmid DNA escape into the cytosol and delivery to the nucleus remains to be resolved.

The use of intracellular bacteria that have access to the host cell cytosol may allow a more specific targeting of DNA vaccine vectors to professional APC. Due to its invasive properties and their preference for macrophages and DC, *Salmonella* are ideally suited for a more direct targeting of DNA vaccines to professional APC [35-36]. Recombinant *Salmonella* should be metabolically attenuated in order to die and lyse in these cells, consequently the plasmid molecules are liberated to transfect host cells [37]. At the same time, the invaded cells are activated by bacterial endotoxins and other bacterial components which stimulate innate immune responses. This might lead to direct presentation of antigen to T cells. In addition, indirect or cross presentation of apoptotic molecules derived from the apoptosis-like reaction in activated macrophage is also induced by *Salmonella* [38]. Neighboring DCs efficiently clear these apoptotic bodies, and re-present these antigens to T cells [39]. DCs migration from Peyer's patches to lymph nodes or spleen, or plasmid-bearing bacteria that disseminate via phagocytes, could be responsible for induction of systemic immune responses [40]. The plasmid DNA is released into the phagosome or subsequently enter the nucleus and plasmid-encoded antigens can be expressed. The mechanism how plasmid DNA escapes into the cytosol and is delivered to the nucleus remains unclear [32, 40]. Finally, these antigens can be presented by APCs in the context of MHC I and MHC II molecules to CD8 and CD4 T cells, thereby eliciting cellular and promoting humoral immune responses.

The strategy of using live attenuated *Salmonella* to deliver plasmid-encoded antigens under the transcriptional control of eukaryotic promoters has been used successfully in vaccination [41]. *Salmonella* carrier DNA vaccines have been employed in prophylactic immunization against bacteria, viruses, fungi, parasites and even against cancer [32, 40, 42].

Many studies have exploited *S.* Typhimurium as a gene delivery vector against bacterial infections inducing protective humoral and cellular immune responses. When β-galactosidase was used as a transgene, specific cytotoxic T-lymphocytes (CTLs) and T-helper (Th) cells, as well as specific antibodies could be detected after a single oral vaccination [6]. Protection of mice against a lethal challenge with *L. monocytogenes* was observed when two virulence factors of *L. monocytogenes* (LLO and ActA) were encoded in *S.* Typhimurium vectors [43]. Partial protective responses against *Chlamydia* were obtained in the lungs of mice after oral administration of *Salmonella* encoding the major outer membrane protein of *Chlamydia trachomatis* [40]. Attenuated *S.* Typhimurium expressing a L7/L12 and BLS fusion antigen of *Brucella* spp. confered protection against Brucellosis [44]. Oral multi-antigen DNA vaccines delivered in attenuated *S.* Typhimurium enhance protection against nasopharyngeal *Streptococcus pneumonia* [45]. In veterinary medicine, live attenuated *S.* Typhimurium vaccines expressing *Campylobacter* antigens are used to control of *Campylobacter jejuni* in poultry [46]. Interestingly, mucosal immunization with attenuated *S.* Typhi strains also introduced as live carriers for DNA vaccines against *Yersinia pestis* [47] and anthrax [48].

Viral infections have also been targeted through DNA vaccination mediated by *Salmonella*. Oral vaccination with *S.* Typhimurium encoding a hepatitis B virus surface antigen (HbsAg) proved successful at inducing CTLs in BALB/c mice [49-50]. Recombinant *S.* Typhimurium administered orally was also reported to induce an immune response against herpes simplex virus 2 [51], human papillomavirus 16 [52] and lethal enterovirus 71 [53]. Vaccination against HIV was attempted using attenuated strains of *Salmonella* carrying eukaryotic expression plasmid encoding HIV gp12 [54]. *S*. Typhimurium expressing HPV16L1 as a model antigen of papillomavirus (HPV) infections can induce both innate and adaptive mucosal immune responses [55]. Surprisingly, oral *Salmonella* carrier DNA vaccines induced immune response against the secreted cell wall antigen Mp1p of the pathogenic *Pencillium marneffei* [56]. *Salmonella* was used for oral DNA vaccination against LACK protein of *Leishmania major* parasites [40]. The *aroA* mutant strain of *S*. Typhimurium was exploited as a DNA delivery system for cruzipain (SCz) and conferred protective immunity against *Trypanosoma cruzi* [57].

Salmonella strains have been used to deliver DNA for therapeutic applications in oncology. Oral administration of model tumor antigens such as β-galactosidase or human gp100 (hgp100), which were encoded in eukaryotic expression vectors carried by strains of *Salmonella,* protected the mice against challenges with fibrosarcoma [58], renal carcinoma [59], and melanoma [60] cells expressing the relevant model antigen. Transgenes including the murine gp100 (mgp100) fused to the invariant chain epitopes of mgp100 and TRP2 fused to ubiquitin, a minigene encoding epitopes of the tyrosine hydroxylase (TH) fused to ubiquitin are used. The complete TH coding sequence was

linked to a virus-derived post-transcriptional regulatory element, and human carcinoembryonic antigen (hCEA) in an hCEA mouse transgenic model which showed successful protection. However, the low rate of somatic gene transfer to the host cells inherent to DNA vaccines limits their potential use for vaccination and calls for the development of new strategies to increase the efficacy of *Salmonella* carrier vaccines [32].

1.6.2. *Salmonella* T3SS-mediated heterologous antigen delivery

The CD4 T cells are specific for peptides presented by MHC class II molecules which translocate antigens from the phagosomal compartment to the cell surface. Therefore, intracellular bacteria remaining in the phagosome such as *Salmonella* are a preferred antigenic target for CD4 T cells. A CD4 T cell population, also termed T helper cells (Th cells), produce various cytokines that have a major influence in ensuing the immune response. The so-called Th2 cells activate eosinophils and basophils and are critical for B-cell maturation into antibody producing plasma cells. Accordingly, Th2 cells are responsible for control of helminthic infections, bacterial infection and for toxin neutralization. The so-called Th1 cells produce cytokines such as interferon-γ (IFN-γ) and interleukin 2 (IL-2), which activates cytolytic T lymphocytes (CTL) and macrophages. In contrast, CD8 T cells recognize antigenic peptides presented by MHC I molecules which transport antigens from the cytoplasm to the cell surface. Accordingly, CD8 T cells are responsible for the response against viral pathogens and also for those intracellular bacteria which egress into the cytoplasm. *L. monocytogenes* also stimulates CD4 T cells, and most microbes remaining in the phagosome can stimulate CD8 T cells [35].

Localization within the SCV prevents delivery of expressed foreign proteins to the MHC class I-restricted antigen presentation pathway and hinders the use of *Salmonella* as vaccine carrier to induce specific CD8 T cells which is crucial for protection against viruses, intracellular bacteria, and tumors. Heterologous antigens can be expressed in *Salmonella* as fusions with recombinant or native proteins. This approach was mainly used to direct the expression of the desired antigen to a particular location of the bacterial cell and increase the immunogenicity of foreign antigens by fusing them to proteins that could exert a carrier effect [61]. Towards this end *Salmonella* T3SS-mediated translocation can be used for efficient delivery of heterologous antigens to the cytosol of antigen-presenting cells leading to prominent CD8 T cell responses [62-64].

Salmonella translocates T3SS effector proteins into the host cell cytoplasm mediated by either the SPI1-T3SS from the extracellular stage and from the SCV at an early stage after entry or the SPI2-T3SS from the SCV at later stages during intracellular life [15, 17, 24]. Efficient immune response mainly requires T cells induction which depends on the strength of antigen presentation. The antigen presentation strength by its role depends on antigen access to the respective processing compartment, the antigen-processing efficacy, and antigen abundance [65]. *Salmonella* T3SS-mediated translocation can be used for efficient delivery of heterologous antigen fusions to SPI1 or SPI2 effector proteins to the cytosol of APC, leading to prominent CD8 T cell priming in orally immunized mice [62].

1.6.2.1. Cytosolic versus phagosomal antigen delivery

Heterologous antigens expressed by recombinant Salmonella carrier strains can be delivered to distinct subcellular locations of the host cells. A critical parameter is the

selection of appropriate carrier proteins. SPI1 effector protein SopE2 is a guanine nucleotide exchange factor for host cellular Rho GTPases [66] and plays a role in the early invasion steps of *Salmonella* into host cells [67].The C-terminal 355 amino acids of P60 (p60130_484) from *L. monocytogenes* served as a model antigenic protein fragment fused to N-terminal portions of SopE2 protein. SopE2/P60 expressed under the control of its native promoter was detected in the cytosolic fraction of macrophages at 2 and 6 h but not 24 h after infection. Vaccination of mice with *Salmonella* strains expressing SopE2/P60 led to the induction of P60-specific CD8 T cells [68]. Also, SPI1 effector protein SopB has been considered as a potential carrier for inducing CD8 and CD4 cells [69].

SPI2 effector proteins, especially those which are expressed only when the *Salmonella* is inside the host cell DCs and macrophage [16, 70] have been thought as promising carrier candidates for antigen delivery to MHC I pathway. SPI2 effector SifA is required for the formation of lysosomal glycoprotein-containing structures, called SIFs, and to maintain the integrity of the phagosomal membrane of the SCV during intracellular proliferation [24]. Vaccination of mice with *Salmonella* strains expressing SifA/P60 led to the induction of P60-specific CD8 T cells [68]. Previously, our group focused on testing and comparing several SPI2-antigen fusions and used different model antigens that include P60, Ovalbumin, LLO and the tumor-associated antigen Survivin. *S.* Typhimurium attenuated strains expressing chimeric proteins engaged by SseF, another SPI2 effector, under control of an intracellular activated promoters efficiently induced both CD4 and CD8 T cells [64, 71-72]. These investigations by our group

resulted in identifying the most efficiently activated promoters of *Salmonella* vaccines [73] and comparison between different SPI2 effectors.

While SseF, SifA, SseJ and other SPI2-effectors are localized inside host cell on the membrane of SCV and SIFs, SpiC and SspH2 are translocated to the cell cytoplasm [24]. Focusing on these cytosolic translocated SPI2 effectors might be beneficial in inducing an efficient immune response. Interestingly, *Yersinia* outer protein E (YopE) for heterologous antigen delivery by the *Salmonella* T3SS was analyzed. YopE was fused to the immuno-dominant T-cell antigens LLO and P60. *Salmonella* secreted and translocated these chimeric proteins into the cytosolic compartments of host cells but not into endosomal compartments. This led to efficient MHC I-restricted antigen presentation of listerial nonamer peptides. Mice orally vaccinated with attenuated *S.* Typhimurium expressing translocated hybrid YopE proteins revealed efficient and specific CD8 T cell response, protecting mice against a challenge with *L. monocytogenes*. These findings suggest that YopE is a versatile carrier molecule for T3SS-mediated foreign antigen delivery by *Salmonella* vaccine strains [74-75].

SspH2 co-localizes with the polymerizing actin cytoskeleton in the host cell and interacts with filamin and profiling, and localized in cytoplasm of the host cell [24]. SspH2/P60 hybrid proteins led to concomitant P60-specific CD4 and CD8 T cell priming, indicating that the SPI2 effector protein SspH2 might be an attractive carrier molecule for antigen delivery when T cell immune responses against complex microbes or tumors are needed [63, 68].

1.7. *Salmonella* for vaccination against cancer

Surgery, radiotherapy, and chemotherapy are conventional anti-cancer therapies which are effective in the treatment of solid tumors. These therapies not only have limited clinical efficacy, but also severe side effects. Use of bacteria as an alternative cancer therapeutics has sporadically been followed over more than a century. At the beginning of the nineteenth century, Vaultier observed a correlation between bacterial infections of cancer patients and the regression of their tumors [76]. To this end, different bacteria showed an efficient targeting and colonization in solid tumors. Such bacteria are either obligate anaerobic bacteria like *Clostridium* or *Bifidobacterium* or facultative anaerobic like *Escherichia coli* or *Salmonella*. Importantly, *S.* Typhimurium were shown not only to colonize large, established tumors but also exhibit the property to invade and affect metastases [77]. Moreover, *Salmonella* preferably colonize solid tumors at tumor to liver ratios of 250:1 to 9,000:1 that usually results in tumor growth retardation [78]. Tumor cells produce compounds that specifically chemoattract bacteria to the necrotic areas of a tumor [79]. There are several receptors which might attract *S.* Typhimurium to peculiar regions of tumors by controlling their chemotaxis towards specific tumor microenvironments [80]. In contrast to obligate anaerobic bacteria, *Salmonella* colonize large solid tumors that contain huge hypoxic cores and accumulate within metastases after systemic administration [81-83]. This renders *Salmonella* an ideal candidate for bacteria-mediated tumor therapy.

Salmonella have been used experimentally in combination with chemotherapeutics such as cisplatin, endostatin, or cyclophosphamide against cancer [84-85]. Also combining *Salmonella* injections and X-ray treatment or low-dose radiation was explored [86].

Such studies showed an enhanced anti-tumor effect when combining chemotherapy or radiotherapy with the application of *Salmonella* in comparison to either treatment alone. There are several successful trails using *Salmonella* as DNA vaccine carrier [42] or *Salmonella* SPI2-T3SS antigen delivery [63-64] to induce specific cellular immunity. For designing *Salmonella* as carrier for vaccination, it is more important to select mutant carrier, tumor antigen and route of administration to ensure an efficient immune response.

In contrast to other tumor-targeting bacteria, *Salmonella* accumulate in solid tumors not only after systemic infection but also after oral administration [84]. *Salmonella* mutant carriers should keep this balance between virulence, to induce efficient immune response, and attenuation, to avoid harm to the host (see above). *Salmonella htrA purD* mutant carrier was employed to deliver survivin antigen and showed an efficient and specific immune response in a murine model of brain tumor glioblastoma [64]. The attenuated *S.* Typhimurium strain VNP20009 as tested in two trials on a total of 28 patients with malignant melanoma and one patient with metastatic renal cell carcinoma in a phase I study. From three patients who exhibited focal bacterial colonization of the tumor, only one responded to the treatment completely and was tumor free 3 months after infection. A second patient showed strong tumor growth after an initial retardation [87-88] may be due to attenuation of the strain leading to impeded entry into the tumor [77].

The cancer immunotherapies designed for direct attacks on tumor cells face longstanding limitations with poor immunogenicity of tumor self-antigens and the genetic instability of tumor cells. Thus the selection of proper tumor antigen is critical for

efficiency of *Salmonella*-based vaccines against cancer. Ideal targets for vaccination are Tumor-Associated Antigens (TAA). TAA for therapeutic vaccination should possess most of the following prosperities that encompass over-expression in tumor but little or no expression in normal tissue, ability to generate endogenous peptides that can be presented on different HLAs, survival of tumor cells and expression in many cancers so that a vaccine could treat many types of cancer.

With 16.5 kDa, survivin is the smallest member of the inhibitor of apoptosis protein family. survivin is considered an excellent TAA, as the vast majority of tumors express survivin protein at high levels while most normal adult tissue do not. Besides tumor cells, survivin is also overexpressed in tumor vascular endothelial cells and is required for both tumor cell survival and tumor induced angiogenesis. survivin is immunogenic and naturally occurring survivin-specific CD8 T cells have been detected at high frequencies in patients with cancer but were not detected in healthy people. CTL reactivity against a number of survivin epitopes has been identified in patients with breast, colon, gastric and esophageal cancer and melanoma [89-91]. *Salmonella* mutant carrier has been used to target survivin antigen for HLA class I pathway. The evaluation of antitumor efficacy of survivin DNA vaccine against pancreatic carcinoma in mouse models resulted in antitumor effects and increased tumor infiltrative lymphocytes [92]. Recently, a T3SS-translocated SPI2 effector-survivin fusion protein was explored to induce specific CTLs [64, 73]. Further, the efficacy of survivin *Salmonella*-based vaccine was enhanced by optimizing codon usage. *Salmonella*-based vaccine expressing codon-optimized survivin enhanced tumor-specific killing of tumor targets [93].

Several attempts were devised to enhance the anti-tumor effect of *Salmonella*-based vaccines, [reviewed in 77]. One of the first strategies aimed at the expression of prodrug-converting enzymes so that *Salmonella* colonizing tumors express an enzyme which converts a non-toxic prodrug into a toxic compound to tumor cells. For example, *S.* Typhimurium expressing the gene encoding thymidine kinase of herpes simplex virus (HSV-tk) showed an enhancement of antitumor activity [94]. Bacterial toxins are used in bacteria-mediated cancer therapy as their expression, secretion, and functionality are easier to realize than the expression of eukaryotic proteins. For example, the bacterial toxin Colicin E3 (ColE3) [95] and cytolysin HlyE [96] were expressed in *S.* Typhimurium and conferred higher antitumor efficacy to the vaccines. Moreover, *S.* Typhimurium expressing different immuno-modulatory ligands, cytokines, and chemokines have been investigated as therapeutic molecules. For example, IL-2 was tested in several studies using several tumor models [64, 97-98].

1.8. Considerations for development of *Salmonella* as live carrier

1.8.1. Attenuated mutant strains as carrier

A mandatory requirement for the use of live recombinant *Salmonella* for vaccination is a sufficient attenuation in virulence to prevent undesired side effects like bacteremia, diarrhea or fever. However, a critical balance between attenuation and over-attenuation must be considered as over-attenuation can lead to poor immunogenicity of the vaccine. *In vivo* studies indicate that various independent genetic defects can yield adequately attenuated *Salmonella* strains. The choice of the attenuating mutations should (i) keep the balance between attenuation and virulence, (ii) carry at least two independent

attenuating mutations to minimize the hypothetical risk of reversion to virulence, and (iii) be genetically stable. For specific applications, it might be necessary to establish systems to minimize the possibility of horizontal gene transfer from the vaccine strain to members of the mucosal flora or environmental microorganisms. Mutants deficient in the biosynthesis of aromatic amino acids or purines, adenylate cyclase or cAMP receptor protein, carrying mutations affecting the global regulatory system *phoPQ* or lacking the DNA adenine methylase Dam have been most widely characterized as carriers. Attenuated strains of *Salmonella* have been approved for use in human and in veterinary medicine [99-100].

1.8.1.1. *S. Typhimurium*-based carrier stains

Infection with *S.* Typhimurium provides a mouse model for systemic infections that resemble typhoid fever in humans. *aroA* mutant strains were proven to be safe and widely used as carrier for vaccination. SL5000 and SL7207 *aroA* mutant carrier strains were widely used to deliver heterologous antigens and induce humoral and cellular responses [99]. SL7207 strain was used to deliver antigens cruzipain, gD and gB from HSVgp100 and TRP-2 epitopes of melanoma, tyrosine hydroxylase and *ureB* for protection against *Trypanosoma cruzi*, HSV-2 virus, melanoma, neuroplastoma and *Helicobacter pylori*, respectively [99]. *aroA* double mutants were also examined, for example *aroA* and *dam* double mutant (Re88) was successfully used to deliver tumor antigen survivin and induce protection against lung cancer [101]. Furthermore, the *aroC ssaV* double mutation of strain WT05 was also found to be safe but only induced humoral responses [102]. Double mutant strains of *S.* Typhimurium defective in *pur* in combination with other genes were also used to induce both humoral and cellular

immune responses. The use of the *htrA purD* mutant strain to deliver tumor associated antigen survivin induced protective CD4 and CD8 responses [64]. LH1 160 (*purB phoP phoQ*) and Vnp20009 (*purI xyl msbB*) mutants induced humoral responses by oral and i.v. routes, respectively [87, 103].

Other trails were done to utilize different mutations to increase the immunogenicity of heterologous antigens and at the same time achieve required attenuation. The *rfaH* mutant strain of *S.* Typhimurium as vaccine carrier enhanced immunogenicity of heterologous antigens and was sufficiently attenuated in mice [104]. Mutations in *Salmonella* virulence genes were tried to develop efficient carriers. Despite reduced inflammatory responses and fluid secretion into the intestinal lumen, inactivation of SopB, a SPI1-T3SS translocated effector protein, also showed similar tissue distribution and promoted similar immune response as a wild-type strain. As SopB is known to aid the bacterial immune escape mechanisms, it can be subverted to optimize carrier performance. Moreover, *sopB* derivatives are less toxic for DCs, which are more efficient in their capacity to process and present antigens expressed by the carrier [69, 99, 105]. Double mutation in *ssaV*, a SPI2-encoded protein, and *aroC* showed variable humoral responses when used in humans [102]. *In vitro* studies in our laboratory showed that *sifA* mutants stimulated specific CD8 T cells proliferation more than *aroA* mutant when used to deliver Ova model antigen to B3Z T cells that have the *lacZ* reporter (Halim and Hensel, unpublished observations). Recently, new systems were developed in *S.* Typhimurium to enhance the safety and immunogenicity including regulated delay in *in vivo* attenuation [106], antigen synthesis [107] and cell lysis [108].

1.8.1.2. *S. Typhi-based* carrier strains

Translating the approaches of *S.* Typhimurium-based vaccines for mice into a *S.* Typhi-based vaccine for humans has led to mixed results. For example, Δ*galE*, Δ*aroC* Δ*aroD*, Δ*cya* Δ*crp* or Δ*phoPQ* mutants of *S.* Typhimurium were completely attenuated in mice and induced protective immunity to challenge with wild-type *S.* Typhimurium. In contrast, *S.* Typhi Δ*galE*, Δ*aroC* Δ*aroD* and Δ*cya* Δ*crp* mutants were not sufficiently attenuated and caused significant reactogenicity in humans [99, 109]. Taking into consideration the higher virulence of *S.* Typhi compared to *S.* Typhimurium, new strategies to sufficiently attenuate *S.* Typhi and overcome the reactogenicity will be essential. In this direction *S.* Typhi triple mutations Δ*cya* Δ*crp* Δ*cdt* or Δa*roC* Δa*roD* Δ*htrA* were reported to be safe and immunogenic in humans [110-111]. It was not a surprising to find that mutations which highly attenuated *S.* Typhimurium were safe and immunogenic when transferred to *S.* Typhi. Furthermore, the Δ*aroC* Δ*ssaV S.* Typhi Ty2 mutant was reported to be safe and immunogenic in humans, while *S.* Typhimurium mutant was strongly attenuated and showed variable humoral response [102]. Another example is the *phoP* mutant of *S.* Typhimurium, which is completely attenuated in mice, led to development of a safe and immunogenic Δ*phoPQ S.* Typhi (Ty800) vaccine strain [112-113].

Non-reactogenic *S.* Typhi strains are often hyper-attenuated and induce poor immune responses, even to *Salmonella* antigens themselves [113]. Using live *S.* Typhi vectors in humans still face major difficulty in balancing between immunogenicity to vectored antigen and reactogenicity. It is believed that the recombinant *S.* Typhi strains do not colonize lymphoid tissues to a sufficient level to stimulate a strong immune response

against the foreign antigens. The host immune system may also be stimulated by the *Salmonella* carrier, inducing a strong response against *Salmonella* antigens instead of the vectored antigen [100]. This anti-vector response can cause severe limitations to prime boost vaccination regimes.

1.8.2. Expression of heterologous antigens and vector stability

Several factors such as the cellular location of the foreign antigen expressed by *Salmonella* carrier strains may affect the magnitude and type of immune response induced against an antigen. This influence may be due to degradation of the antigen in certain cellular compartments or modification of the antigen presentation to the immune system. While efficient antigen display is an indispensable requirement for induction of T cells, the strength of antigen presentation might depend on antigen abundance. Thus it is hypothesized that the higher the antigen expression, the higher the immunogenicity of antigens. Surprisingly, the highest levels of expression are not necessarily required for an elevated immune response to heterologous antigens [73, 114]. As with any foreign antigen expression system in bacteria, certain proteins may be toxic to carrier bacteria especially, when they are expressed at high levels.

The foreign genes inserted into the chromosome or foreign plasmid vectors carried by *Salmonella*-based vaccines should possess some containment features to minimize the possibility of transfer to and maintenance in other bacterial species. There are also other considerations that should be considered for successful construction of heterologous antigen expression cassette. For example, the stability of plasmid, enzymatic degradation, resistance to any antibiotic and incorrect folding of the expressed antigen(s) are some of the most common problems encountered in the

development of *Salmonella*-based recombinant strains. In the following section, we will discuss some ideas to overcome these limitations.

1.8.2.1. Low-level or unstable expression of heterologous antigens

Although the highest expression level may not induce the highest immune response [73], there is a necessity to express foreign antigen at sufficient levels to induce immune response. Plasmids with different replicons and greatly differing copy number have been used for antigen expression in *Salmonella*. Although, high expression level of high-copy number plasmids can improve the immunogenicity of the vaccine [115], unfortunately, high-level expression of the heterologous antigen has been found to be toxic to the *Salmonella* carrier. Lowering the copy number of the plasmid often results in increased stability of the construct and in reduction of the expression of the heterologous antigen to non-toxic levels [71, 116]. A possible solution is to construct expression cassettes in which promoters are located on inverting sequences, ensuring that expression of heterologous antigen only occurs when the promoters are in frame with the antigen-encoding sequences [117-118].

1.8.2.2. In-vivo inducible promoters

An alternative technique involves the use of *in vivo*-inducible promoters that allow expression of heterologous antigens at particular cellular location. Using these promoters, the level of antigen expression will be low until the *Salmonella* carrier recognizes an environmental stimulus, which then results in increased antigen expression. Ideally, the promoters should allow increased expression in APCs following uptake of the *Salmonella* carriers. For example, the *nirB in vivo*-inducible promoter is known to be induced by entry of *Salmonella* into cells in anaerobic environments [119].

The expression of genes within SPI2 as well as large number of loci outside of SPI2 is under control of the SPI2-encoded two regulatory system SsrAB [120]. Promoters of the SsrAB virulon appear to be ideal *in vivo*-induced promoters, as they can be used for the temporally and spatially controlled expression of heterologous antigens by live attenuated bacterial carrier strains. P_{sseA} of the SsrAB virulon, that is active after the uptake of *Salmonella* by antigen-presenting cells, was used to generate recombinant vaccines using attenuated *S.* Typhimurium bacteria as carriers [71, 116, 121]. Upon testing other SsrAB promoters for *in-vivo* activity, P_{sifB} and P_{sseJ} were found to be activated efficiently for generation of *Salmonella*-based vaccines [73, 122]. In addition to the low expression level of heterologous antigens in *Salmonella*, unstable expression of the antigens may also be encountered, particularly *in vivo*. Such problems may occur as a result of instability of the genes encoding the antigens. The attempts to increase plasmid stability and to reduce the toxic effects of the expressed proteins on the bacterial carrier are not always successful and in some cases integration of the foreign genes in the chromosome may be needed.

1.8.2.3. Chromosomal integration of foreign genes

To solve the problem of instability, a system was developed by which DNA encoding a heterologous antigen could be incorporated by recombination into the *Salmonella* chromosome. The foreign gene is introduced into the *Salmonella* vaccine on a suicide-vector (unable of replicate in the bacterial host) with flanking regions that allow incorporation into the *Salmonella* chromosome by homologous recombination into a pre-determined site [123]. This is an attractive solution which has several advantages such as elimination of the unstable plasmid and removal of the antibiotic resistance

marker [123]. In addition, the insertion of the foreign gene into the chromosome itself introduces an attenuating mutation into the vaccine strains [124-125]. This system has since been used to insert DNA encoding various heterologous antigens into the *Salmonella* chromosome [72, 126-127].

Chromosomal integration of a heterologous gene can result in complete stabilization of expression. However, the antigen will be expressed from only a single copy of the foreign gene, compared to multiple copies of the gene present in *Salmonella* harboring a multicopy expression plasmid. This may result in low expression of the antigen and, as a result, low immunogenicity. Possible ways to avoid this problem may involve the integration of several copies of the foreign gene into the chromosome or the use of stronger promoters.

1.8.2.4. Use of selectable markers

The development of recombinant *Salmonella* vaccines usually requires the use of selectable markers, often conferring antibiotic resistance. However, there is the concern that resistance genes may spread to pathogenic organisms in the environment, possibly rendering them refractory to antibiotic treatment. Because, the use of plasmids carrying antibiotic resistance genes for human use is not ideal, it is needed to develop *Salmonella* vaccine systems that do not contain antibiotic resistance markers. In this direction, chromosomal integration of foreign genes can overcome the disadvantage of expressing antigens from recombinant plasmids which otherwise require antibiotic selection for maintenance [72, 126].

1.8.2.5. Balanced-lethal stabilization systems

An alternative approach to genetic stability is the use of balanced-lethal plasmid stabilization systems. This involves the incorporation of the gene encoding the heterologous antigen into a plasmid containing gene that complements a metabolic defect in the *Salmonella* carrier strain. As an example, a plasmid vector carrying the gene of the aspartate β-semialdehyde dehydrogenase (*asd*) was generated. The enzyme is common to the biosynthetic pathways of several amino acids, and also diaminopimelic acid (DAP), an essential constituent of the peptidoglycan of the cell wall of Gram-negative bacteria. DAP is not present in mammals that leads to lysis of the bacteria. *Salmonella asd* mutant strains were complemented with The *asd*+ plasmid, constituting a balanced lethal combination in that all surviving cells would have to possess the recombinant *asd*+ plasmid [128]. Expression plasmids carrying the thymidylate synthetase (*thyA*) gene provide another balanced-lethal system, used to complement a *Salmonella* vaccine strain with a thymidine requirement, caused by a mutation in the chromosomal *thyA* gene [129].

1.8.2.6. Incompatible plasmids carrying promoter and repressor

This system was used to control the expression of recombinant proteins that are toxic to *Salmonella* [130]. In this system the foreign antigen is expressed from one plasmid under the control of *trc* promoter, whereas a second incompatible plasmid carries the LacI repressor. In These conditions, expression of the foreign antigen will be repressed when the bacteria are grown *in vitro* under antibiotic selection for both plasmids. But the growth of *Salmonella* within the host will result in loss of one of the two incompatible plasmids. The one which carries the foreign antigen encodes also Asd and will be

essential for survival of an *asd Salmonella* mutant. The overall effect is the repression of *in vitro* expression of the antigen is (avoiding a possible deleterious effect to the carrier) and after replication within the host tissues occurs, the incompatible plasmid carrying the repressor will segregate leading to constitutive expression of the foreign antigen [130].

1.8.2.7. The ON/OFF expression system

An “on-off” promoter controlled by a randomly invertible sequence derived from the bacteriophage Mu Gin invertase generates a non-expressing bacterial population that continually yields producing bacteria. When the system was used to the expression of the B-subunit of cholera toxin in an *aroA Salmonella* vaccine, an increase in specific systemic and secretory antibody responses was obtained [117].

1.9. Adjuvants

Adjuvants are molecules, compounds or macromolecular complexes that boost the potency and longevity of specific immune response to antigens, causing long lasting immune effects. Adjuvants functions to enhance, prolong and direct the immunogenicity of antigens that result in reducing the amount of antigen or number of immunizations required and improving the efficacy of vaccines [131]. As vehicles or delivery system is considered an important adjuvant in modern vaccination as they present vaccine antigen to the immune system in an optimal manner and control release of antigen [131]. Recently, immuno-stimulants that directly act on the immune system, such as

Toll-like receptors (TLR) ligands, cytokines, saponins and bacterial exotoxins, were introduced as potent adjuvants.

Alum, an aluminum salt-based adjuvant, is widely used in several licensed human vaccines [132]. *Cholera* toxin B subunit (CTB) is used to enhance mucosal responses of orally delivered vaccines [133]. To further increase immunogenicity of *Salmonella* DNA vaccine, the use of molecular adjuvants such as chemokine and cytokines have been employed [97]. Molecular adjuvants are administered typically as plasmids encoding chemokines, cytokines, or costimulatory molecules. They are administered in conjunction with a given DNA vaccine and serve as immune modulators. Several studies focused on proinflammatory cytokines, such as GM-CSF, IL-1α, TNF-α; Th1 cytokines, such as IFN-γ, IL-2, and IL-18; Th2 cytokines, such as IL-4, IL-6, and IL-10; and chemokines, such as CCL5 or CCL21 [134].

For therapeutic vaccination against tumors, specific adjuvants have been experimentally evaluated. α-Galactosylceramide, a glycolipid with an alpha anomeric-linked galactose moiety, was originally derived from the marine sponge *Agelas mauritianus* and was shown to possess potent antitumor properties in mice, which required the presence of both CD1d as the antigen-presenting molecule and natural killer T (NKT) cells [135]. NKTs are activated during *Salmonella* infection by interleukin (IL)-12 and IL-18 produced by LPS-activated DCs [136] and, possibly, by an endogenous ligand isoglobotrihexosylceramide (iGb3)[137]. NKT play a crucial role in immunity against viral, cancer and intracellular bacterial infection. NKT ligands Galactosylceramide were used with great success as adjuvants for *Salmonella*-based cancer vaccines by

triggering CD1d- and TLR-mediated programs in DCs and inducing effector responses by NKT and T cells [64].

1.10. Aim of the work

Our detailed molecular understanding of the virulence mechanisms of *S. enterica*, in conjunction with broad array for molecular tools for the genetic manipulation of this species provide an excellent basis future development of live vaccine carriers. Whether DNA vaccine delivery by attenuated *Salmonella* strains or the presentation or translocation of heterologous vaccine antigens is the more efficient, has to be shown by comparative, quantitative analyses.

Although many of the new strategies that deploy live attenuated *Salmonella* as vaccine carriers lead to promising results in animal models of vaccination, the transfer of these approaches to human vaccination is likely to be a major obstacle. The current limitations include the limited number of *S*. Typhi-based carrier strains that are approached for applications in humans, the differences in the repertoire of virulence determinants and the difference in the immunity of murine systemic *Salmonella* infections and human typhoid fever. The delicate balance in life attenuated vaccines between immunostimulatory potential and safety requires careful selection of new *S*. Typhi carrier strains. If these requirements have been met, the life carrier approach should allow a very versatile, rapid and cost efficient way to respond to future needs for new vaccines.

Our aim of the work is to optimize *Salmonella* as a live carrier for vaccination vaccines not only against Bacterial, Viral and Parasitic infections but also against Cancer. And we are looking for optimizing the action of these vaccines through (**Fig 1.3**):

1- Optimal expression: find most active promoters, tight regulation, which in consequence provide strong immune response.
2- Optimal translocation: by selection of effectors for delivery and subcellular localization which provide a strong immune response against antigen.
3- Optimal carrier: which confer safety and provide sufficient survival in the host.
4- Stability of constructs.

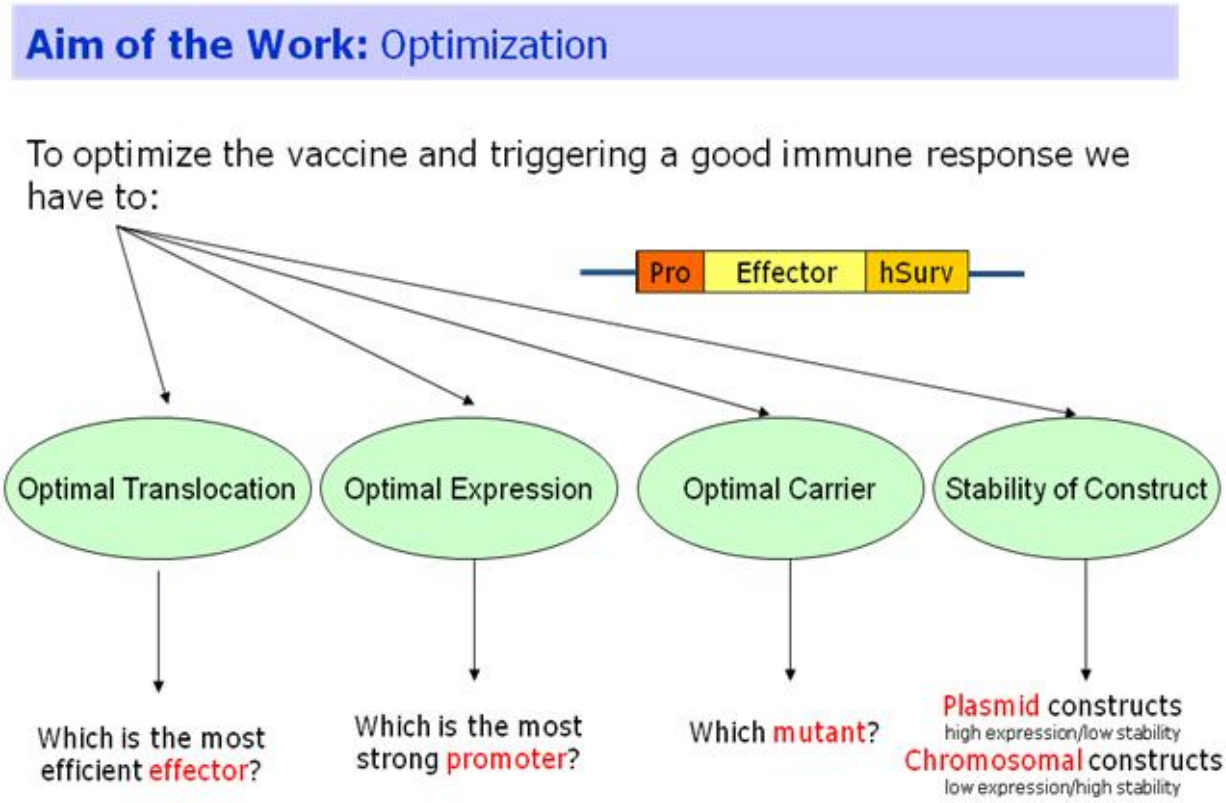


Fig. 1.3. Optimization of *Salmonella* as carrier for vaccination.

1.11. References

1. Mollenkopf, H., G. Dietrich, and S.H. Kaufmann, *Intracellular bacteria as targets and carriers for vaccination.* Biol. Chem., 2001. **382**(4): p. 521-532.
2. Dietrich, G., et al., *Delivery of antigen-encoding plasmid DNA into the cytosol of macrophages by attenuated suicide Listeria monocytogenes.* Nat Biotechnol, 1998. **16**(2): p. 181-5.
3. Weiss, S. and S. Krusch, *Bacteria-mediated transfer of eukaryotic expression plasmids into mammalian host cells.* Biol Chem, 2001. **382**(4): p. 533-41.
4. Fennelly, G.J., et al., *Mucosal DNA vaccine immunization against measles with a highly attenuated Shigella flexneri vector.* J Immunol, 1999. **162**(3): p. 1603-10.
5. Catic, A., et al., *Introduction of protein or DNA delivered via recombinant Salmonella typhimurium into the major histocompatibility complex class I presentation pathway of macrophages.* Microbes Infect, 1999. **1**(2): p. 113-21.
6. Darji, A., et al., *Oral somatic transgene vaccination using attenuated S. typhimurium.* Cell, 1997. **91**(6): p. 765-775.
7. Pasetti, M.F., et al., *Attenuated deltaguaBA Salmonella typhi vaccine strain CVD 915 as a live vector utilizing prokaryotic or eukaryotic expression systems to deliver foreign antigens and elicit immune responses.* Clin Immunol, 1999. **92**(1): p. 76-89.
8. Grillot-Courvalin, C., et al., *Functional gene transfer from intracellular bacteria to mammalian cells.* Nat Biotechnol, 1998. **16**(9): p. 862-6.
9. Miller, L.A., et al., *Oral vaccination of white-tailed deer using a recombinant Bacillus Calmette-Guerin vaccine expressing the Borrelia burgdorferi outer surface protein A: prospects for immunocontraception.* Am J Reprod Immunol, 1999. **41**(4): p. 279-85.
10. O'Gaora, P., et al., *Yersinia enterocolitica aroA mutants as carriers of the B subunit of the Escherichia coli heat-labile enterotoxin to the murine immune system.* Microb Pathog, 1990. **9**(2): p. 105-16.
11. Rüssmann, H., *Inverted pathogenicity: the use of pathogen-specific molecular mechanisms for prevention or therapy of disease.* Int J Med Microbiol, 2004. **293**(7-8): p. 565-9.
12. Boyd, E.F., et al., *Molecular genetic relationships of the salmonellae.* Appl Environ Microbiol, 1996. **62**(3): p. 804-808.
13. Crump, J.A., S.P. Luby, and E.D. Mintz, *The global burden of typhoid fever.* Bull World Health Organ, 2004. **82**(5): p. 346-53.
14. DeRoeck, D., L. Jodar, and J. Clemens, *Putting typhoid vaccination on the global health agenda.* N Engl J Med, 2007. **357**(11): p. 1069-71.
15. Hansen-Wester, I. and M. Hensel, *Salmonella pathogenicity islands encoding type III secretion systems.* Microbes Infect, 2001. **3**(7): p. 549-59.
16. Hensel, M., *Salmonella pathogenicity island 2.* Mol Microbiol, 2000. **36**(5): p. 1015-23.
17. Hensel, M., *Evolution of pathogenicity islands of Salmonella enterica.* Int J Med Microbiol, 2004. **294**(2-3): p. 95-102.

18. Haraga, A., M.B. Ohlson, and S.I. Miller, *Salmonellae interplay with host cells.* Nat. Rev. Microbiol., 2008. **6**: p. 53-66.
19. Hueck, C.J., *Type III protein secretion systems in bacterial pathogens of animals and plants.* Microbiol Mol Biol Rev, 1998. **62**(2): p. 379-433.
20. Ghosh, P., *Process of protein transport by the type III secretion system.* Microbiol Mol Biol Rev, 2004. **68**(4): p. 771-95.
21. Aizawa, S.I., *Bacterial flagella and type III secretion systems.* FEMS Microbiol Lett, 2001. **202**(2): p. 157-64.
22. Cornelis, G.R., *The type III secretion injectisome.* Nat Rev Microbiol, 2006. **4**(11): p. 811-25.
23. Gerlach, R.G. and M. Hensel, *Salmonella pathogenicity islands in host specificity, host pathogen-interactions and antibiotics resistance of Salmonella enterica.* Berl Munch Tierarztl Wochenschr, 2007. **120**(7-8): p. 317-27.
24. Kuhle, V. and M. Hensel, *Cellular microbiology of intracellular Salmonella enterica: functions of the type III secretion system encoded by Salmonella pathogenicity island 2.* Cell Mol Life Sci, 2004. **61**(22): p. 2812-26.
25. Waterman, S.R. and D.W. Holden, *Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system.* Cell Microbiol, 2003. **5**(8): p. 501-11.
26. Hobbie, S., et al., *Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by Salmonella typhimurium in cultured intestinal epithelial cells.* J Immunol, 1997. **159**(11): p. 5550-9.
27. Schlumberger, M.C. and W.D. Hardt, *Salmonella type III secretion effectors: pulling the host cell's strings.* Curr. Opin. Microbiol., 2006. **9**(1): p. 46-54.
28. Kuhle, V. and M. Hensel, *Cellular microbiology of intracellular Salmonella enterica: functions of the type III secretion system encoded by Salmonella pathogenicity island 2.* Cell. Mol. Life Sci., 2004. **61**(22): p. 2812-26.
29. Deiwick, J., et al., *Environmental regulation of Salmonella pathogenicity island 2 gene expression.* Mol Microbiol, 1999. **31**(6): p. 1759-73.
30. Ochman, H., et al., *Identification of a pathogenicity island required for Salmonella survival in host cells.* Proc Natl Acad Sci U S A, 1996. **93**(15): p. 7800-4.
31. Shea, J.E., et al., *Influence of the Salmonella typhimurium pathogenicity island 2 type III secretion system on bacterial growth in the mouse.* Infect Immun, 1999. **67**(1): p. 213-9.
32. Vassaux, G., et al., *Bacterial gene therapy strategies.* J Pathol, 2006. **208**(2): p. 290-8.
33. Hohlfeld, R. and A.G. Engel, *The immunobiology of muscle.* Immunol Today, 1994. **15**(6): p. 269-74.
34. Deck, R.R., et al., *Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine.* Vaccine, 1997. **15**(1): p. 71-8.
35. Mollenkopf, H., G. Dietrich, and S.H. Kaufmann, *Intracellular bacteria as targets and carriers for vaccination.* Biol Chem, 2001. **382**(4): p. 521-32.
36. Loessner, H., et al., *Improving live attenuated bacterial carriers for vaccination and therapy.* Int J Med Microbiol, 2008. **298**(1-2): p. 21-6.

37. Darji, A., et al., *Oral delivery of DNA vaccines using attenuated Salmonella typhimurium as carrier.* FEMS Immunol Med Microbiol, 2000. **27**(4): p. 341-9.
38. Monack, D.M., et al., *Salmonella exploits caspase-1 to colonize Peyer's patches in a murine typhoid model.* J Exp Med, 2000. **192**(2): p. 249-58.
39. Yrlid, U., et al., *Salmonella infection of bone marrow-derived macrophages and dendritic cells: influence on antigen presentation and initiating an immune response.* FEMS Immunol Med Microbiol, 2000. **27**(4): p. 313-20.
40. Loessner, H. and S. Weiss, *Bacteria-mediated DNA transfer in gene therapy and vaccination.* Expert Opin Biol Ther, 2004. **4**(2): p. 157-68.
41. Schoen, C., et al., *Bacteria as DNA vaccine carriers for genetic immunization.* Int J Med Microbiol, 2004. **294**(5): p. 319-35.
42. Shahabi, V., et al., *Live, attenuated strains of Listeria and Salmonella as vaccine vectors in cancer treatment.* Bioeng Bugs, 2010. **1**(4): p. 235-9.
43. Bauer, H., et al., *Salmonella-mediated oral DNA vaccination using stabilized eukaryotic expression plasmids.* Gene Ther, 2005. **12**(4): p. 364-72.
44. Zhao, Z., et al., *Protection of mice from Brucella infection by immunization with attenuated Salmonella enterica serovar typhimurium expressing A L7/L12 and BLS fusion antigen of Brucella.* Vaccine, 2009. **27**(38): p. 5214-9.
45. Zhang, Q., et al., *Enhanced protection against nasopharyngeal carriage of Streptococcus pneumoniae elicited by oral multiantigen DNA vaccines delivered in attenuated Salmonella typhimurium.* Mol Biol Rep, 2011. **38**(2): p. 1209-17.
46. Buckley, A.M., et al., *Evaluation of live-attenuated Salmonella vaccines expressing Campylobacter antigens for control of C. jejuni in poultry.* Vaccine, 2010. **28**(4): p. 1094-105.
47. Ramirez, K., et al., *Mucosally delivered Salmonella typhi expressing the Yersinia pestis F1 antigen elicits mucosal and systemic immunity early in life and primes the neonatal immune system for a vigorous anamnestic response to parenteral F1 boost.* J Immunol, 2009. **182**(2): p. 1211-22.
48. Galen, J.E., et al., *Mucosal immunization with attenuated Salmonella enterica serovar Typhi expressing protective antigen of anthrax toxin (PA83) primes monkeys for accelerated serum antibody responses to parenteral PA83 vaccine.* J Infect Dis, 2009. **199**(3): p. 326-35.
49. Woo, P.C., et al., *Unique immunogenicity of hepatitis B virus DNA vaccine presented by live-attenuated Salmonella typhimurium.* Vaccine, 2001. **19**(20-22): p. 2945-54.
50. Zheng, B.J., et al., *A single dose of oral DNA immunization delivered by attenuated Salmonella typhimurium down-regulates transgene expression in HBsAg transgenic mice.* Eur J Immunol, 2002. **32**(11): p. 3294-304.
51. Flo, J., S. Tisminetzky, and F. Baralle, *Oral transgene vaccination mediated by attenuated Salmonellae is an effective method to prevent Herpes simplex virus-2 induced disease in mice.* Vaccine, 2001. **19**(13-14): p. 1772-82.
52. Baud, D., et al., *Improved efficiency of a Salmonella-based vaccine against human papillomavirus type 16 virus-like particles achieved by using a codon-optimized version of L1.* J Virol, 2004. **78**(23): p. 12901-9.
53. Chiu, C.H., et al., *Protection of neonatal mice from lethal enterovirus 71 infection by maternal immunization with attenuated Salmonella enterica serovar*

Typhimurium expressing VP1 of enterovirus 71. Microbes Infect, 2006. **8**(7): p. 1671-8.
54. Shata, M.T., et al., *Mucosal and systemic HIV-1 Env-specific CD8(+) T-cells develop after intragastric vaccination with a Salmonella Env DNA vaccine vector.* Vaccine, 2001. **20**(3-4): p. 623-9.
55. Echchannaoui, H., et al., *Intravaginal immunization of mice with recombinant Salmonella enterica serovar Typhimurium expressing human papillomavirus type 16 antigens as a potential route of vaccination against cervical cancer.* Infect Immun, 2008. **76**(5): p. 1940-51.
56. Wong, L.P., et al., *DNA immunization using a secreted cell wall antigen Mp1p is protective against Penicillium marneffei infection.* Vaccine, 2002. **20**(23-24): p. 2878-86.
57. Cazorla, S.I., et al., *Oral vaccination with Salmonella enterica as a cruzipain-DNA delivery system confers protective immunity against Trypanosoma cruzi.* Infect Immun, 2008. **76**(1): p. 324-33.
58. Paglia, P., et al., *Gene transfer in dendritic cells, induced by oral DNA vaccination with Salmonella typhimurium, results in protective immunity against a murine fibrosarcoma.* Blood, 1998. **92**(9): p. 3172-6.
59. Zoller, M. and O. Christ, *Prophylactic tumor vaccination: comparison of effector mechanisms initiated by protein versus DNA vaccination.* J Immunol, 2001. **166**(5): p. 3440-50.
60. Cochlovius, B., et al., *Oral DNA vaccination: antigen uptake and presentation by dendritic cells elicits protective immunity.* Immunol Lett, 2002. **80**(2): p. 89-96.
61. Mastroeni, P., et al., *Salmonella: immune responses and vaccines.* Vet J, 2001. **161**(2): p. 132-64.
62. Rüssmann, H., *Bacterial type III translocation: a unique mechanism for cytosolic display of heterologous antigens by attenuated Salmonella.* Int J Med Microbiol, 2003. **293**(1): p. 107-112.
63. Panthel, K., et al., *Salmonella type III-mediated heterologous antigen delivery: a versatile oral vaccination strategy to induce cellular immunity against infectious agents and tumors.* Int J Med Microbiol, 2008. **298**(1-2): p. 99-103.
64. Xiong, G., et al., *Novel cancer vaccine based on genes of Salmonella pathogenicity Island 2.* Int J Cancer, 2010. **126**(11): p. 2622-2634.
65. Yewdell, J.W. and J.R. Bennink, *Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses.* Annu Rev Immunol, 1999. **17**: p. 51-88.
66. Stender, S., et al., *Identification of SopE2 from Salmonella typhimurium, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell.* Mol Microbiol, 2000. **36**(6): p. 1206-21.
67. Fu, Y. and J.E. Galan, *A Salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion.* Nature, 1999. **401**(6750): p. 293-7.
68. Panthel, K., et al., *Salmonella pathogenicity island 2-mediated overexpression of chimeric SspH2 proteins for simultaneous induction of antigen-specific CD4 and CD8 T cells.* Infect Immun, 2005. **73**(1): p. 334-41.

69. Nagarajan, A.G., et al., *SopB of Salmonella enterica serovar Typhimurium is a potential DNA vaccine candidate in conjugation with live attenuated bacteria.* Vaccine, 2009. **27**(21): p. 2804-11.
70. Abrahams, G.L. and M. Hensel, *Manipulating cellular transport and immune responses: dynamic interactions between intracellular Salmonella enterica and its host cells.* Cell Microbiol, 2006. **8**(5): p. 728-37.
71. Husseiny, M.I. and M. Hensel, *Evaluation of an intracellular-activated promoter for the generation of live Salmonella recombinant vaccines.* Vaccine, 2005. **23**(20): p. 2580-2590.
72. Husseiny, M.I. and M. Hensel, *Evaluation of Salmonella live vaccines with chromosomal expression cassettes for translocated fusion proteins.* Vaccine, 2009. **27**(28): p. 3780-3787.
73. Xu, X., et al., *Efficacy of intracellular activated promoters for generation of Salmonella-based vaccines.* Infect Immun, 2010. **78**(11): p. 4828-38.
74. Russmann, H., et al., *Protection against murine listeriosis by oral vaccination with recombinant Salmonella expressing hybrid Yersinia type III proteins.* J Immunol, 2001. **167**(1): p. 357-65.
75. Igwe, E.I., G. Geginat, and H. Russmann, *Concomitant cytosolic delivery of two immunodominant listerial antigens by Salmonella enterica serovar typhimurium confers superior protection against murine listeriosis.* Infect Immun, 2002. **70**(12): p. 7114-9.
76. Barbe, S., L. Van Mellaert, and J. Anne, *The use of clostridial spores for cancer treatment.* J Appl Microbiol, 2006. **101**(3): p. 571-8.
77. Leschner, S. and S. Weiss, *Salmonella-allies in the fight against cancer.* J Mol Med, 2010. **88**(8): p. 763-73.
78. Pawelek, J.M., K.B. Low, and D. Bermudes, *Tumor-targeted Salmonella as a novel anticancer vector.* Cancer Res, 1997. **57**(20): p. 4537-44.
79. Kasinskas, R.W. and N.S. Forbes, *Salmonella typhimurium specifically chemotax and proliferate in heterogeneous tumor tissue in vitro.* Biotechnol Bioeng, 2006. **94**(4): p. 710-21.
80. Kasinskas, R.W. and N.S. Forbes, *Salmonella typhimurium lacking ribose chemoreceptors localize in tumor quiescence and induce apoptosis.* Cancer Res, 2007. **67**(7): p. 3201-9.
81. Minton, N.P., *Clostridia in cancer therapy.* Nat Rev Microbiol, 2003. **1**(3): p. 237-42.
82. Theys, J., et al., *Tumor-specific gene delivery using genetically engineered bacteria.* Curr Gene Ther, 2003. **3**(3): p. 207-21.
83. Yam, C., et al., *Monotherapy with a tumor-targeting mutant of S. typhimurium inhibits liver metastasis in a mouse model of pancreatic cancer.* J Surg Res, 2010. **164**(2): p. 248-55.
84. Jia, L.J., et al., *Tumor-targeting Salmonella typhimurium improves cyclophosphamide chemotherapy at maximum tolerated dose and low-dose metronomic regimens in a murine melanoma model.* Int J Cancer, 2007. **121**(3): p. 666-74.

85. Jia, L.J., et al., *Enhanced therapeutic effect by combination of tumor-targeting Salmonella and endostatin in murine melanoma model.* Cancer Biol Ther, 2005. **4**(8): p. 840-5.
86. Avogadri, F., et al., *Intra-tumoral Salmonella typhimurium induces a systemic anti-tumor immune response that is directed by low-dose radiation to treat distal disease.* Eur J Immunol, 2008. **38**(7): p. 1937-47.
87. Toso, J.F., et al., *Phase I study of the intravenous administration of attenuated Salmonella typhimurium to patients with metastatic melanoma.* J Clin Oncol, 2002. **20**(1): p. 142-52.
88. Heimann, D.M. and S.A. Rosenberg, *Continuous intravenous administration of live genetically modified salmonella typhimurium in patients with metastatic melanoma.* J Immunother, 2003. **26**(2): p. 179-80.
89. Altieri, D.C., *Validating survivin as a cancer therapeutic target.* Nat Rev Cancer, 2003. **3**(1): p. 46-54.
90. Guha, M. and D.C. Altieri, *Survivin as a global target of intrinsic tumor suppression networks.* Cell Cycle, 2009. **8**(17): p. 2708-10.
91. Johnson, M.E. and E.W. Howerth, *Survivin: a bifunctional inhibitor of apoptosis protein.* Vet Pathol, 2004. **41**(6): p. 599-607.
92. Zhu, K., et al., *Survivin DNA vaccine generated specific antitumor effects in pancreatic carcinoma and lymphoma mouse models.* Vaccine, 2007. **25**(46): p. 7955-61.
93. Manuel, E.R., et al., *Enhancement of Cancer Vaccine Therapy by Systemic Delivery of a Tumor Targeting Salmonella-based STAT3 shRNA Suppresses the Growth of Established Melanoma Tumors.* Cancer Res, 2011.
94. Friedlos, F., et al., *Attenuated Salmonella targets prodrug activating enzyme carboxypeptidase G2 to mouse melanoma and human breast and colon carcinomas for effective suicide gene therapy.* Clin Cancer Res, 2008. **14**(13): p. 4259-66.
95. Lancaster, L.E., W. Wintermeyer, and M.V. Rodnina, *Colicins and their potential in cancer treatment.* Blood Cells Mol Dis, 2007. **38**(1): p. 15-8.
96. Ryan, R.M., et al., *Bacterial delivery of a novel cytolysin to hypoxic areas of solid tumors.* Gene Ther, 2009. **16**(3): p. 329-39.
97. Sorenson, B.S., et al., *Attenuated Salmonella typhimurium with IL-2 gene reduces pulmonary metastases in murine osteosarcoma.* Clin Orthop Relat Res, 2008. **466**(6): p. 1285-91.
98. al-Ramadi, B.K., et al., *Potent anti-tumor activity of systemically-administered IL2-expressing Salmonella correlates with decreased angiogenesis and enhanced tumor apoptosis.* Clin Immunol, 2009. **130**(1): p. 89-97.
99. Becker, P.D., M. Noerder, and C.A. Guzman, *Genetic immunization: bacteria as DNA vaccine delivery vehicles.* Hum Vaccin, 2008. **4**(3): p. 189-202.
100. Shi, H., et al., *Live recombinant Salmonella Typhi vaccines constructed to investigate the role of rpoS in eliciting immunity to a heterologous antigen.* PLoS One, 2010. **5**(6): p. e11142.
101. Xiang, R., et al., *A dual-function DNA vaccine encoding carcinoembryonic antigen and CD40 ligand trimer induces T cell-mediated protective immunity*

against colon cancer in carcinoembryonic antigen-transgenic mice. J Immunol, 2001. **167**(8): p. 4560-5.
102. Hindle, Z., et al., *Characterization of Salmonella enterica derivatives harboring defined aroC and Salmonella pathogenicity island 2 type III secretion system (ssaV) mutations by immunization of healthy volunteers.* Infect Immun, 2002. **70**(7): p. 3457-67.
103. Angelakopoulos, H. and E.L. Hohmann, *Pilot study of phoP/phoQ-deleted Salmonella enterica serovar typhimurium expressing Helicobacter pylori urease in adult volunteers.* Infect Immun, 2000. **68**(4): p. 2135-41.
104. Kong, Q., et al., *Regulated delayed expression of rfaH in an attenuated Salmonella enterica serovar typhimurium vaccine enhances immunogenicity of outer membrane proteins and a heterologous antigen.* Infect Immun, 2009. **77**(12): p. 5572-82.
105. Link, C., et al., *An SopB-mediated immune escape mechanism of Salmonella enterica can be subverted to optimize the performance of live attenuated vaccine carrier strains.* Microbes Infect, 2006. **8**(8): p. 2262-9.
106. Curtiss, R., 3rd, et al., *Salmonella enterica serovar typhimurium strains with regulated delayed attenuation in vivo.* Infect Immun, 2009. **77**(3): p. 1071-82.
107. Xin, W., et al., *Analysis of type II secretion of recombinant pneumococcal PspA and PspC in a Salmonella enterica serovar Typhimurium vaccine with regulated delayed antigen synthesis.* Infect Immun, 2008. **76**(7): p. 3241-54.
108. Kong, W., et al., *Regulated programmed lysis of recombinant Salmonella in host tissues to release protective antigens and confer biological containment.* Proc Natl Acad Sci U S A, 2008. **105**(27): p. 9361-6.
109. Bumann, D., et al., *Recombinant live Salmonella spp. for human vaccination against heterologous pathogens.* FEMS Immunol Med Microbiol, 2000. **27**(4): p. 357-64.
110. Tacket, C.O. and M.M. Levine, *CVD 908, CVD 908-htrA, and CVD 909 live oral typhoid vaccines: a logical progression.* Clin Infect Dis, 2007. **45 Suppl 1**: p. S20-3.
111. Tacket, C.O., et al., *Phase 2 clinical trial of attenuated Salmonella enterica serovar typhi oral live vector vaccine CVD 908-htrA in U.S. volunteers.* Infect Immun, 2000. **68**(3): p. 1196-201.
112. Galan, J.E. and R. Curtiss, 3rd, *Virulence and vaccine potential of phoP mutants of Salmonella typhimurium.* Microb Pathog, 1989. **6**(6): p. 433-43.
113. Hohmann, E.L., C.A. Oletta, and S.I. Miller, *Evaluation of a phoP/phoQ-deleted, aroA-deleted live oral Salmonella typhi vaccine strain in human volunteers.* Vaccine, 1996. **14**(1): p. 19-24.
114. Pathangey, L., et al., *Effect of expression level on immune responses to recombinant oral Salmonella enterica serovar Typhimurium vaccines.* Vaccine, 2009. **27**(20): p. 2707-11.
115. Covone, M.G., et al., *Levels of expression and immunogenicity of attenuated Salmonella enterica serovar typhimurium strains expressing Escherichia coli mutant heat-labile enterotoxin.* Infect Immun, 1998. **66**(1): p. 224-31.

116. Husseiny, M.I., F. Wartha, and M. Hensel, *Recombinant vaccines based on translocated effector proteins of Salmonella Pathogenicity Island 2.* Vaccine, 2007. **25**(1): p. 185-93.
117. Tijhaar, E.J., et al., *Construction and evaluation of an expression vector allowing the stable expression of foreign antigens in a Salmonella typhimurium vaccine strain.* Vaccine, 1994. **12**(11): p. 1004-11.
118. Yan, Z.X. and T.F. Meyer, *Mixed population approach for vaccination with live recombinant Salmonella strains.* J Biotechnol, 1996. **44**(1-3): p. 197-201.
119. Everest, P., et al., *Expression of LacZ from the htrA, nirB and groE promoters in a Salmonella vaccine strain: influence of growth in mammalian cells.* FEMS Microbiol Lett, 1995. **126**(1): p. 97-101.
120. Deiwick, J. and M. Hensel, *Regulation of virulence genes by environmental signals in Salmonella typhimurium.* Electrophoresis, 1999. **20**(4-5): p. 813-7.
121. Jantsch, J., et al., *Intracellular activities of Salmonella enterica in murine dendritic cells.* Cell Microbiol, 2003. **5**(12): p. 933-45.
122. Xu, X. and M. Hensel, *Systematic analysis of the SsrAB virulon of Salmonella enterica.* Infect Immun, 2010. **78**(1): p. 49-58.
123. Hone, D., et al., *A chromosomal integration system for stabilization of heterologous genes in Salmonella based vaccine strains.* Microb Pathog, 1988. **5**(6): p. 407-18.
124. Chatfield, S.N., et al., *Evaluation of Salmonella typhimurium strains harbouring defined mutations in htrA and aroA in the murine salmonellosis model.* Microb Pathog, 1992. **12**(2): p. 145-51.
125. Strugnell, R.A., et al., *Stable expression of foreign antigens from the chromosome of Salmonella typhimurium vaccine strains.* Gene, 1990. **88**: p. 57-63.
126. Lee, E., et al., *Chromosomal integration and expression of the Escherichia coli K88 gene cluster in Salmonella enterica ser. Choleraesuis strain 54 (SC54).* Vet Microbiol, 2001. **83**(2): p. 177-83.
127. Husseiny, M.I. and M. Hensel, *Construction of highly attenuated Salmonella enterica serovar Typhimurium live vectors for delivering heterologous antigens by chromosomal integration.* Microbiol Res, 2008. **163**(6): p. 605-15.
128. Zhang, T. and S.L. Stanley, Jr., *Expression of the serine rich Entamoeba histolytica protein (SREHP) in the avirulent vaccine strain Salmonella typhi TY2 chi 4297 (delta cya delta crp delta asd): safety and immunogenicity in mice.* Vaccine, 1997. **15**(12-13): p. 1319-22.
129. McNeill, H.V., et al., *Development of a nonantibiotic dominant marker for positively selecting expression plasmids in multivalent Salmonella vaccines.* Appl Environ Microbiol, 2000. **66**(3): p. 1216-9.
130. Ervin, S.E., P.A. Small, Jr., and P.A. Gulig, *Use of incompatible plasmids to control expression of antigen by Salmonella typhimurium and analysis of immunogenicity in mice*, in *Microb Pathog*. 1993. p. 93-101.
131. Reed, S.G., et al., *New horizons in adjuvants for vaccine development.* Trends Immunol, 2009. **30**(1): p. 23-32.
132. Clements, C.J. and E. Griffiths, *The global impact of vaccines containing aluminium adjuvants.* Vaccine, 2002. **20 Suppl 3**: p. S24-33.

133. Ryan, E.T. and S.B. Calderwood, *Cholera vaccines.* Clin Infect Dis, 2000. **31**(2): p. 561-5.
134. Abdulhaqq, S.A. and D.B. Weiner, *DNA vaccines: developing new strategies to enhance immune responses.* Immunol Res, 2008. **42**(1-3): p. 219-32.
135. Lu, X., et al., *Synthesis and evaluation of an alpha-C-galactosylceramide analogue that induces Th1-biased responses in human natural killer T cells.* Chembiochem, 2006. **7**(11): p. 1750-6.
136. Berntman, E., et al., *The role of CD1d-restricted NK T lymphocytes in the immune response to oral infection with Salmonella typhimurium.* Eur J Immunol, 2005. **35**(7): p. 2100-9.
137. Brigl, M., et al., *Mechanism of CD1d-restricted natural killer T cell activation during microbial infection.* Nat Immunol, 2003. **4**(12): p. 1230-7.

Chapter 2

2.1. Introduction

The use of live attenuated bacterial pathogens is a promising approach for the rational design of new recombinant vaccines. Several attenuated carrier strains have been deployed for expression and delivery of various viral, bacterial or parasitic antigens for vaccination [1]. Live attenuated strains of *Salmonella* that synthesize and secrete foreign antigens are being developed as vaccines for a number of infectious diseases and cancer [2]. Moreover, the use of live attenuated *Salmonellae* to deliver recombinant antigens to the immune system is an attractive strategy for the construction of multivalent vaccines [3]. *Salmonella*-based vaccines provide a number of advantages over other antigen delivery strategies including low cost of production, oral delivery, the absence of animal products, genetic stability and safety [4]. In addition, *Salmonella* vaccines delivering heterologous antigens elicit efficient immune responses via stimulation of both innate and adaptive immunity [4]. The existence of the serovar Typhimurium for preclinical work in mouse models and favorable clinical experience with the live attenuated vaccines *S*. Typhi Ty21a, CVD908 and CVD909 [5] further justify the development of *Salmonella*-based vaccine carriers.

The pathogenic *S. enterica* is a facultative intracellular pathogen that inhabits a unique membrane-bound host cell compartment, termed *Salmonella*-containing vacuole or SCV [reviewed in 6]. Localization within the SCV prevents delivery of expressed foreign proteins to the MHC class I-restricted antigen presentation pathway and hinders the use of *Salmonella* as vaccine carrier to induce specific CD8 T cells which are crucial for protection against viruses, intracellular bacteria, and tumors.

The majority of Gram-negative pathogens deploy complex virulence factor termed type III secretion systems (T3SS). T3SS mediate distinct functions that include anti-phagocytic and cytotoxic effects on host cells (Ysc/Yop system of *Yersinia* spp.), invasion of host cells (*S. enterica* SPI1 system, *Shigella* spp. Mxi/Spa system) and intracellular pathogenesis (*S. enterica* SPI2 system, *Chlamydia* spp. T3SS) [7]. T3SS consist of at least 20 different subunits which enable these bacteria to translocate specific effectors directly into the host cell cytoplasm in order to exert a broad range of virulence functions. The T3SS assembles needle-like appendages, which share similarity with the flagellar basal body and some of its proteins, including those which form the core of the central channel and are highly conserved between the two systems [8]. The T3SS apparatus, also referred to as injectisome, spans the inner and outer membranes of the bacterial envelope and secretes translocon and effector proteins. Translocon proteins allow access of effector proteins to the eukaryotic cells by forming pores in the host cell membrane and forming a connecting channel-like complex between the bacterium and the eukaryotic membrane. The effector proteins further subvert different aspects of host cell physiology and immunity thereby promoting bacterial virulence [9].

Several important virulence factors of *S. enterica* are encoded by genes within *Salmonella* Pathogenicity Islands (SPI) and two important loci are termed SPI1 and SPI2 [10]. SPI1 and SPI2 genes encode distinct T3SS that translocate bacterial effectors during different phases of pathogenesis [11]. *Salmonella* translocates T3SS effector proteins into the host cell cytoplasm mediated by either the SPI1-T3SS from the

extracellular stage and from within the SCV during the early stage after entry, or by the SPI2-T3SS from within the SCV at later stages during intracellular life [10].

Salmonella T3SS-mediated translocation was used for efficient delivery of heterologous antigens into the cytosol of antigen-presenting cells leading to prominent CD8 T cell-responses [2, 12]. *Salmonella* T3SS-mediated translocation can be used for efficient delivery of heterologous antigen fusions to SPI2 effector proteins to the cytosol of APC, leading to prominent CD8 T cell-priming in orally immunized mice [13]. SPI2 effector proteins, especially those which are synthesized only when the *Salmonella* is inside host cells such as dendritic cells (DCs) or macrophage [14] have been thought to be promising carrier candidates for antigen delivery to the MHC-I pathway.

Our previous studies used the SPI2-T3SS effector protein SseF as fusion partner for the translocation of heterologous antigens [12, 15]. In addition to SseF, 20 or more effector proteins are translocated by the SPI2-T3SS. We hypothesized that the translocation efficacy of effector-antigen fusions might be directly correlated to the immune response triggered by the vaccine. In this study, we used five membrane-associated SPI2 effectors under control of P_{sseA} promoter for the generation of expression cassettes with model antigens. Heterologous antigens can be expressed in *Salmonella* as fusions with recombinant or native proteins. This approach was mainly used to direct the expression of the desired antigen to a particular location in the bacterial cell and increase the immunogenicity of foreign antigens by fusing them to proteins that could exert a carrier effect [16]. We examined the efficacy of antigen translocation into APC cytosol and in vivo immunogenicity of fusion constructs with effectors SseJ, SifA, SseL or SteC in comparison with that of the previously described SseF effector. We observed that the

highest immune response in vitro and in vivo was induced by constructs containing effector SseJ.

2.2 Materials and Methods

2.2.1. Bacterial strains and growth conditions

Salmonella enterica serovar Typhimurium NCTC 12023 was used as wild-type strain. The *purD htrA* double-deficient strain MvP728 [12] was used as attenuated carrier strain. For the generation of recombinant plasmids, *E. coli* DH 5α was used as host. Plasmids were introduced into *Salmonella* strains by electroporation and recombinant strains were cultured in media containing 50 μg x ml^{-1} Carbenicillin in order to maintain the plasmids.

2.2.2. Generation of plasmids

For generation of expression cassettes with gene fusions consisting of *sseJ, sifA or sseL*, OVA and HA tag under control of SPI2 promoter P_{sseA}, the gene encoding OVA and carry its CD4 and CD8 epitopes was amplified by PCR using Ova-For-NaeI, OVA-HA-Rev-XbaI and pOMP as a template, which later digested with NaeI and XbaI. P_{sseJ}::s*seJ*::hSurvivin (p3550), P_{sifA}::s*ifA*::hSurvivin (p3551) or P_{sseL}::s*seL*::hSurvivin (p3552) plasmid was digested with EcoRV and XbaI. Digested OVA was ligated to large fragment of digested plasmids to obtain plasmids P_{sseJ}::s*seJ*::OVA::HA (p3554), P_{sifA}::s*ifA*::OVA::HA (p3556) or P_{sseL}::*sseL*::OVA::HA (p3555), these plasmids which are used to amplify *sseJ*::OVA::HA, s*ifA*::OVA::HA or *sseL*::OVA::HA using forward primers SseJ-For-EcoRI, SifA-For-Effector-EcoRI, or SseL-Effector-For-EcoRI and reverse primer OVA-HA-Rev-XbaI, which were digested later by EcoRI and XbaI. Plasmid

p3342 harboring P_{sseA}::*sscB sseF*::hSurvivin::HA [17] was digested with EcoRI and XbaI and the large fragment was ligated to digested *sseJ*::OVA::HA, *sifA*::OVA::HA or *sseL*::OVA::HA fragements resulting in P_{sseA}::*sseJ*::OVA::HA (p3631), P_{sseA}::*sifA*::OVA::HA (p3632), or P_{sseA}::sseL::OVA::HA (p3633). The obtained plasmids are confirmed by colony PCR, diagnostic digestion and sequenced using T7-Seq and T3-Seq primers.

For generation of expression cassette consisting of gene fusion SteC::OVA::HA under control of SPI2 promoter P_{sseA}, pWSK29 Low copy plasmid was digested with EcoRI and XbaI and OVA was amplified using primers OVA595-For-EcoRI and OVA-HA-Rev-XbaI and pOMP plasmid as template. Digested amplified OVA with EcoRI and XbaI was ligated to digested pWSK29 resulting in plasmid pWSK29::OVA::HA. The *sseA* promoter will be amplified using primers ProB-For-KpnI and ProB-Rev-EcoRI and plasmid p3342 as template. Plasmid pWSK29::OVA::HA was digested with KpnI and EcoRI and ligated to amplified SseA promoter which digested with KpnI and EcoRI obtaining plasmid pWSK29::P_{sseA}::OVA::HA. The gene for effector SteC was amplified using SteC-Effector-For-EcoRI and SteC-Rev-EcoRI. pWSK29 P_{sseA}::OVA::HA and amplified SteC effector both are digested by EcoRI and ligated obtaining or P_{sseA}::*steC*::OVA::HA (p3634). The resulting plasmid was confirmed by colony PCR for the right orientation and sequenced using T3-Seq and T7-Seq.

For generation of expression cassette consisting of gene fusion *sseJ, sifA, sseL* or *steC* to *lisA* and HA tag under control of SPI2 promoter P_{sseA}, the fragments P_{sseA}::*sseJ*, *PsseA*::*sifA*, *PsseA*::*sseL* or P_{sseA}::SteC were amplified using ProB-For-KpnI as forward primer and SseJ-Rev-EcoRV, SifA-Rev-EcoRV, SseL-Rev-EcoRV or SteC-Rev-NaeI as

reverse primer, which were digested with KpnI and EcoRV or NaeI. Plasmid p2810 harboring P_{sseA}::*sseF*::*lisA*::HA (Xu et al, 2010) was digested with KpnI and EcoRV, the large fragment was ligated to amplified digested P_{sseA}::*sseJ*, P_{sseA}::*sifA*, P_{sseA}::*sseL* or P_{sseA}::*steC,* obtaining plasmids P_{sseA}::*sseJ*::*lisA*::HA (p3635), P_{sseA}::*sifA*::*lisA*::HA (p3636), P_{sseA}::*sseL*::*lisA*::HA (p3637) or P_{sseA}::*steC*::*lisA*::HA (p3638). All the plasmids are confirmed with colony PCR, diagnostic digestion and sequenced using T3-Seq and T7-Seq.

Table 1. Plasmids used in this study

Designation	relevant characteristics	reference
pWSK29	Low copy number vector, Amp^R	Lab stock
pOMP-OVA	*lacZ*::OVA	Lab stock
p2629	pWSK29 P_{sseA} *sscB sseF*::OVA::M45	[15]
p2810	pWSK29 P_{sseA} *sscB sseF*::$lisA_{51\text{-}363}$::HA	[15]
p3550	pWSK29 P_{sseJ} *sseJ*::hSurvivin	This study
p3551	pWSK29 P_{sifA} *sifA*::hSurvivin	This study
p3552	pWSK29 P_{sseL} *sseL*::hSurvivin	This study
p3553	pWSK29 P_{steC} *steC*::hSurvivin	This study
p3554	pWSK29 P_{sseJ} *sseJ*::OVA::HA	This study
p3556	pWSK29 P_{sifA} *sifA*::OVA::HA	This study
p3555	pWSK29 P_{sseL} *sseL*::OVA::HA	This study
p3626	pWSK29 P_{steC} *steC*::OVA::HA	This study

p3631	pWSK29 P_{sseA} *sseJ*::OVA::HA	This study
p3632	pWSK29 P_{sseA} *sifA*::OVA::HA	This study
p3633	pWSK29 P_{sseA} *sseL*::OVA::HA	This study
p3634	pWSK29 P_{sseA} *steC*::OVA::HA	This study
p3635	pWSK29 P_{sseA} *sseJ*::*lisA*$_{51\text{-}363}$::HA	This study
p3636	pWSK29 P_{sseA} *sifA*::*lisA*$_{51\text{-}363}$::HA	This study
p3637	pWSK29 P_{sseA} *sseL*::*lisA*$_{51\text{-}363}$::HA	This study
p3638	pWSK29 P_{sseA} *steC*::*lisA*$_{51\text{-}363}$::HA	This study

Table 2. Oligonucleotides used in this study

Designation	**Sequence 5' – 3'**
SseJ-Rev-EcoRV	acggatatcttcagtggaataatgatgagc
SseJ-Pro-For-KpnI	tacggtacctcacataaaacactagcac
SseJ-For-EcoRI	ccggaattcgtaaggaggacactatgcc
SifA-Rev-EcoRV	acggatatcaaaacaacataaacagccgc
SifA-Pro-For-Kpn	tacggtacctcataagcgattaattgcgcaac
SifA–EcoRI-eff-For	GGCGAATTCATTTTTACTCCAGTATAAGTG
SseL-Rev-EcoRV	tatgatatcCTGGAGACTGTATTCATATATTTG
SseL-For-KpnI	attggtaCCATCAGACATATACCCTTC
SseL-effector-For-EcoRI	ggaGAATTCcagagcaaaatgaatatatgtgt
SteC-Rev-NaeI	tatgccggcTTTTTTTAATTCATCCTTTAATAC
SteC-Rev-EcoRI	tatGAATTCTTTTTTTAATTCATCCTTTAATAC

SteC-For-KpnI	attggtaCCAAGGTTCTGTAGGAAGCCTG
SteCeffector-For-EcoRI	ggaGAATTCcagaggatgagacatatgccg

2.2.3. Analysis of synthesis and translocation of recombinant vaccine antigens

In order to quantify the amounts of recombinant protein produced by the various strains, Western blots were analyzed using the Odyssey detection system and signal for model antigens were normalized by the levels of constitutively synthesized control protein DnaK.

For analyses of translocation, HeLa cells were infected with various *Salmonella* strains harboring plasmids for the expression of *effector*::*lisA*::HA, or *effector*::*OVA*::HA under the control of P_{sseA} promoter. As carrier strains a double mutant strain MvP728 were used. At 16 h after infection, the cells were fixed and processed for immuno-staining of *Salmonella* LPS (rabbit anti-*Salmonella* O1,4,5, Difco, BD) and the HA epitope tag (Roche) The cells were analyzed by microscopy using a Zeiss LSM700 laser-scanning confocal microscope.

2.2.4. Dendritic cell infection

The preparation and culture of bone marrow cells from C57BL/6 mice for generation of bone marrow-derived dendritic cells (BM-DC) has been previously described [18]. After 6 d of culture in RPMI 1640 medium (PAA, Colbe) containing 10 % heat-inactivated fetal calf serum (Gibco-BRL, Grand Island, NY) and GMCSF at 37°C in an atmosphere of 5 % CO_2, the BM-DC were suspended in small flasks for experiments at the density of 8 x 10^6 cells per flask and allowed to adhere for at least 12 h.

Bacterial strains were grown overnight in LB and the OD at 600 nm of the cultures was adjusted to 0.2 in 1 ml of PBS. Aliquots of this suspension were added to flasks in order

to yield a multiplicity of infection (MOI) of approximately 100. The flask were centrifuged at 500 x g for 5 min to synchronize the infection and subsequently incubated at 37°C in an atmosphere of 5 % CO_2 for 30 min. Thereafter, the flasks were washed thrice with RPMI 1640 medium and incubated with RPMI 1640 medium containing 100 μg x ml^{-1} gentamicin for 1 h, followed by RPMI 1640 medium containing 25 μg x ml^{-1} gentamicin for the rest of the experiment.

At 16 h after infection, the cells were fixed and processed for immuno-staining of *Salmonella* LPS (rabbit anti-*Salmonella* O1,4,5, Difco, BD), the HA epitope tag (Roche) and the dendritic cell-specific marker CD11c (Armenian hamster anti-CD11c, BD). The cells were analyzed by microscopy using a Zeiss LSM700 laser-scanning confocal microscope.

2.2.5. Quantification of T cell stimulation

The SL-H2-Kb-specific, murine CD8+ T cell hybridoma B3Z express the *lacZ* reporter gene under the control of the NFAT enhancer [19]. The cell line was kindly provided by Nilabh Shastri at the University of California at Berkeley (CA, USA). Briefly, 10^5 BM-DC from C57BL/6 mice per well of 96-well plates were infected with bacterial strains grown to stationary phase. Infection was performed for 1 h at an MOI of 25 or 100 for WT or MvP728 strains, respectively. The plate was centrifuged for 5 min at 500 x g to synchronize the infection. After the infection period, non-internalized bacteria were removed by two washes with phosphate-buffered saline (PBS). To kill remaining extracellular bacteria, infected cells were incubated for 1 h in medium containing 100 μg x ml^{-1} of gentamicin. After a washing step, medium containing 25 μg x ml^{-1} gentamicin was added. B3Z T cells were added to plate and co-cultured with a DC:T cell-ratio

ranging from 1:8 to 1:0.125 in a total volume of 200 µl per well for 24 h. Cells were centrifuged at maximal speed and lysed by addition of 100 µl substrate solution (0.15 mM chlorophenyl red β-galactopyranoside, 0.5% (v/v) Nonidet P-40 in PBS). After incubation for 6 - 8 h at 37°C, the absorbance was determined at 595 nm.

For stimulation assays, 10^5 BM-DCs from C57BL/6 mice were seeded per well of a 96-well plate. The cell were infected with bacterial strains at a MOI of 25 or stimulated with 50 µg x ml^{-1} of ovalbumin or 1 µg x ml^{-1} concanavalin A (both from Sigma, Deisenhofen). All experiments were performed in parallel for 1 h. The cells were washed twice with PBS and incubated with RPMI1640 medium containing 100 µg x ml^{-1} gentamicin for 1 h. After infection, plates were γ-irradiated (3,600 rad) prior to co-culture with either 2×10^5 spleen cells from OT-I [20] or OT-II mice in a final ratio of 4:1. These mice which express a transgenic TCR (Vα2/Vβ5) specific for the OVA-derived peptides presented either in the MHC-I or MHC-II context. The transgene status was confirmed by FACS with anti-mouse CD4 (clone RM4-5), anti-mouse CD8α (clone 5H10), and anti-mouse Vα2 (clone B20.1) from BD Biosciences. After 48 h of co-incubation, the proliferation was measured by ^3H uptake for additional 24 h. All experiments were performed in triplicates and repeated at least three times.

2.2.6. Vaccination experiments

The immune response to vaccination with *Salmonella* live carriers translocating fusion protein of Llo to various effectors were analyzed basically as described before [17]. Briefly, BALB/c female mice of age 6 weeks (Jackson Laboratory) were maintained under standard conditions at Baylor College animal facility and were treated according to an IACUC approved protocol. For immunization, cohorts of 7 mice were immunized

by orogastric application of 1 × 10^9 CFU per mouse of *Salmonella* MvP728 or MvP728 harboring plasmid p2810 (*sseF*::*lisA*), p3635 (*sseJ*::*lisA*), p3636 (*sifA*::*lisA*), p3637 *sseL*::*lisA* or p3638 (*steC*::*lisA*). The inocula were applied in 200 µl of 5 % sodium bicarbonate using 20 G gastric gavage needles (Popper & Sons, Inc.). Booster immunizations were applied at day 14 and 28 after primary vaccination using the same conditions. The inoculum used was always verified by serial dilution and plating on LB-agar plates in the presence of the appropriate antibiotic.

2.2.7. MHC pentamer staining and FACS analysis

Blood samples were collected 10, 21 and 35 days after immunization from vaccinated mice or control groups. After erythrocyte lysis with ammonium chloride solution, cells were stained with PE-labeled H-2Kd/LLO91-99 GYKDGNEYI Pro5 pentamer (ProImmune, Inc), rat anti-mouse CD8-FITC KT15 mAb (Proimmune, Inc). APC-hamster-Anti-mouse-CD3e (BD Biosciences) and Percp Rat Anti-mouse-CD45 (BD Biosciences). BD-suggested fluorochrome and isotype-matching mAbs were used as negative controls. The analysis was performed on a LSR-II flow cytometer (BD Biosciences) using BD FACDiva software v. 6.0.

2.3. Results

2.3.1. Generation and evaluation of expression cassettes

In order to test the efficacy of various SPI2-effector antigen fusions in vaccination approaches with live attenuated carrier strains, we generated expression cassettes containing promoter of *sseA* and a genes encoding a hybrid protein consisting of SPI2-T3SS translocated effector proteins SseJ, SseL, SteC, SifA and model antigens

ovalbumin (OVA) or listeriolysin (Llo). For either model antigen, a large panel of tools is available for the characterization of immune responses. The expression cassettes were placed on low copy number plasmid pWSK29 that has previously been shown to be compatible with SsrAB-regulated *in vivo* expression. The modular design of the various expression cassettes is presented in **Fig.2. 1**.

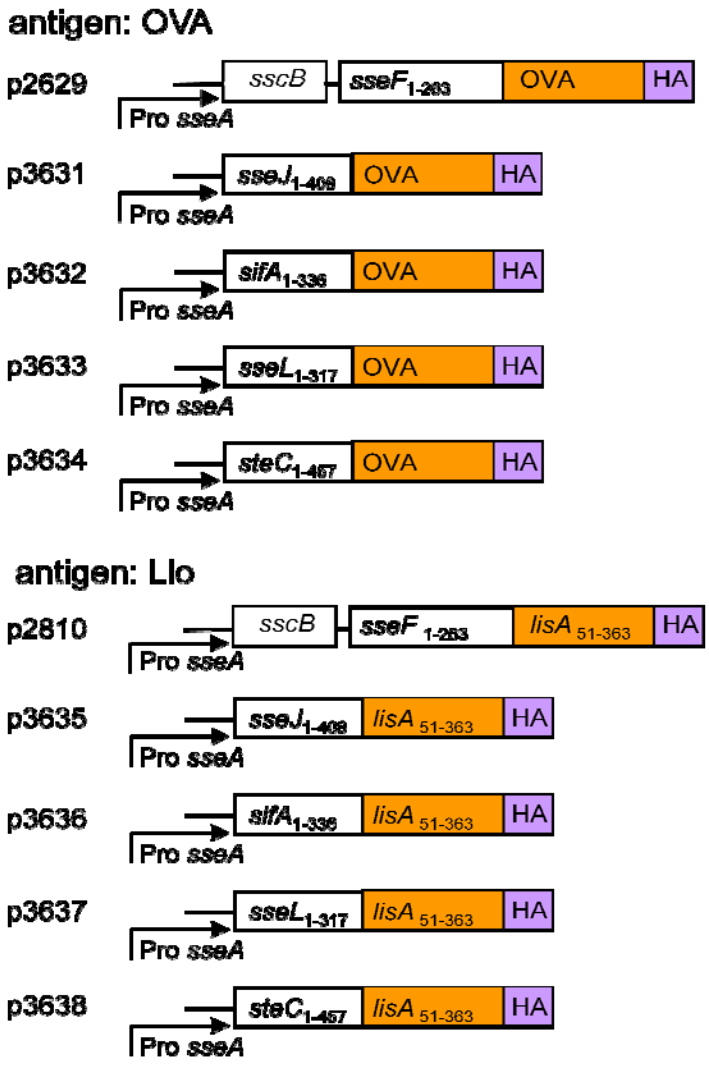


Fig. 2.1. Generation of expression cassettes for heterologous vaccine antigens. Expression cassettes consist of hybrid genes for the expression of fusion proteins comprising SPI2 effector proteins SseJ, SifA, SseL or SteC, the model antigens Llo or OVA and a C-terminal epitope tag HA for the standardized detection of the amounts of fusion protein. Expression cassettes with fusions to *sseF* have been previously

described [15]. In all cassettes, the expression is controlled by the *in vivo*-activated promoter P_{sseA} of the SsrAB regulon. All plasmids were generated on the basis of low copy number vector pWSK29, and plasmid designations are indicated by p3631, etc.

Next, strains harboring the expression cassettes were tested for the levels of recombinant fusion proteins. We first used *in vitro* culture conditions known to induce the expression of genes of the SsrAB regulon and the synthesis of SPI2 effector proteins. The synthesis of SPI2 effector-OVA-HA (**Fig. 2.2.A**) and SPI2-effector-Llo-HA (**Fig. 2.2.B**) fusion proteins was observed for all constructs analyzed here. In order to quantify the amounts of recombinant protein produced by the various strains, Western blots were analyzed using the Odyssey detection system and signal for model antigens were normalized by the levels of constitutively synthesized control protein DnaK. For both model antigens, SseJ or SteC fusions were expressed at significantly higher levels than SseL or SifA fusions. SteC fusion provided the highest level of the recombinant protein synthesis.

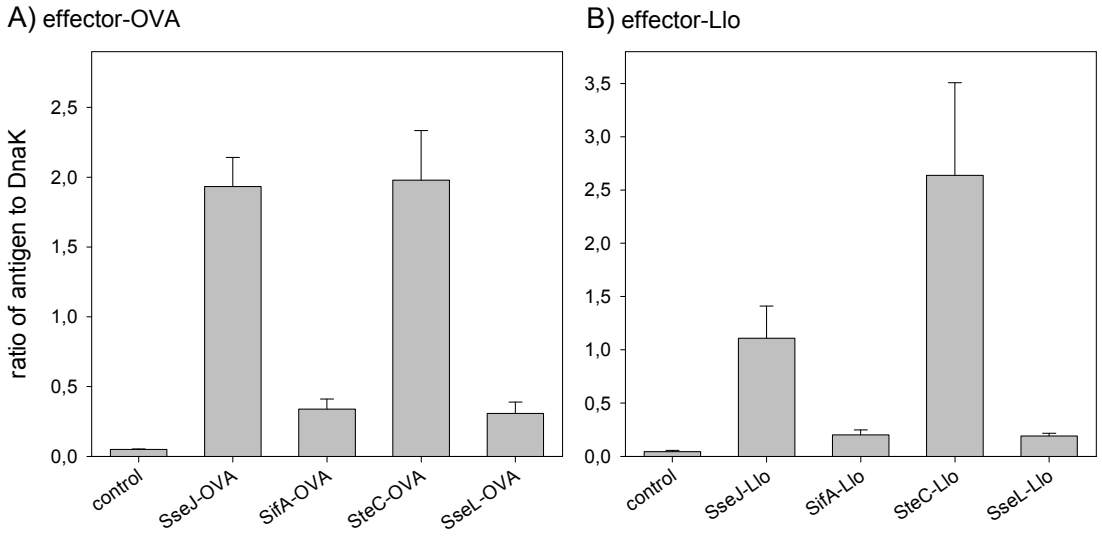


Fig. 2.2. Synthesis of fusion proteins with model antigens. The *S. enterica* serovar Typhimurium wild-type strain without plasmid (blank) or harboring plasmids of the of fusion proteins consisting of SPI2 effector proteins SseJ, SifA, SseL or SteC model antigens OVA (A) or Llo (B), each with a C-terminal epitope tag HA. The strains were grown in SPI2-inducing minimal media (PCN-P, pH 5.8) and bacteria were harvested after 6 h of culture under inducing conditions. Equal amounts of bacteria as adjusted by OD_{600} were lysed and subjected to SDS-PAGE and Western blot analyses for the detection of the HA epitope tag was performed. Blots were probed with fluorescently labeled secondary antibodies and signal intensities were quantified using the Odyssey system (Li-Cor). As loading controls, the cytosolic heat shock protein DnaK was detected on the same blot and signals were quantified. The ratios of the HA to DnaK signals were calculated and means and standard deviations for three samples were shown. The experiment was performed at least three times and means and standard deviations are shown.

Since all fusion constructs were expressed by episomal genes, we tested the plasmid stability in the absence of antibiotic selection and under conditions that mimic the intracellular environment of *Salmonella* in host cell (**Fig. 2.3**). These experiments indicated that 80-90 % of the bacterial cells maintained the plasmids over 7 days of repeated subculture in the absence of antibiotics. Similar numbers of plasmid-harboring bacteria were obtained by subculture in non-inducing minimal media without antibiotics, while 60-70 % plasmid-harboring cells were obtained after subculture in SPI2-inducing minimal media without antibiotics. Under these conditions, the highest loss of plasmids was observed for fusion constructs with SseJ and SseF, while construct based on SseL were maintained in more than 80 % of the cells. These data suggest that the expression cassettes are sufficiently stable for potential in vivo applications.

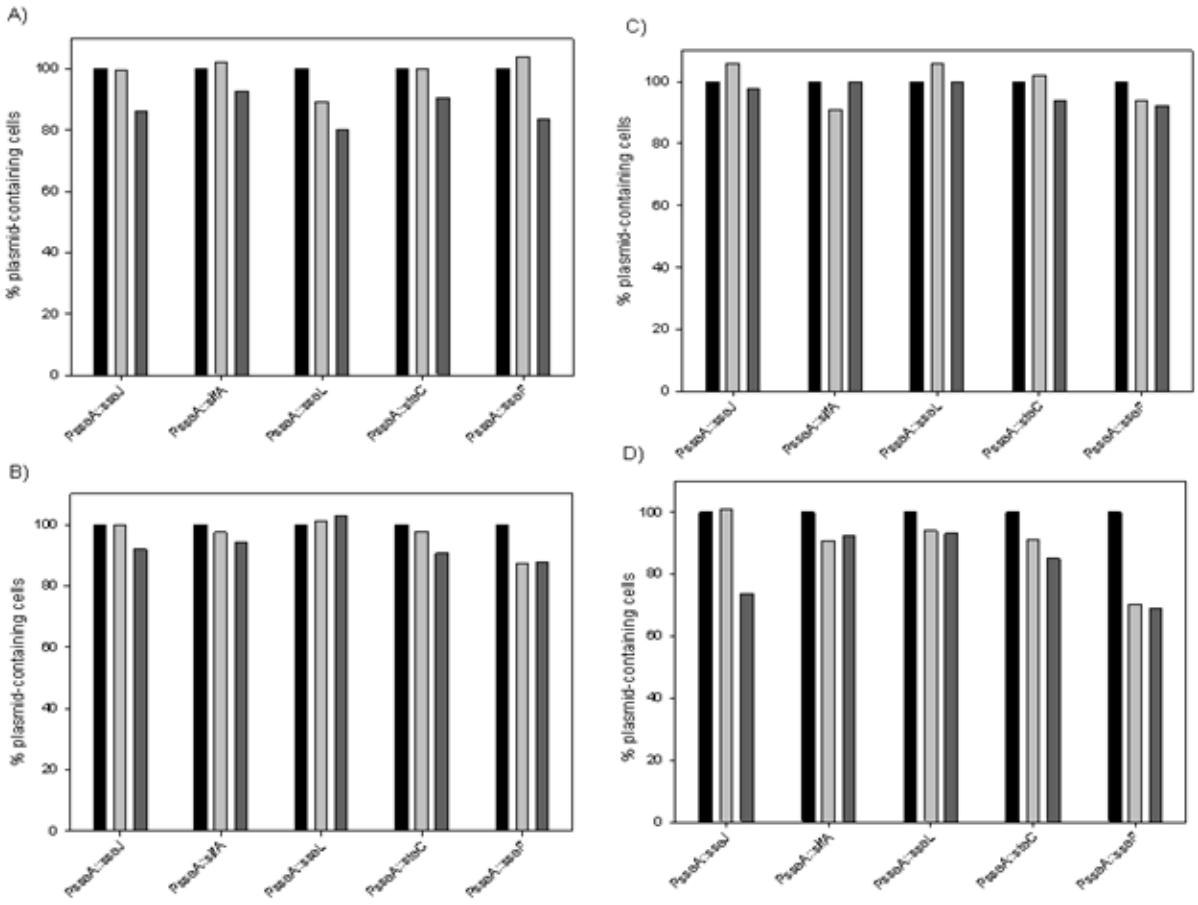
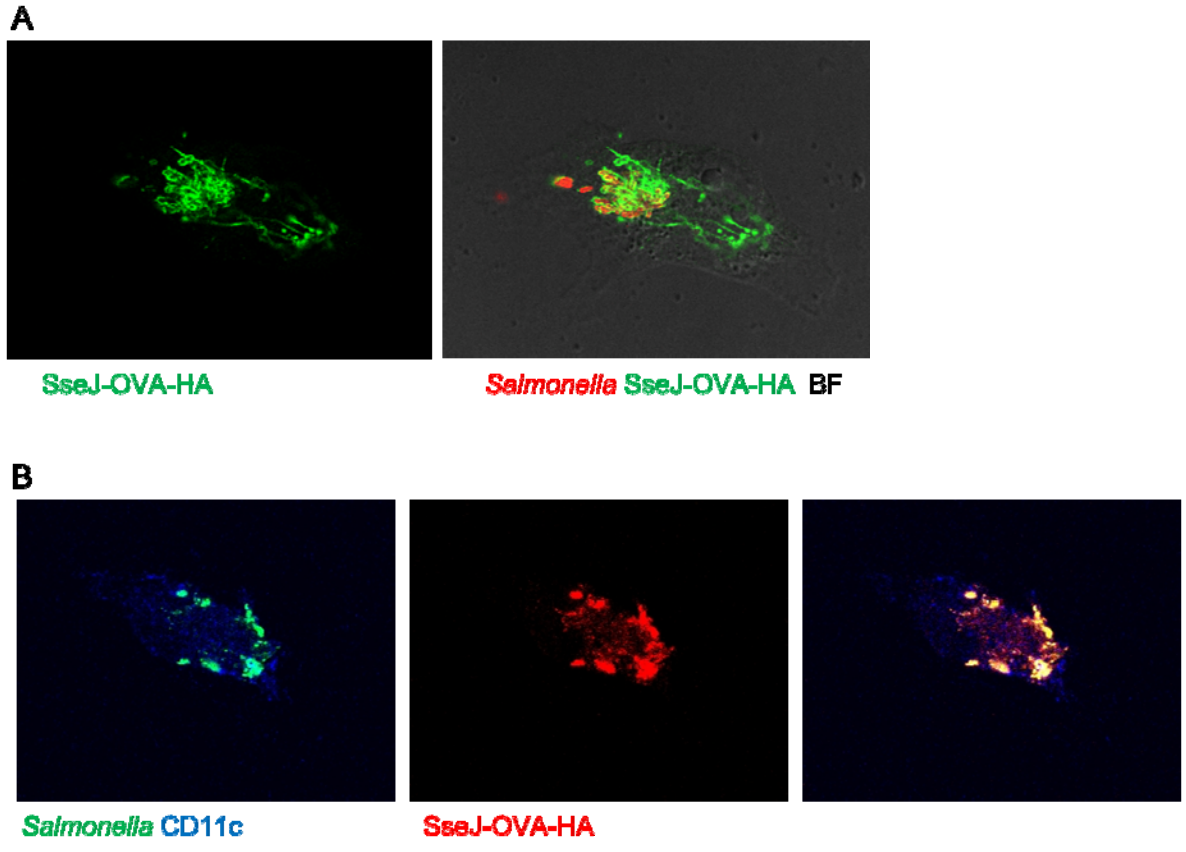


Fig. 2.3: In vitro stability of low copy number plasmids harboring expression cassettes in recombinant *Salmonella.* The *S*. Typhimurium *htrA purD* carrier strain harbor plasmids with expression cassettes consisting of P_{sseA} for the control of expression of *lisA* fused to various genes for SPI2-T3SS effector proteins. The bacteria were cultured in various media and subcultures were prepared each day over a period of 7 days. Bacterial strains were cultured in LB medium without antibiotics (A), LB medium containing 50 µg/ml carbenicillin (B), synthetic PCN medium containing 1 mM Pi at pH 7.4 (C), or PCN medium containing 0.4 m Pi at pH 5.8 (D). At 1 day (black bars), 3 days (grey bars), or 7 days (dark grey bar) of subculture, dilutions of the cultures were plated onto LB agar plates with or without carbenicillin. The percentage of antibiotic-resistant bacterial colonies was calculated.

Next, we analyzed the translocation of the model antigens by intracellular *Salmonella* in BM-DC. We investigated the translocation of OVA-HA (**Fig. 2.4**) and Llo-HA fusion proteins using expression cassettes with the P_{sseA} promoter. All expression cassettes mediated translocation of fusion proteins into HeLa cells (**Fig. 2.4.A**) and BM-DC (**Fig. 2.4.B**). However, the intensities of the HA tag immuno-fluorescence staining varied, indicating different amounts of the translocated protein. For quantification, we used infected HeLa cells harboring similar numbers of intracellular *Salmonella* and compared the signal intensities of the HA tagof the examined constructs. The SPI2 effectors were ranked in the following order depending on the level of the fusion protein expression: SteC>SseJ>SseF>SseL>SifA OVA-HA (**Fig. 2.4.C**).



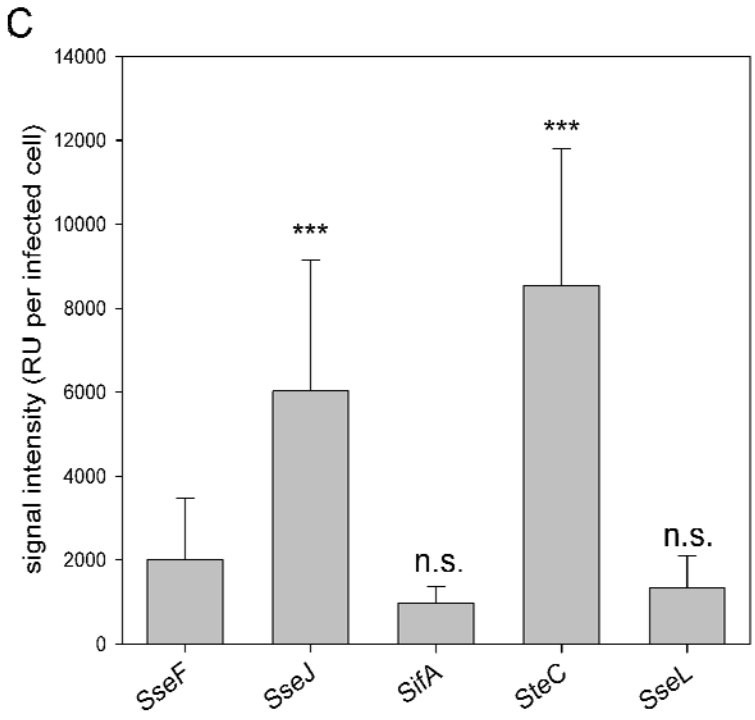


Fig. 2.4. Translocation of fusion proteins by intracellular *Salmonella*. Wild-type *S.* Typhimurium serovar *enterica* harboring plasmids with cassettes for the expression of fusion proteins consisting of SPI2 effector proteins SseJ, SifA, SseL or SteC model antigens Llo and a C-terminal epitope tag HA under control of P_{sseA} promoter were used to infect the epithelial cell line HeLa at an MOI of 10, or BM-DC at an MOI of 25. A) HeLa cells were fixed 16 h after infection and processed for immune-staining for intracellular *Salmonella* (red) and translocated fusion protein SseJ-OVA-HA (green) was performed. B) For BM-DC, DC marker CD11c was labeled (blue) in addition to *Salmonella* (green) and fusion protein SseJ-OVA-HA (red). Cells were analyzed by confocal laser-scanning microscopy using the ZEN software package (Zeiss). Representative infected HeLa cells (A) and BM-DC (B) are shown. C) For quantification, attenuated *purD htrA* carrier strain (MvP728) harboring plasmids with expression cassettes for fusion proteins consisting of SPI2 effector proteins SseJ, SifA, SseL or SteC to OVA and the HA tag under control of P_{sseA} promoter were used to infect HeLa cells at MOI of 100. The cells were fixed 16 h

after infection and processed for immune-staining. Infected cells with similar amounts of intracellular *Salmonella* were selected for the various conditions and the signal intensities of the Cy3 channel for the anti-HA stain were measured with identical exposure times. The mean signal intensity per cell and standard deviations for at least 50 infected cells per condition are shown. Statistical analysis was performed by One way ANOVA and is indicated as: n.s., not significant; ***, $P < 0.001$.

2.3.2. Quantification of T cell-responses to antigens presented by intracellular *Salmonella*

We next compared the antigen-dependent simulation of T cells after uptake of recombinant *Salmonella* strains with expression of recombinant antigens under control of P_{sseA} promoter. BM-DC were infected with *S. enterica* serovar Typhimurium attenuated carrier strain MvP728 deficient in *purD* and *htrA* [12] harboring plasmids for the expression of SPI2-effector::*OVA*::HA under the control of P_{sseA}. Subsequently, infected BM-DC were incubated with the B3Z T cell line. B3Z is a T cell hybridoma that recognizes the OVA-derived SIINFEKL epitope in the context of H2Kb and express *lacZ* reporter gene under control of the NFAT enhancer. The β-galactosidase activity thus is a measure of the antigen-dependent stimulation [17]. Very low stimulation was observed with the vector controls without expression cassettes, while addition of the SIINFEKL peptide was used as positive control resulting in maximal stimulation. The use of the *Salmonella purD htrA* strain with plasmids harboring various expression cassettes resulted in highly increased stimulation of the T cell hybridoma. Highest stimulation was obtained with expression cassettes with SseJ and SteC fusion proteins (**Fig. 2.5.**). These findings were compatible with the quantification of translocation signal

which also showed that SteC and SseJ fusion proteins were the highest.

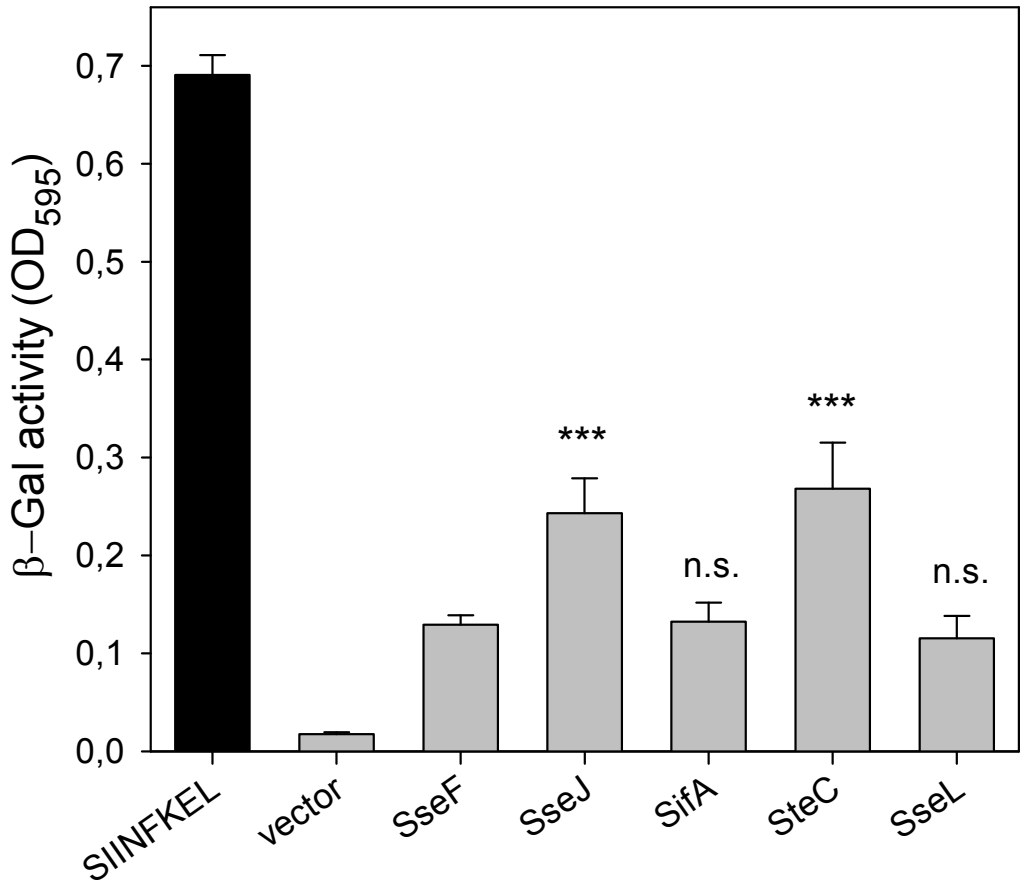


Fig. 2.5. Efficiency of SPI2 effector fusion proteins in stimulation of T cells. Murine BM-DC were infected at an MOI of 25 with the *purD htrA*-deficient carrier strain. Strains harbored the empty plasmid vector or plasmids with expression of fusion proteins consisting of cassettes for SPI2 effector proteins SseF, SseJ, SifA, SseL or SteC to OVA and the HA tag. As positive control, BM-DC were infected with *Salmonella* and stimulated with the SIINFEKL peptide or infected with *Salmonella* only (vector). Infected BM-DC were incubated with B3Z reporter cell line and after co-culture for 24 h, the □-galactosidase substrate chlorophenyl red β-galactopyranoside was added. After additional incubation for 6 h, the reaction was stopped and the □-galactosidase product was quantified colorimetrically by measurement

the extinction at 595 nm. T cell stimulation was analyzed at ratio of infected BM-DC to T cells 1:4. The means and standard deviations of triplicate samples are shown and the data sets are representative for 4 independent experiments. Statistical significance of stimulation by SseJ, SifA, SteC or SseL fusion proteins compared to the SseF fusion protein was determined by One way ANOVA and is indicated as: n.s., not significant; ***, $P < 0.001$.

As a second approach, we used the OVA-specific T cells isolated from OT-I or OT-II transgenic mice [17]. OT-I mice are transgenic for CD8+ T cells that recognize OVA epitope SIINFEKL ($OVA_{257-276}$) in the context of MHC class I molecules, while OT-II mice are transgenic for CD4+-cells that respond to the $OVA_{323-339}$ peptide ISQAVHAAHAEINEAGR in the context of the MHC class II I-A^2 molecule. BM-DC were infected with various combinations of carrier strains and expression cassettes and the proliferation of OT-I- and OT-II-derived T cells was determined as a measure of antigen-dependent stimulation (**Fig. 2.6)**. Compared to the control stimulation of T cells by concanavalin A, high levels of stimulation of OT-I cells were obtained with *Salmonella purD htrA* carrier strain with the various expression cassettes for *SPI2-effector*::OVA::HA. In contrast, stimulation of OT-II cells was rather low under the various conditions investigated in this assay. This indicates that peptides derived from translocated fusion proteins are predominantly presented in a MHC class I-restricted manner. The highest stimulation was observed with strains harboring SteC and SseJ fusion proteins.

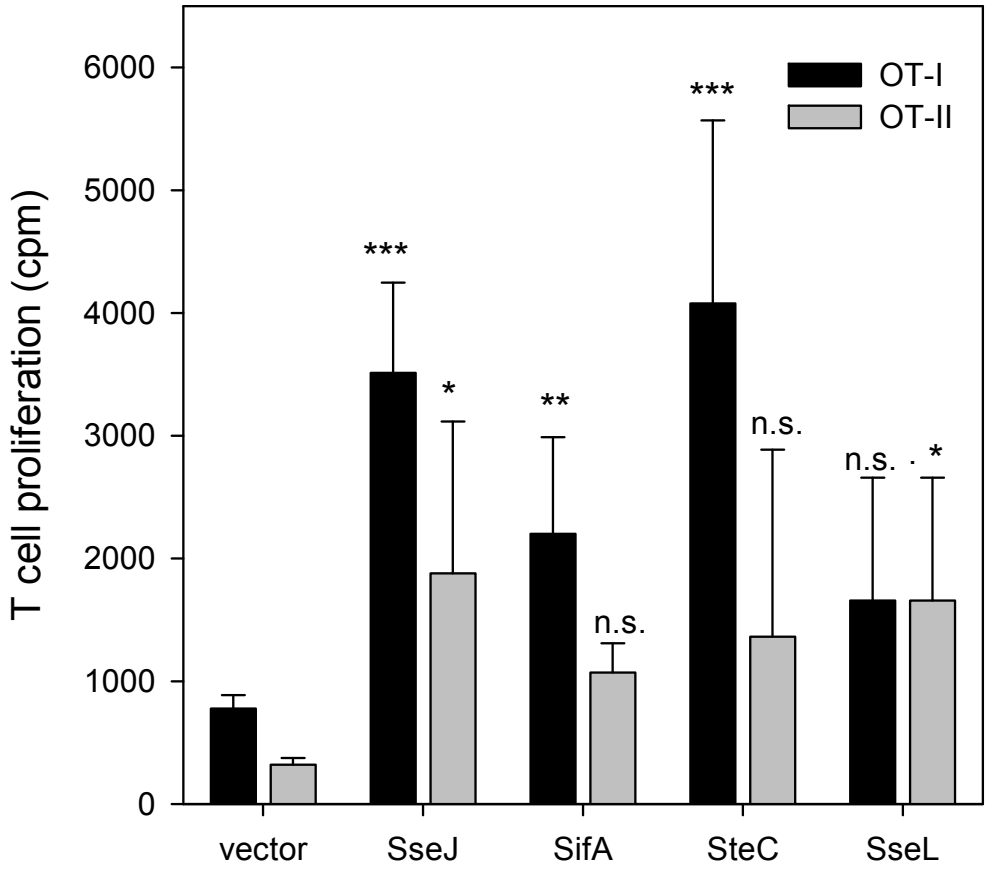


Fig. 2.6. Efficiency of SPI2 effector fusion proteins in stimulation of OT-I or OT-II cells. Murine BM-DC were infected as described for **Fig. 2.5**. Infected BM-DC were incubated with T cells obtained from transgenic mouse strains OT-I or OT-II as indicated. The proliferation of T cell proliferation was quantified by determination of ^{3}H thymidine incorporation as is expressed as counts per minute (cpm). The means and standard deviations of triplicate samples are shown and the data sets are representative for 3 independent experiments. Statistical significance of stimulation by SseJ, SifA, SteC or SseL fusion proteins compared to the vector control was determined by One way ANOVA and is indicated as: n.s,. not significant; *, $P < 0.05$; ***, $P < 0.001$.

2.3.3. Fusion proteins with SseJ and SifA efficiently induce T cell-responses after vaccination in mice

To examine the effect of SPI2 effectors on the in vivo immunogenicity of the recombinant vaccines, we used the *purD htrA* double-deficient strain MvP728 that harbored expression cassettes for the SPI2 effector-Llo fusion proteins under the control of promoter P_{sseA}. Mice were immunized by oral administration of the vector or vaccine strains followed by two booster immunizations that were performed on days 14 and 28. In order to quantify the immune response to vaccination, blood samples were analyzed on days 10, 21 and 35 after the initial immunization. The frequency of Llo-specific cytotoxic T lymphocytes (CTL) was determined using specific pentamers (**Fig. 2.7**). As it has previously been reported, the immunization with SseF-Llo fusion induced specific CTL responses. The quantification of Llo-specific CTL at the various time points indicted that strains translocating SteC-Llo or SseL-Llo fusion proteins did not trigger specific immune responses in mice. In contrast, immunization with strains translocating SseJ-Llo or SifA-Llo fusion proteins induced increased numbers of CTL compared with SseF-Llo. The highest CTL frequency was detected in the group immunized with the SseJ fusion protein. These results indicate that effector proteins SteC and SseL are suboptimal for the use in recombinant vaccines. In contrast, SseJ and SifA are superior to the previously characterized effector protein SseF and could be used in the design of potent recombinant vaccines to intracellular antigens.

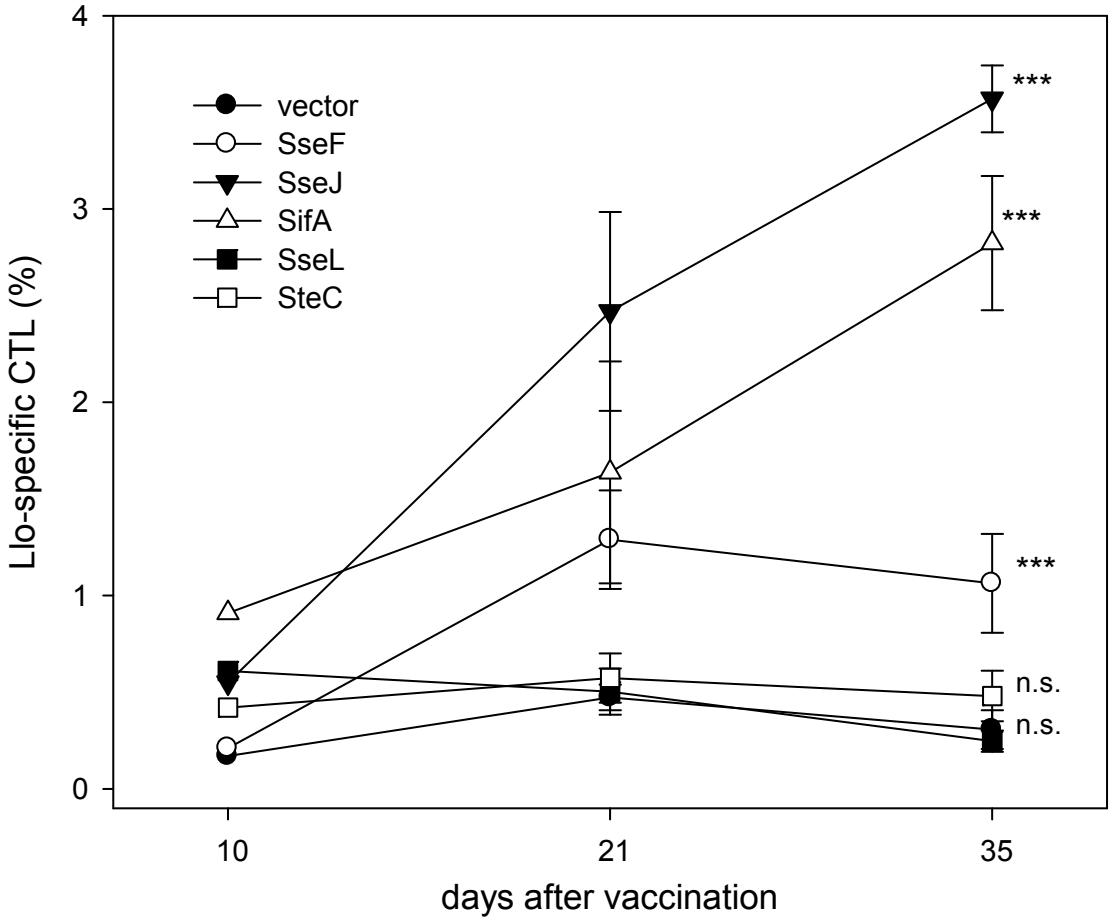


Fig. 2.7. Efficiency of SPI2 effector fusion proteins in vaccination. Cohorts of mice were vaccinated with the *purD htrA*-deficient carrier strain harboring plasmids for the synthesis of fusion proteins consisting of various SPI2 effector proteins and a listerolysin fragment. Blood samples were collected at day 10, 21 and 35 after initial vaccination. The amount of Llo-specific cytotoxic T cells (CTL) was determined by staining with the PE-labeled H-2Kd/LLO91-99 GYKDGNEYI Pro5 pentamer and flow cytometry. Statistical significance of stimulation by SseF, SseJ, SifA, SteC or SseL fusion proteins compared to the vector control was determined by One way ANOVA for the final time point and is indicated as: n.s., not significant; ***, $P < 0.001$.

2.4. Discussion

Previous work from others and our labs has demonstrated the broad potential applicability of bacterial T3SS for the translocation of heterologous vaccine antigens by live attenuated bacterial carrier strains [2]. Among multiple factors that contribute to the vaccine efficacy, the choice of a T3SS translocation signal or a T3SS effector protein as fusion partner for the target antigens of particular importance. Here, we compared five SPI2 effectors SseJ, SifA, SteC, SseL and SseF in their ability to translocate model antigens in APCs and to elicit antigen-specific immune response in vivo. We observed that the amount of translocated protein, as well as the stimulation of T-cell responses under *in vitro* conditions correlated well with the translocation efficacy of the various effectors. However, the T cell responses triggered in a vaccination experiment were only in partial agreement with the effects observed in vitro. Only one effector SseJ consistently demonstrated the strongest activities both in vitro and in vivo.

While stimulation of T cell responses by fusion proteins based on SseJ were efficient in all assay systems applied here, effector protein SseL did not appear to be useful for the generation of the fusion proteins with heterologous antigens in the same settings. The results from in vitro and in vivo experiments were only in partial agreement for fusion proteins based on SteC or SifA. While fusion proteins with SteC were efficiently translocated and stimulated T cell responses in □-Gal or OVA responsive T cells, the stimulation of Llo-responsive T cells in vaccinated mice was not detectable.

The reasons for the different levels of immune stimulation observed for the studied effector proteins are still poorly understood. Synthesis level of the effector proteins under intracellular conditions may vary considerably, but this feature is difficult to

quantify for bacterial strains in their intracellular environment. Furthermore, the recognition of effector proteins by dedicated chaperones may vary, as well as the efficacy of translocation by the SPI2-T3SS has to be investigated. Other parameters that will likely affect the efficacy of translocated recombinant antigens are the subcellular localization of the translocated protein in APC and the route of processing for presentation. The proteins of the subgroup of SPI2-T3SS effectors investigated in this study are all characterized by their association with endosomal membranes after translocation into host cells and the rather long half-life in host cells. Further work is required to test if SPI2-T3SS effectors that are predominantly localized in the cytosol and more rapidly degraded by the host confer different characteristics to vaccine fusion proteins.

Dendritic cells as professional APC are the key link between innate and adaptive immunity. Immature DC can internalize and process *Salmonella* for peptide presentation on MHC-II as well as MHC-I, initiating an immune response [21]. Interestingly, although *Salmonella* remains confined to the vacuolar compartments, it can be processed for MHC-I presentation of bacterial antigens to CD8+ T cells [22]. Because of the cytotoxicity to infected cells, DC can either be direct or indirect presenters of *Salmonella* antigens. Direct presentation of bacterial antigens to T cells upon phagocytic processing of *Salmonella* that does not induce their death and also indirectly present bacterial antigens to T cells as by engulfing antigenic material from neighboring cells that have undergone *Salmonella*-induced apoptotic death as a professional APC [21]. Our results for the stimulation of OT-I and OT-II T cells indicate that translocation of all fusion constructs result in MHC-I as well as MHC-II restricted presentation. However, it is still

unknown how SPI2-T3SS effectors differ in their subcellular localization and the route of processing of heterologous antigens in DC. A recent study indicated that a subgroup of SPI2-T3SS effector proteins has DC-specific functions, such as the interference with MHC-II presentation [23].

The expression levels of genes encoding SPI2 effector proteins are highly divergent and this is likely to affects the amount of the translocated effector protein [24]. By comparison of various promoters of the SsrAB regulon for expression of heterologous antigen fusions to SSPI2-T3SS effector protein SseF, we recently identified P_{sifB} as a promoter with superior stimulation of immune responses to vaccination [17]. In this study we compared various SPI2 effector proteins with high levels of synthesis for the translocation of fusion proteins. Here, the expression of the vaccine antigen fusions was controlled by P_{sseA}, a promoter that has been utilized in our previous approaches [12, 15]. The comparison of effector proteins SifA, SseJ, SteC and SseL to the previously deployed effector SseF clearly revealed high level of stimulation of immune responses by SifA and SseJ. Based on these results, we propose that optimized vectors for vaccination should comprise SseJ or SifA as an effector protein and P_{sifB} as promoter. The vaccination efficacy of such combinations of promoter and SPI2-T3SS effectors is currently being investigated.

In addition to the selection of promoters for the controlled in vivo-expression of heterologous antigens and the SPI2-T3SS effector protein, other parameters may affect vaccination efficacy. Our recent work showed that adaptation of the codon usage of foreign antigens to the codon usage of *Salmonella* as carrier strain can strongly affect levels of protein synthesis and in turn the immune response to the antigen [12]. Another

important aspect is the selection of a suitable attenuated carrier strain. In the previous analysis and in this study, *S. enterica* serovar Typhimurium strain MvP728 harboring deletions of *purD* and *htrA* was used. If other attenuating mutants are more compatible with SPI2-T3SS-dependent translocation of heterologous antigens needs to be investigated by future work.

From results of this study we conclude that the choice of a translocated SPI2 effector is an important parameter for the construction of efficient recombinant vaccines. However, the in vitro efficacy of the various SPI2-effectors only partly correlated with the immunogenicity observed in vivo. This discordance may be explained by several factors. i) Carrier strains with high level of expression of heterologous antigens (e.g. with SteC effector) could be more rapidly eliminated in vivo, resulting in reduced immune responses. ii) The levels of synthesis of certain effector are different of vivo and under the in vitro conditions applied here. iii) The subcellular location of the fusion protein under in vivo conditions is not favorable for efficient stimulation of immune response.

In conclusion, our analysis showed that the choice of SPI2 effector protein as a vehicle for intracellular delivery of a target antigen is a critical parameter for the optimal design of *Salmonella*-based recombinant vaccines. The in vivo vaccination efficacy of such SPI2 effectors was not completely matching their in vitro performance. These results indicate the complex interplay between expression level, attenuation of live carrier strains and properties of the recombinant antigen in a vaccination of host organisms. Further mechanistic studies are required for the comprehensive evaluation of these critical parameters for the rational design of recombinant vaccines

2.5. References

1. Medina, E. and C.A. Guzman, *Use of live bacterial vaccine vectors for antigen delivery: potential and limitations.* Vaccine, 2001. **19**(13-14): p. 1573-1580.
2. Panthel, K., et al., *Salmonella type III-mediated heterologous antigen delivery: a versatile oral vaccination strategy to induce cellular immunity against infectious agents and tumors.* Int. J. Med. Microbiol., 2008. **298**(1-2): p. 99-103.
3. Mollenkopf, H., G. Dietrich, and S.H. Kaufmann, *Intracellular bacteria as targets and carriers for vaccination.* Biol. Chem., 2001. **382**(4): p. 521-532.
4. Cheminay, C. and M. Hensel, *Rational design of Salmonella recombinant vaccines.* Int. J. Med. Microbiol., 2008. **298**(1-2): p. 87-98.
5. Pasetti, M.F., et al., *Immunology of gut mucosal vaccines.* Immunol. Rev., 2011. **239**(1): p. 125-48.
6. Ibarra, J.A. and O. Steele-Mortimer, *Salmonella - the ultimate insider. Salmonella virulence factors that modulate intracellular survival.* Cell. Microbiol., 2009. **11**(11): p. 1579-86.
7. Ghosh, P., *Process of protein transport by the type III secretion system.* Microbiol. Mol. Biol. Rev., 2004. **68**(4): p. 771-95.
8. Aizawa, S.I., *Bacterial flagella and type III secretion systems.* FEMS Microbiol. Lett., 2001. **202**(2): p. 157-64.
9. Cornelis, G.R., *The type III secretion injectisome.* Nat. Rev. Microbiol., 2006. **4**(11): p. 811-25.
10. Haraga, A., M.B. Ohlson, and S.I. Miller, *Salmonellae interplay with host cells.* Nat. Rev. Microbiol., 2008. **6**: p. 53-66.
11. Gerlach, R.G. and M. Hensel, *Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens.* Int. J. Med. Microbiol., 2007. **297**(6): p. 401-15.
12. Xiong, G., et al., *Novel cancer vaccine based on genes of Salmonella pathogenicity Island 2.* Int. J. Cancer., 2010. **126**(11): p. 2622-2634.
13. Rüssmann, H., *Bacterial type III translocation: a unique mechanism for cytosolic display of heterologous antigens by attenuated Salmonella.* Int. J. Med. Microbiol., 2003. **293**(1): p. 107-112.
14. Abrahams, G.L. and M. Hensel, *Manipulating cellular transport and immune responses: dynamic interactions between intracellular Salmonella enterica and its host cells.* Cell. Microbiol., 2006. **8**(5): p. 728-37.
15. Husseiny, M.I., F. Wartha, and M. Hensel, *Recombinant vaccines based on translocated effector proteins of Salmonella Pathogenicity Island 2.* Vaccine, 2007. **25**(1): p. 185-93.
16. Mastroeni, P., et al., *Salmonella: immune responses and vaccines.* Vet. J., 2001. **161**(2): p. 132-64.
17. Xu, X., et al., *Efficacy of intracellular activated promoters for generation of Salmonella-based vaccines.* Infect. Immun., 2010. **78**(11): p. 4828-38.
18. Lutz, M.B., et al., *An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow.* J. Immunol. Methods, 1999. **223**(1): p. 77-92.

19. Karttunen, J., S. Sanderson, and N. Shastri, *Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens.* Proc. Natl. Acad. Sci. U S A, 1992. **89**(13): p. 6020-4.
20. Hogquist, K.A., et al., *T cell receptor antagonist peptides induce positive selection.* Cell, 1994. **76**(1): p. 17-27.
21. Sundquist, M., A. Rydstrom, and M.J. Wick, *Immunity to Salmonella from a dendritic point of view.* Cell. Microbiol., 2004. **6**(1): p. 1-11.
22. Yrlid, U. and M.J. Wick, *Antigen presentation capacity and cytokine production by murine splenic dendritic cell subsets upon Salmonella encounter.* J. Immunol., 2002. **169**(1): p. 108-116.
23. Halici, S., et al., *Functional analysis of the Salmonella Pathogenicity Island 2-mediated inhibition of antigen presentation in dendritic cells.* Infect. Immun., 2008. **76**: p. 4924-4933.
24. Xu, X. and M. Hensel, *Systematic analysis of the SsrAB virulon of Salmonella enterica.* Infect. Immun., 2010. **78**(1): p. 49-58.

Chapter 3

3.1. Introduction

Salmonellae are Gram-negative, facultative anaerobic and infects humans and animals generally by the oro-faecal route. Very diverse disease outcomes following *Salmonella enterica* infection can be observed with human diseases ranging from a general mild, self-limiting gastroenteritis, caused mainly by *S. enteric* serovar Typhimurium and serovar Enteritidis (*S. enteritidis*), to the severe, systemic infection of typhoid fever caused by *S. enterica* serovar Typhi or serovar Paratyphi. Typhoid fever is an acute, life threatening febrile illness with 16 million to 33 million cases and 500,000 to 600,000 deaths every year according to a World Health Organization (WHO) report [1].

Type III secretion systems (T3SS) are specialized organelles of Gram-negative bacterial pathogens that deliver effector proteins to host cell membranes and cytosol [2]. The genes for virulence factors of *Salmonella* cluster in pathogenicity islands (PI) owing to foreign genome integration into the bacterial chromosome. Two major SPI encode TTSS (SPI1 and SPI2) that translocates bacterial effectors [3]. The SPI2-TTSS is a multifunctional virulence system, the most important is the ability to cause systemic infections and to proliferate within host organs (Kuhle and Hensel 2004). Most of the characterized SPI2-phenotypes are linked to the manipulation of host-cell vesicle trafficking, thus ensuring nutrient supply and evading bactericidal activities [4].

In the last decade live attenuated strains of *Salmonella* that synthesize and secrete foreign antigens were being developed as vaccines for a number of infectious bacterial, viral and parasitic diseases and cancer. Live attenuated *Salmonellae* were used to deliver recombinant antigens to the immune system to stimulate innate immunity and also activate both the arms of the adaptive immune system by which they exert efficient immune response [5-7]. As a consequence of intracellular location of *Salmonella*,

proteins delivered are trapped in the phagolysosome and presented to the immune system preferentially in the context of MHC class II molecules. There have been several approaches in the literature for bioengineering *Salmonella* strains, which could deliver antigens directly to the intracellular compartment instead of the phagosome. One of these approaches using live attenuated *Salmonella* to deliver plasmid-encoded antigens under the transcriptional control of eukaryotic promoters has been used successfully in vaccination [8]. *Salmonella* carrier DNA vaccines have been employed in prophylactic immunization against bacteria, viruses, fungi, parasites and even against cancer [6-7, 9].

Heterologous antigens can be expressed in *Salmonella* as fusions with recombinant or native proteins under control of in vivo inducible promoters. This approach has been used mainly to direct the expression of the desired antigen to a particular location of the bacterial cell and increase the immunogenicity of foreign antigens by fusing them to proteins that could exert a carrier effect [10]. Our group and other groups used TTSS-mediated translocation for efficient delivery of heterologous antigens to the cytosol of antigen-presenting cells leading to prominent CD8 T-cell [11-13]. Efficient immune response mainly requires T cells induction which depends on the strength of antigen presentation.

The level of antigen presentation depends on antigen access to the respective processing compartment, the antigen-processing efficacy, and antigen abundance [14]. We hypothesized that the higher expression levels and the efficient translocation of protein fusion may lead to enhancement of the immune response. But our and other's finding did not consistent with our hypothesis. The highest levels of expression are not

necessarily required for an elevated immune response to heterologous antigens (Xu [15-16]. Inspite of its moderate expression level, P_{sifB} prompter showed the most in vivo efficiency in induction of specific CD8 T cells [15]. Our results (In previous chapter of this work) showed how the efficient translocation of fusion proteins is needed for induction of efficient immune response. We showed that the efficacy of translocation of SPI2 effector proteins is a conditionally required for an elevated immune response to heterologeous antigens. SseJ effector fusions were the most efficient in vitro translocated proteins and most efficient induction of specific CD8 T cells.

In this work we are looking for optimizing *Salmonella* as a carrier for vaccination. Toward this aim we tested several membrane associated SpI2 effectors SseJ, SseL, SifA, SteC or SseF fusions under the control of different promoters P_{sseA}, P_{sifA}, P_{sseJ} or P_{sifB}. We found that P_{sifB}*::sseJ* construct was the most efficient in induction of specific Cd8 T cells in vitro. This finding is consistent with the in vivo results; the systematic in vivo analysis of the immune response to several constructs is currently in progress.

3.2. Materials and Methods

3.2.1. Bacterial strains

Salmonella enterica serovar Typhimurium NCTC 12023 was used as wild-type strain. The *purD htrA*-deficient strain MvP728 [13] was used as attenuated carrier strain. For the generation of recombinant plasmids, *E. coli* DH 5α was used as host.

3.2.2. Generation of plasmids

For generation of expression cassette consisting of gene fusion *sseJ* ,*sifA, sseL* or *steC*, hSurvivin and HA tag under control of their own promoters. The gene encoding hSurvivin was amplified by PCR using hSurv-For-EcoRV, hSurvivin-HA-Rev-XbaI and

p3342 as a template, which later digested with EcoRV and XbaI. The digested hSurvivin was ligated to EcoRV and XbaI digested low copy plasmid pWSK29. The resulting plasmid pWSK29::*hsurvivin* was digested with KpnI and EcoRV. The genes *sseJ, sifA, sseL* or *steC* were PCR amplified using SseJ-Pro-For-KpnI and SseJ-Rev-EcoRV, SifA-Pro-For-KpnI and SifA-Rev-EcoRV, SseL- For-KpnI and SseL-Rev-EcoRV or SteC -For-KpnI and SteC-Rev-NaeI, respectively. The resulting amplified *sseJ* ,*sifA,* or *sseL* were digested with KpnI and EcoRV and ligated to digested pWSK29::*hsurvivin* resulting in plasmids pWSK29 P_{sseJ} *sseJ*::hSurvivin::HA (p3550), Pwsk29 P_{sifA} *sifA*::hSurvivin::HA(p3551) and pWSK29 P_{sseL} *sseL*::hSurvivin::HA (p3551). PCR amplified SteC gene was digested with KpnI and NaeI and ligated to digested pWSK29::*hsurvivin* resulting in plasmids pWSK29 P_{steC} *steC*::hSurvivin::HA (p3553). The obtained plasmids were confirmed by colony PCR, diagnostic digestion and sequenced using T7-Seq and T3-Seq primers.

For generation of expression cassette consisting of gene fusion *sseJ* ,*sifA or sseL*, OVA and HA tag under control of SPI2 promoter P_{sseJ}, P_{sifA} or P_{sifB} the gene encoding OVA and carry its CD4 and CD8 epitopes was amplified by PCR using Ova-For-NaeI, OVA-HA-Rev-XbaI and pOMP as a template , which later digested with NaeI and XbaI . P_{sseJ} *sscB sseJ*::hSurvivin (p3550), P_{sifA} *sifA*::hSurvivin (p3551) or P_{sseL} *sseL*::hSurvivin (p3552) plasmid was digested with EcoRV and XbaI. Digested OVA was ligated to Large fragment of digested plasmids to obtain plasmids P_{sseJ} *sseJ*::OVA::HA (p3554), P_{sifA} *sifA*::OVA::HA (p3556) or P*sseL*::SseL::OVA::HA (p3555), these plasmids which are used to amplify SseJ::OVA::HA, SifA::OVA::HA or SseL::OVA::HA using forward primers SseJ-For-EcoRI, SifA-For-Eff-EcoRI, or SseL-Effector-For-EcorI and reversal

primer OVA-HA-Rev-XbaI, which were digested later by EcoRI and XbaI. Plasmids p3524, p3526 or p3527 harboring P_{sifB} *sscB*::*sseF*::hSurvivin::HA, P_{sifA} *sscB*::*sseF*::hSurvivin::HA or P_{sseJ} *sscB*::*sseF*::hSurvivin::HA repetitively were digested with EcoRI and XbaI and the large fragment was ligated to digested SseJ::OVA::HA, SifA::OVA::HA or SseL::OVA::HA . The obtained plasmids were confirmed by colony PCR, diagnostic digestion and sequenced using T7-Seq and T3-Seq primers.

For generation of expression cassette consisting of gene fusion SteC::OVA::HA under control of SPI2 promoters P_{sifB}, P_{sifA} or P_{sseJ} . Plasmid pWSK29 Low copy plasmid was digested with EcoRI and XbaI and OVA was amplified using primers OVA595-For-EcoRI and OVA-HA-Rev-XbaI and pOMP plasmid as template. Digested amplified OVA with EcoRI and XbaI was ligated to digested pWSK29 resulting in plasmid pWSK29::OVA::HA. P_{sifB}, P_{sifA} or P_{sseJ} promoters will be amplified using forward primers Pro-sifB-For-KpnI, SifA-Pro-For-KpnI or SseJ-Pro-For-KpnI and reversal primers Pro-SifB-Rev-EcoRI, Pro-SifA-Rev-EcoRI or Pro-SseJ-ReV-EcoRI and plasmids p3524, p3526 or p3527 as template, respectively. Plasmid pWSK29::OVA::HA was digested with KpnI and EcoRI and ligated to amplified *sifB*, *sifA* or *sseJ* promoters which digested with KpnI and EcoRI obtaining plasmid pWSK29::P_{sifB}::OVA::HA, pWSK29::P_{sifA}::OVA::HA or pWSK29::P_{sseJ}::OVA::HA. Effector gene *steC* was amplified using SteC-Effector-For-EcoRI and SteC-Rev-EcoRI. pWSK29::P_{sifB}::OVA::HA, pWSK29::P_{sifA}::OVA::HA or pWSK29::P_{sseJ}::OVA::HA and amplified *steC* both are digested by EcoRI and ligated obtaining P_{sifB} *sscB steC*::OVA::HA(p3646), P_{sifA} *sscB steC*::OVA::HA (p3666) or P_{sseJ} *sscB steC*::OVA::HA (p3656). The resulting plasmids

were confirmed by colony PCR for the right construction and sequenced using T3-Seq and T7-Seq.

For generation of expression cassette consisting of gene fusion *sseJ ,sifA ,sseL* or *steC*, LisA and HA tag under control of SPI2 promoters P_{sifB}, P_{sifA} *or* P_{sseJ}. P_{sifB}::SseJ, P_{sifB}::SifA, P_{sifB}::SseL, P_{sifB}::SteC, P_{sifA}::SseJ,..etc. were amplified using Pro-SifB-For-KpnI, Pro-SifA-For-KpnI or SseJ-Pro-For-KpnI and SseJ-Rev-EcoRV, SifA-Rev-EcoRV, SseL-Rev-EcorV or SteC-Rev-NaeI, which were digested with KpnI and EcoRV or NaeI. Plasmid p2810 harboring P_{sseA} *sscB sseF*::*lisA*::HA was digested with KpnI and EcoRV, the large fragment was ligated to PCR-generateed and digested P_{sifB}::SseJ, P_{sifB}::SifA, P_{sifB}::SseL, P_{sifB}::SteC etc. Optained plasmids expressing SseJ:: LisA, SifA:: LisA, SseL:: LisA or SteC:: LisA were confirmed with colony PCR, diagnostic digestion and sequenced using T3-Seq and T7-Seq.

3.2.3. Western blotting

In order to quantify the amounts of recombinant protein produced by the various strains, Western blots were analyzed using the Odyssey detection system and signal for model antigens were normalized by the levels of constitutively synthesized control protein DnaK [15].

Table 1. Plasmids used in this Study

Designation	Relvant characteristics	Reference
pWSK29	Low copy number vector, Amp^R	Lab stock
p2629	pWSK29 P_{sseA} *sscB sseF*::OVA::M45	Lab stock
p2810	pWSK29 P_{sseA} *sscB sseF*::$lisA_{51\text{-}363}$::HA	Lab stock
pOMP-OVA	*lacZ*::OVA	Lab stock
p3342	pWSK29 P_{sseA} *sscB sseF*::hSurvivin::HA	Lab stock
p3524	pWSK29 P_{sifB} *sscB sseF*::hSurvivin::HA	Lab stock
p3526	pWSK29 P_{sifA} *sscB sseF*::hSurvivin::HA	Lab stock
p3527	pWSK29 P_{sseJ} *sscB sseF*::hSurvivin::HA	Lab stock
p3550	pWSK29 P_{sseJ} *sseJ*::hSurvivin::HA	This study
p3551	pWSK29 P_{sifA} *sifA*:: hSurvivin::HA	This study
p3552	pWSK29 P_{sseL}*sseL*:: hSurvivin::HA	This study
p3553	pWSK29 P_{steC} *steC*:: hSurvivin::HA	This study
p3554	pWSK29 P_{sseJ} *sseJ*:: OVA::HA	This study
p3556	pWSK29 P_{sifA} *sifA*:: OVA::HA	This study
p3557	pWSK29 P_{sseJ} *sseJ*:: $lisA_{51\text{-}363}$::HA	This study
p3559	pWSK29 P_{sifA} *sifA*:: $lisA_{51\text{-}363}$::HA	This study
p3627	pWSK29 P_{sseA} *sseJ*:: hSurvivin::HA	Lab stock
p3628	pWSK29 P_{sseA} *sifA*:: hSurvivin::HA	Lab stock
p3629	pWSK29 P_{sseA} *sseL*:: hSurvivin::HA	Lab stock
p3630	pWSK29 P_{sseA} *steC*:: hSurvivin::HA	Lab stock
p3631	pWSK29 P_{sseA} *sseJ*::OVA::HA	Lab stock
p3632	pWSK29 P_{sseA} *sifA*:: OVA::HA	Lab stock
p3633	pWSK29 P_{sseA} *sseL*:: OVA::HA	Lab stock
p3634	pWSK29 P_{sseA} *steC*:: OVA::HA	Lab stock
p3635	pWSK29 P_{sseA} *sseJ*:: $lisA_{51\text{-}363}$::HA	Lab stock

p3636	pWSK29 P_{sseA} *sifA*:: *lisA*$_{51\text{-}363}$::HA	Lab stock
p3637	pWSK29 P_{sseA} *sseL*:: *lisA*$_{51\text{-}363}$::HA	Lab stock
p3638	pWSK29 P_{sseA} *steC*:: *lisA*$_{51\text{-}363}$::HA	Lab stock
p3639	pWSK29 P_{sifB} *sseJ*:: hSurvivin::HA	This study
p3640	pWSK29 P_{sifB} *sfA*:: hSurvivin::HA	This study
p3641	pWSK29 P_{sifB} *sseL*:: hSurvivin::HA	This study
p3642	pWSK29 P_{sifB} *steC*:: hSurvivin::HA	This study
p3643	pWSK29 P_{sifB} *sseJ*:: OVA::HA	This study
p3644	pWSK29 P_{sifB} *sfA*:: OVA::HA	This study
p3645	pWSK29 P_{sifB} *sseL*:: OVA::HA	This study
p3646	pWSK29 P_{sifB} *steC*:: OVA::HA	This study
p3647	pWSK29 P_{sifB} *sseJ*::*lisA*$_{51\text{-}363}$::HA	This study
p3648	pWSK29 P_{sifB} *sifA*::*lisA*$_{51\text{-}363}$::HA	This study
p3649	pWSK29 P_{sifB} *sseL*:: *lisA*$_{51\text{-}363}$::HA	This study
p3650	pWSK29 P_{sifB} *steC*:: *lisA*$_{51\text{-}363}$::HA	This study
p3651	pWSK29 P_{sseJ} *sifA*:: hSurvivin::HA	This study
p3652	pWSK29 P_{sseJ} *sseL*:: hSurvivin::HA	This study
p3653	pWSK29 P_{sseJ} *steC*:: hSurvivin::HA	This study
p3654	pWSK29 P_{sseJ} *sifA*:: OVA::HA	This study
p3655	pWSK29 P_{sseJ} *sseL*:: OVA::HA	This study
p3656	pWSK29 P_{sseJ} *steC*:: OVA::HA	This study
p3657	pWSK29 P_{sseJ} *sifA*:: *lisA*$_{51\text{-}363}$::HA	This study
p3658	pWSK29 P_{sseJ} *sseL*:: *lisA*$_{51\text{-}363}$::HA	This study
p3659	pWSK29 P_{sseJ} *steC*:: *lisA*$_{51\text{-}363}$::HA	This study
p3660	pWSK29 P_{sifA} *sseJ*:: hSurvivin::HA	This study
p3661	pWSK29 P_{sifA} *sseL*:: hSurvivin::HA	This study
p3662	pWSK29 P_{sifA} *steC*:: hSurvivin::HA	This study

p3663	pWSK29 P_{sifA} *sseJ*:: OVA::HA	This study
p3664	pWSK29 P_{sifA} *sseL*:: OVA::HA	This study
p3665	pWSK29 P_{sifA} *steC*:: OVA::HA	This study
p3666	pWSK29 P_{sifA} *sseJ*:: *lisA*$_{51\text{-}363}$::HA	This study
p3667	pWSK29 P_{sifA} *sseL*:: *lisA*$_{51\text{-}363}$::HA	This study
p3668	pWSK29 P_{sifA} *steC*:: *lisA*$_{51\text{-}363}$::HA	This study

Table 2. Oligonucleotides used in this study

Designation	**sequence 5'-3'**
SseJ-Rev-EcoRV	ACGGATATCTTCAGTGGAATAATGATGAGC
SseJ-Pro-For-KpnI	TACGGTACCTCACATAAAACACTAGCAC
SseJ-For-EcoRI	CCGGAATTCGTAAGGAGGACACTATGCC
SifA-Rev-EcoRV	ACGGATATCAAAACAACATAAACAGCCGC
SifA-Pro-For-KpnI	TACGGTACCTCATAAGCGATTAATTGCGCAAC
SifA –EcoRI-eff-For	GGCGAATTCATTTTTACTCCAGTATAAGTG
SseL-Rev-EcoRV	TATGATATCCTGGAGACTGTATTCATATATTTG
SseL-For-KpnI	ATTGGTACCATCAGACATATACCCTTC
SseL-effector-For-EcoRI	GGAGAATTCCAGAGCAAAATGAATATATGTGT
SteC-Rev-NaeI	TATGCCGGCTTTTTTTAATTCATCCTTTAATAC
SteC-Rev-EcoRI	TATGAATTCTTTTTTTAATTCATCCTTTAATAC
SteC-For-KpnI	ATTGGTACCAAGGTTCTGTAGGAAGCCTG
Stec effector-For-EcoRI	GGAGAATTCCAGAGGATGAGACATATGCCG
ProB-For-KpnI	CTAGGTACCAGAAGAGAACAACGGCAAG
ProB-Rev-EcoRI	CACGAATTCACGATAAGATAATTAACGTGC
Pro-SifB-For-KpnI	ATGGGTACCTGCCCTACCGCTAAACATC
Pro-SifB-Rev-EcoRI	TCCGAATTCCACAAGTGATTATATGATAC

Pro-SseJ-Rev-EcoRI	ACGGAATTCCTCCTTACTTTATTAAACACGC
Pro-sifA-Rev-EcoRI	ACGGAATTCGGCATATTAATCTCACTTATACTG
LisA-51-For-EcoRV	CTAGATATCACGCCAATCGAAAAGAAAC
LisA-363-HA-Rev-XbaI	GAGTCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTAGA GGTTGCCGTCGATGATTTG
Ova-For-NaeI	ATAGCCGGCGCAATGCCTTTCAGAGTGAC
Ova595-For-EcoRI	GGAGAATTCGCAATGCCTTTCAGAGTGACTGAG
OVA-HA-Rev-XbaI	AGATCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTAAG GGGAAACACATCTGCCAAAG
hSurvivin-HA-Rev-XbaI	ATTTCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTAAT CCATAGCAGCCAGCTGCTC
hSurvivin-For-EcoRV	TACGATATCGGTGCCCCGACGTTGCCCCC
T3-Seq	AATTAACCCTCACTAAAGG
T7-Seq	TAATACGACTCACTATAGGG

3.2.4. SPI2-T3SS-dependent translocation of fusion proteins by intracellular *Salmonella.*

HeLa cells were infected with various *Salmonella* strains harboring plasmids for the expression of *effector*::*lisA*::HA, or *effector*::OVA::HA under the control of P_{sseA}, P_{sifB}, P_{sifA} or P_{sseJ} promoter. As carrier strains a double mutant strain MvP728 were used. At 16 h after infection, the cells were fixed and processed for immuno-staining of *Salmonella* LPS (rabbit anti-*Salmonella* O1,4,5, Difco, BD) and the HA epitope tag (Roche) The cells were analyzed by microscopy using a Zeiss LSM700 laser-scanning confocal microscope.

3.2.5 Dendritic cell infection

The preparation and culture of bone marrow cells from C57BL/6 mice for generation of bone marrow-derived dendritic cells (BM-DC) has been previously described (Xu et al, 2010). After 6 d of culture in RPMI 1640 medium (PAA, Colbe) containing 10 % heat-inactivated fetal calf serum (Gibco-BRL, Grand Island, NY) and GMCSF at 37°C in an atmosphere of 5 % CO_2, the BM-DC were suspended in small flasks for experiments at the density of 8 x 10^6 cells per flask and allowed to adhere for at least 12 h.

Bacterial strains were grown overnight in LB and the OD at 600 nm of the cultures was adjusted to 0.2 in 1 ml of PBS. Aliquots of this suspension were added to flasks in order to yield a multiplicity of infection (MOI) of approximately 100. The flask were centrifuged at 500 x g for 5 min to synchronize the infection and subsequently incubated at 37°C in an atmosphere of 5 % CO_2 for 30 min. Thereafter, the flasks were washed thrice with RPMI 1640 medium and incubated with RPMI 1640 medium containing 100 mg x ml^{-1} gentamicin for 1 h, followed by RPMI 1640 medium containing 25 mg x ml^{-1} gentamicin for the rest of the experiment.

At 16 h after infection, the cells were fixed and processed for immuno-staining of *Salmonella* LPS (rabbit anti-*Salmonella* O1,4,5, Difco, BD), the HA epitope tag (Roche) and the dendritic cell-specific marker CD11c (Armenian hamster anti-CD11c, BD). The cells were analyzed by microscopy using a Zeiss LSM700 laser-scanning confocal microscope.

3.2.6. Quantification of T cell stimulation

The SL-H2-K^b-specific, murine $CD8^+$ T cell hybridoma B3Z express the *lacZ* reporter gene under the control of the NFAT enhancer (Karttunen et al, 1992). The cell line was

kindly provided by Nilabh Shastri at the University of California at Berkeley (CA, USA). Briefly, 10^5 BM-DC from C57BL/6 mice per well of 96-well plates were infected with bacterial strains grown to stationary phase. Infection was performed for 1 h at MOI of 25 or 100 for WT or MvP728 strains, respectively. The plate was centrifuged for 5 min at 500 x *g* to synchronize the infection. After the infection period, non-internalized bacteria were removed by two washes with phosphate-buffered saline (PBS). To kill remaining extracellular bacteria, infected cells were incubated for 1 h in medium containing 100 µg x ml^{-1} of gentamicin. After a washing step, medium containing 25 µg x ml^{-1} gentamicin was added. B3Z T-cells were added to plate and co-cultured with a DC: T cell ratio ranging from 1:8 to 1:0.125 in a total volume of 200 µl per well for 24 h. Cells were centrifuged at maximal speed and lysed by addition of 100 µl substrate solution (0.15 mM chlorophenyl red β-galactopyranoside, 0.5% (v/v) Nonidet P-40 in PBS). After incubation for 6 - 8 h at 37°C, the absorbance was determined at 595 nm.

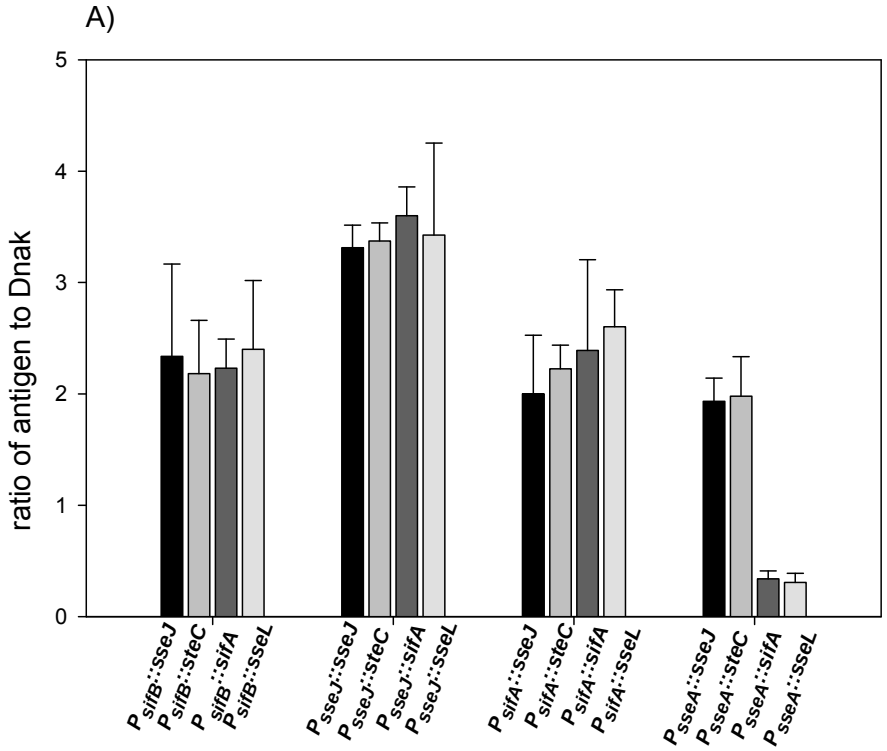
3.3. Results:

3.3.1. Generation and evaluation of expression cassettes

To test the efficacy of various SPI2-effector antigen fusions in vaccination approaches with live attenuated carrier strains, expression cassettes containing promoter of *sifB, ssifA, sseJ* or *sseA* and a genes encoding a hybrid protein consisting of SPI2-T3SS translocated effector proteins SseJ, SseL, SteC, SifA and model antigens ovalbumin (OVA) or listeriolysin (Llo) were generated. For either model antigen, a large panel of tools is available for the characterization of immune responses. The expression cassettes were located on low copy number plasmids that have previously was shown

to be compatible with SsrAB-regulated *in vivo* expression. The modular design of the various expression cassettes is presented in Table. 1.

Next, strains harboring the expression cassettes were tested for the levels of recombinant fusion proteins. We first used *in vitro* culture conditions known to induce the expression of genes of the SsrAB regulon and the synthesis of SPI2 effector proteins. The synthesis of SPI2 effector-OVA-HA and SPI2-effector-Llo-HA fusion proteins was observed for all constructs analyzed here (**Fig.3.1.**). In order to quantify the amounts of recombinant protein produced by the various strains, Western blots were analyzed using the Odyssey detection system and signals for recombinant antigen fusions were normalized by the levels of constitutively synthesized control protein DnaK. For both model antigens, highest amounts of recombinant proteins were detected if P_{sseJ} was used. Constructs with P_{sifA} and P_{sifB} were at a comparable level protein, while expression under control of P_{sseA} was lowest.



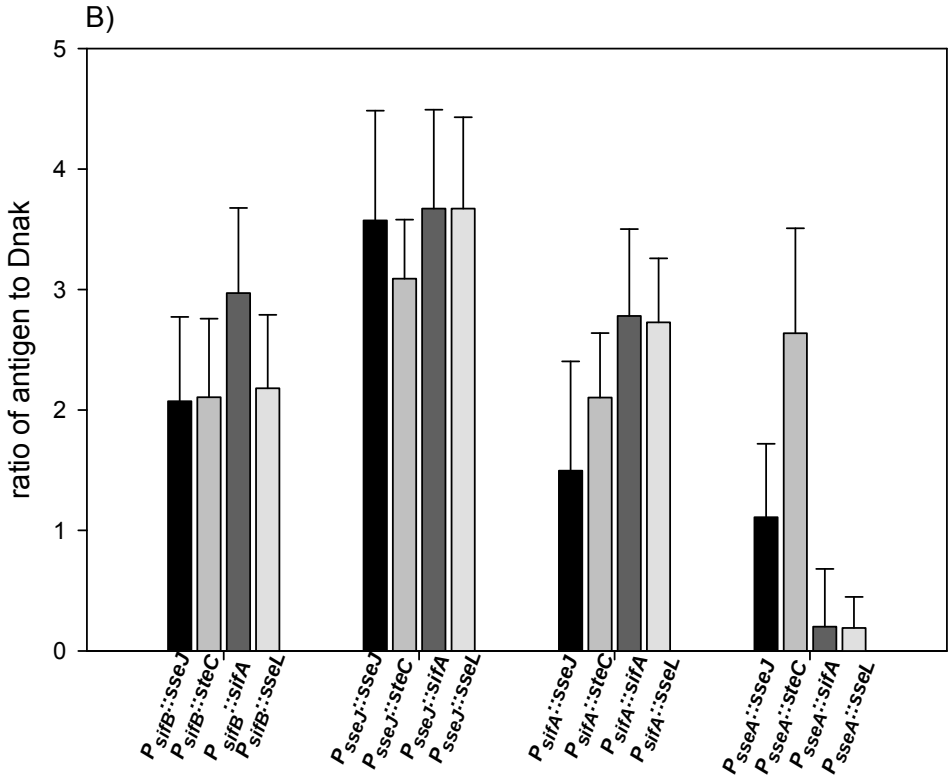
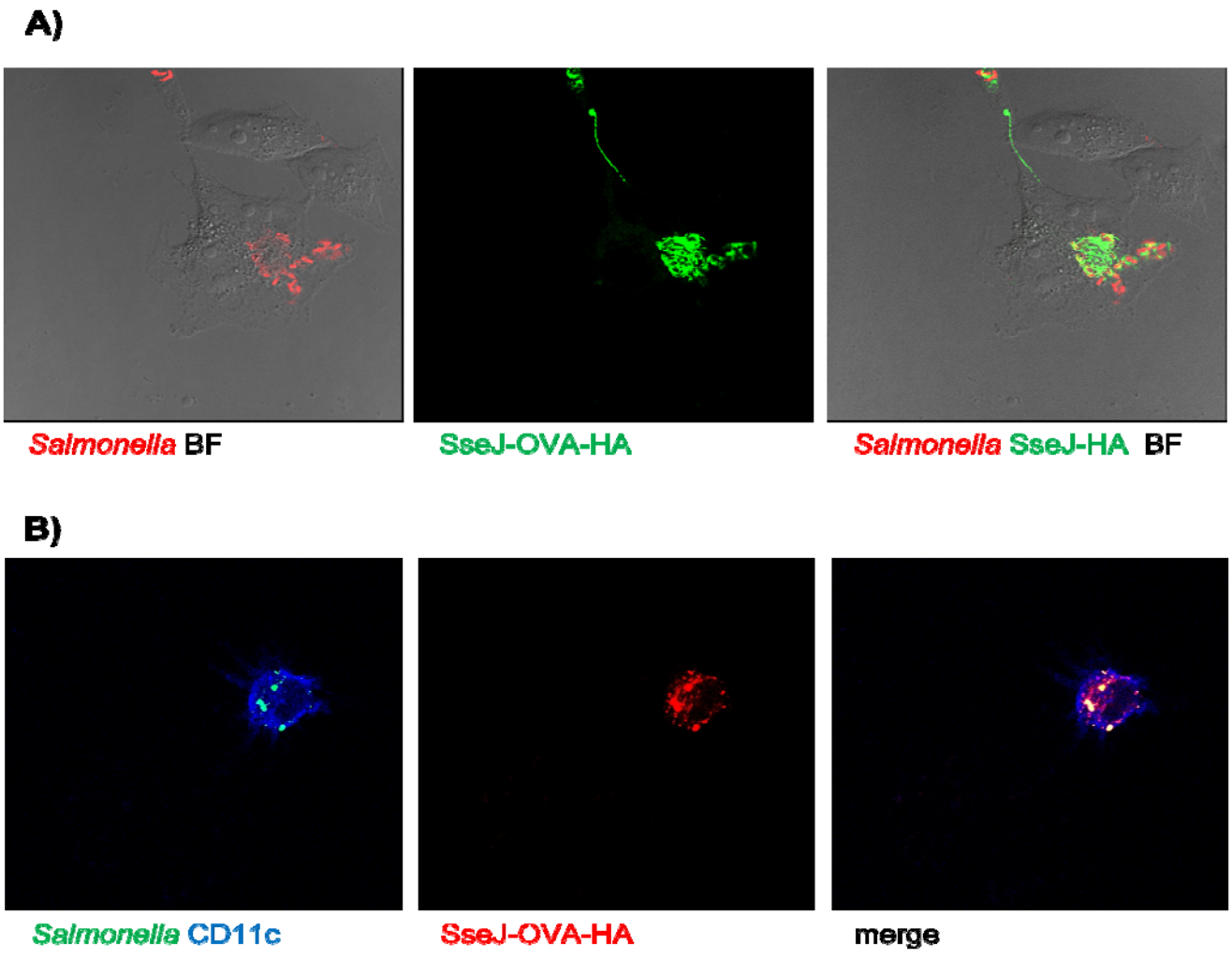


Fig.3.1. Synthesis of fusion proteins with model antigens. The *S. enterica* serovar Typhimurium wild-type strain harboring plasmids of the of fusion proteins consisting of SPI2 effector proteins SseJ, SifA, SseL or SteC model antigens OVA (A) or Llo (B) and a C-terminal epitope tag HA under control of $P_{sifB,}$ P_{sseJ}, P_{sifA} or P_{sseA} promoters were grown in SPI2-inducing minimal media (PCN-P, pH 5.8). Bacteria were harvested after 6 h of culture under inducing conditions and equal amounts of bacteria as adjusted by OD_{600} were lysed and subjected to SDS-PAGE and Western blot analyses for the detection of the HA epitope tag was performed. Blots were probed with fluorescently labeled secondary antibodies and signal intensities were quantified using the Odyssey system (Li-Cor). As loading controls, the cytosolic heat shock protein DnaK was detected on the same blot and signals were quantified. The ratios of the HA to DnaK signals were calculated and means and standard deviations for three samples were shown. The Experiement was performed at least three times and means and standard deviations are shown.

As a further control of the function of the expression cassettes, the translocation of the model antigens by intracellular *Salmonella* in BM-DC was analyzed. We investigated the translocation of OVA-HA and Llo-HA fusion proteins by strains harboring expression

cassettes with P_{sifB}, P_{sseJ}, P_{sifA} *or* P_{sseA} promoters. The use of the various expression cassettes resulted in the translocation of fusion proteins into BM-DC (as example, see **Fig. 3.2)**. The intensities of the immuno-fluorescence staining were variable, indicating different amounts of translocated protein. For quantification, infected HeLa cells harboring similar numbers of intracellular *Salmonella* were selected and the signal intensities for the fluorescence channel for SPI2-Effector-Ova-HA staining were quantified. The comparison of expression cassettes with various effectors indicated that SseJ-Ova-HA fusion proteins were the most efficient translocated under control of different promoters (**Fig. 3.2.C**).



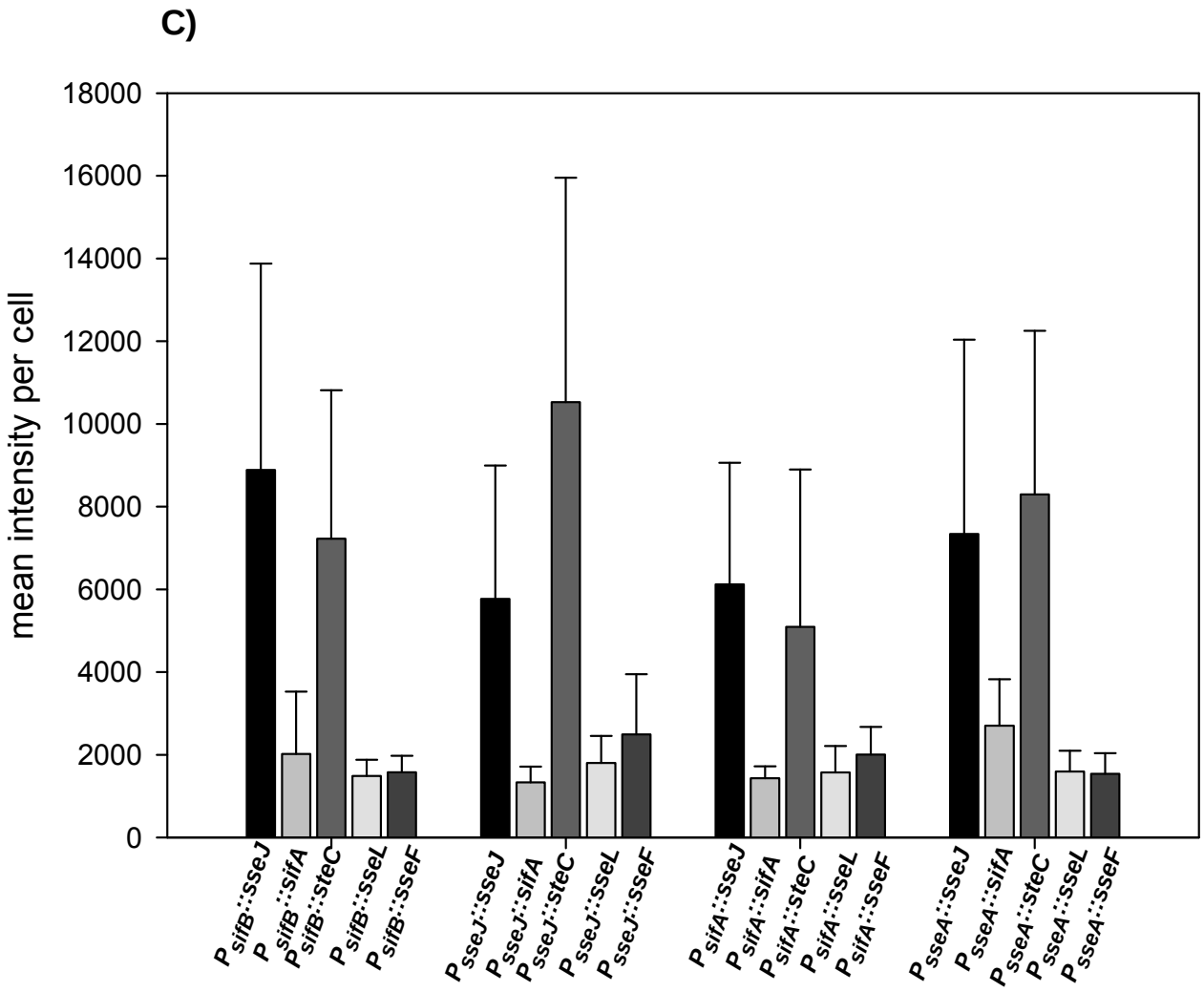


Fig. 3.2. Translocation of fusion proteins by intracellular *Salmonella*. Wild-type *S.* Typhimurium serovar *enterica* harboring plasmids with cassettes for the expression of fusion proteins consisting of SPI2 effector proteins SseJ, SifA, SseL or SteC model antigens Llo and a C-terminal epitope tag HA under control of $P_{sifB,}$ P_{sseJ} , P_{sifA} or P_{sseA} promoters promoters were used to infect the epithelial cell line HeLa at an MOI of 10, or BM-DC at an MOI of 25. A) HeLa cells were fixed 16 h after infection and processed for immune-staining for intracellular *Salmonella* (red) and translocated fusion protein effector-Llo-HA (green) was performed. B) For BM-DC, DC marker CD11c was labeled (blue) in addition to *Salmonella* (green) and fusion protein SPI2 effector-OVA-HA (red). Cells were analyzed by confocal laser-scanning microscopy using the ZEN software package (Zeiss). Representative infected HeLa cells (A) and BM-DC

(B) are shown. C) For quantification, attenuated *purD htrA* carrier strain (MvP728) harboring plasmids with expression cassettes for fusion proteins consisting of SPI2 effector proteins SseJ, SifA, SseL or SteC to OVA and the HA tag under control of P_{sseA} promoter were used to infect HeLa cells at MOI of 100. The cells were fixed 16 h after infection and processed for immune-staining. Infected cells with similar amounts of intracellular *Salmonella* were selected for the various conditions and the signal intensities of the Cy3 channel for the anti-HA stain were measured with identical exposure times. The mean signal intensity per cell and standard deviations for at least 50 infected cells per condition are shown.

3.3.2. Quantification of T-cell responses to antigens presented by intracellular *Salmonella*

We next compared the antigen-dependent simulation of T-cells after uptake of recombinant *Salmonella* strains with expression of recombinant antigens under control of with P_{sifB}, P_{sseJ}, P_{sifA} *or* P_{sseA} promoters. BM-DC were infected with *S. enterica* serovar Typhimurium attenuated carrier strain MvP728 deficient in *purD* and *htrA* [13] harboring plasmids for the expression of SPI2-effector::OVA::HA under the control of with P_{sifB}, P_{sseJ}, P_{sifA} *or* P_{sseA} promoters. Subsequently, infected BM-DC were incubated with the B3Z T-cell line. B3Z is a T cell hybridoma that recognizes the OVA-derived SIINFEKL epitope in the context of H2Kb and express *lacZ* reporter gene under control of the NFAT enhancer. The β-galactosidase activity thus is a measure of the antigen-dependent stimulation [15]. Very low stimulation was observed with the vector controls without expression cassettes, while addition of the SIINFEKL peptide was used as positive control resulting in maximal stimulation. The use of *Salmonella purD htrA* strains with plasmids harboring various expression cassettes resulted in highly

increased stimulation of the T-cell hybridoma. Highest stimulation was obtained with P_{sifB}::*sseJ* expression cassette (**Fig 3.4**). The P_{sifB}::SseJ fusion construct stimulated specific T-cell stimulation significantly more than P_{sseA}::SseJ (P<0.05), P_{sifB}::SseF (P<0.01) and P_{sseA}::SseF (P<0.001).

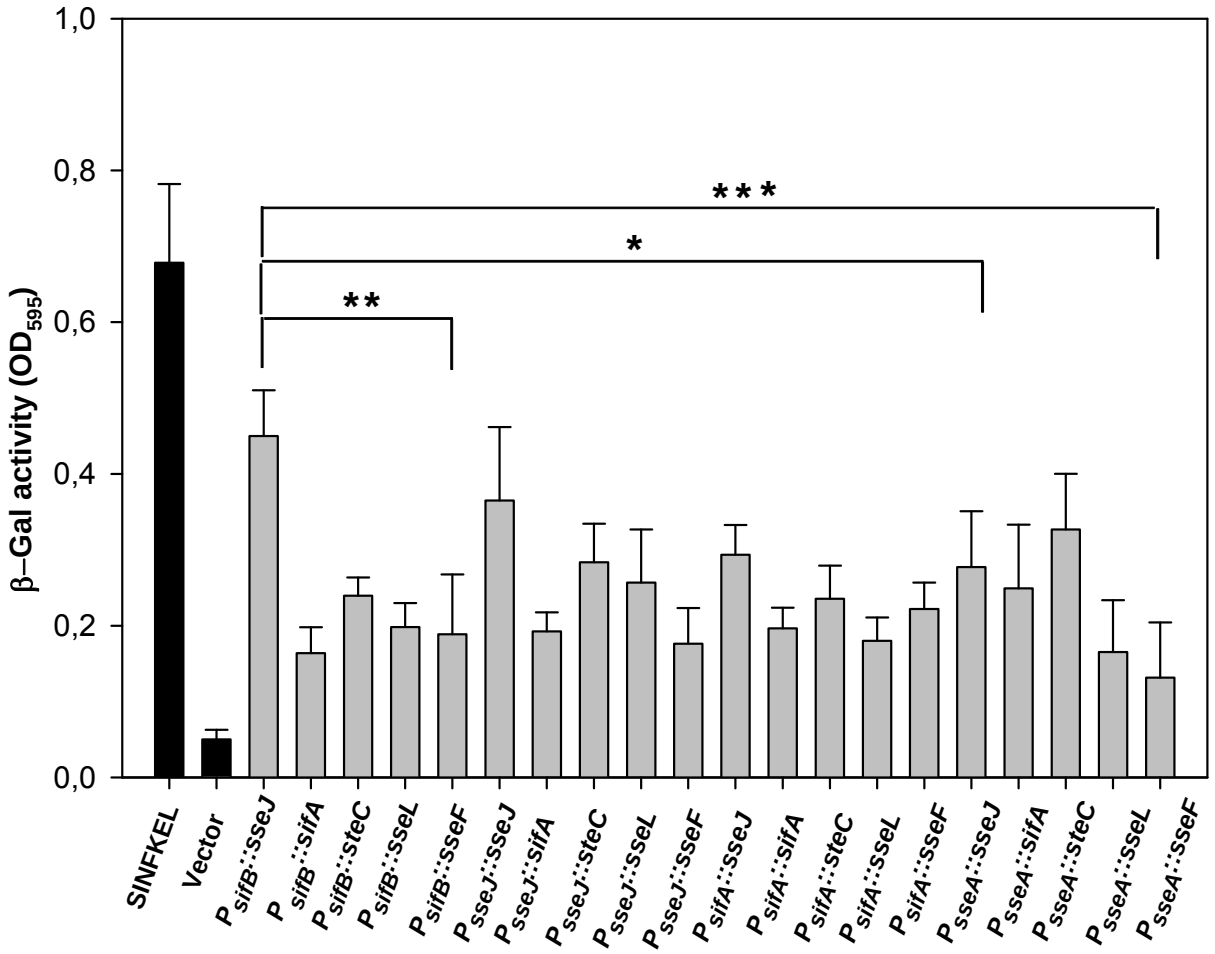


Fig. 3.4. The effect of various SPI2 promoter and effector fusions on the stimulation of T-cells. Murine BM-DC were infected at an MOI of 25 with the *purD htrA*-deficient carrier strain. Strains harbored the empty plasmid vector or plasmids with expression of fusion proteins consisting of cassettes for SPI2 effector proteins SseJ, SifA, SseL or SteC model antigens OVA and a C-terminal epitope tag HA. As positive control, BM-DC were infected with WT *Salmonella* and stimulated with the SIINFEKL peptide. The infected BM-DC were incubated with B3Z reporter cell line and after co-culture for 24 h, the

β-galactosidase substrate chlorophenyl red β-galactopyranoside was added. After additional incubation for 6 h, the reaction was stopped and the β-galactosidase product was quantified photometrically by measurement of the absorbance at 595 nm. T-cell stimulation was analyzed at ratio of infected BM-DC to T-cells 1:4. The means and standard deviations of triplicate samples are shown and the data sets are representative for 4 independent experiments. The experiments were performed in triplicates and repeated at least three times and means and standard deviations are shown. Statistical significance was determined by Student's t-test and are indicated as: N.S. not significant,*P< 0.05, **P< 0.01; ***P< 0.001.

3.4. Discussion

Salmonella carrier DNA vaccines have been employed successfully in prophylactic immunization against bacteria, viruses, fungi, parasites and even against cancer [6-7, 9]. The strategy of *Salmonella* T3SS-mediated heterologous antigen delivery was used by our group and other groups [11, 13]. SseF membrane-associated SPI2 fusion protein expressed under control of P_{sseA} showed and efficient induction of specific CD8 T cells [13]. We hypothesized that the efficacy of immune response may depend on: (i) the expression level of recombinant antigen, as the higher expression levels may lead to a more efficient processing and presentation to MHC class I, (ii) the translocation efficacy of SPI2 fusion protein, as the more translocation to the cytoplasm the more processing and presentation by MHC class I pathway which in return will lead to elevated specific CD8 T cells, (iii) The proper choice of mutant carrier strain and (iv) The stability of construct.

From our previous results, we found that the higher expression levels did not show efficient in vivo stimulation of CTLs [15]. These results were consistent with other results from independent groups [16]. P_{sseJ} and P_{sifA} showed the higher expression levels in vitro while P_{sifB} with moderate expression level was the most efficient promoter in vivo.

We hypothesised that the presentation of endogenous antigens in the cytosol to MHC-I may depends on its abundance. In this direction we tested several SPI2 effectors which are known to be translocated in APC. Our results “in the previous chapter” partialy agree with this hypothesis, we found that SseJ SPI2 effector was the most efficent in vitro and in vivo. On the other side SteC was efficently in vitro translocated while was not efficent in vivo.

In this work we constructed different SPI2 effectors under control of different promoters. As expected the expression levels are mainly due to the strength of promoter, we found the expression levels of various fusion proteins under control of P_{sseJ}>P_{sifA}>P_{sifB}>P_{sseA}. This finding is consistent with our previous results [17]. Also the translocation of different SPI2 effectors were consistent to our previous finding invitro, that the SseJ and SteC fusion proteins were significantly translocated more than othe SPI2 effector fusions. Moreover, SseJ fusion proteins showed the higer levels of CD8 T cells stimulation in vitro. SteC fusion proteins were also stimulated specific CD8 T cells but lesser than SseJ fusion proteins . This finding is consistent with our previous results. Interestingly, we found that P_{sifB}*::sseJ* construct was, in vitro, the most efficient in stimulation of specific CD8 T cells, which is completely in agreement with our previous results. P_{sifB}*::sseJ* stimulated specfic CD8 T-cells significantly more than P_{sifB}*::sseF* or P_{sseA}*::sseJ*, these results are compatible with in vivo results. Currently, a systematic in vivo analysis of the immune response of P_{sifB}*::sseJ* using different antigen models is in progress.

As a conclusion of this work, the efficacy of in vitro translocation of fusions proteins is dependnent to the selection of SPI 2 effectors. Moreover, the expression level is mainly

due to the promoter strength. A combination of the most efficient promoter P_{sifB} and most efficient translocated SPI2 effector SseJ, P_{sifB}*::sseJ* is the most efficient construct stimulating specific CD8 T cells in vitro.

3.5. References

1. DeRoeck, D., L. Jodar, and J. Clemens, *Putting typhoid vaccination on the global health agenda.* N Engl J Med, 2007. **357**(11): p. 1069-71.
2. Hueck, C.J., *Type III protein secretion systems in bacterial pathogens of animals and plants.* Microbiol Mol Biol Rev, 1998. **62**(2): p. 379-433.
3. Gerlach, R.G. and M. Hensel, *Salmonella pathogenicity islands in host specificity, host pathogen-interactions and antibiotics resistance of Salmonella enterica.* Berl Munch Tierarztl Wochenschr, 2007. **120**(7-8): p. 317-27.
4. Waterman, S.R. and D.W. Holden, *Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system.* Cell Microbiol, 2003. **5**(8): p. 501-11.
5. Mollenkopf, H., G. Dietrich, and S.H. Kaufmann, *Intracellular bacteria as targets and carriers for vaccination.* Biol Chem, 2001. **382**(4): p. 521-32.
6. Loessner, H. and S. Weiss, *Bacteria-mediated DNA transfer in gene therapy and vaccination.* Expert Opin Biol Ther, 2004. **4**(2): p. 157-68.
7. Vassaux, G., et al., *Bacterial gene therapy strategies.* J Pathol, 2006. **208**(2): p. 290-8.
8. Schoen, C., et al., *Bacteria as DNA vaccine carriers for genetic immunization.* Int J Med Microbiol, 2004. **294**(5): p. 319-35.
9. Shahabi, V., et al., *Live, attenuated strains of Listeria and Salmonella as vaccine vectors in cancer treatment.* Bioeng Bugs, 2010. **1**(4): p. 235-43.
10. Mastroeni, P., et al., *Salmonella: immune responses and vaccines.* Vet J, 2001. **161**(2): p. 132-64.
11. Panthel, K., et al., *Salmonella type III-mediated heterologous antigen delivery: a versatile oral vaccination strategy to induce cellular immunity against infectious agents and tumors.* Int J Med Microbiol, 2008. **298**(1-2): p. 99-103.
12. Russmann, H., *Bacterial type III translocation: a unique mechanism for cytosolic display of heterologous antigens by attenuated Salmonella.* Int J Med Microbiol, 2003. **293**(1): p. 107-12.
13. Xiong, G., et al., *Novel cancer vaccine based on genes of Salmonella pathogenicity island 2.* Int J Cancer, 2010. **126**(11): p. 2622-34.
14. Yewdell, J.W. and J.R. Bennink, *Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses.* Annu Rev Immunol, 1999. **17**: p. 51-88.
15. Xu, X., et al., *Efficacy of intracellular activated promoters for generation of Salmonella-based vaccines.* Infect Immun, 2010. **78**(11): p. 4828-38.
16. Pathangey, L., et al., *Effect of expression level on immune responses to recombinant oral Salmonella enterica serovar Typhimurium vaccines.* Vaccine, 2009. **27**(20): p. 2707-11.
17. Xu, X. and M. Hensel, *Systematic analysis of the SsrAB virulon of Salmonella enterica.* Infect Immun, 2010. **78**(1): p. 49-58.

Chapter 4

4.1. Introduction

Salmonellae are Gram-negative, a causative agent for very diverse disease outcomes ranging from a general mild, self-limiting gastroenteritis, to systemic infection of typhoid fever. Several clusters of virulence genes are important for the adhesion to specific cell types of the mucosa, the invasion of enterocytes and for the triggering of fluid secretion leading to the diarrheal symptoms. Pathogenesis of *Salmonella* depends on its ability to survive and replicate inside host cells. This virulence trait is linked to the ability to cause systemic infections [1]. A large number of genes are required to enable *Salmonella* to cope with nutritional limitations (*aro*, *pur,* etc.) and to avoid clearance by the host immune system. Further virulence factors enable *Salmonella* to avoid or survive damage by antimicrobial peptides [2-3].

The T3SS apparatus, also referred to as injectisome, spans the inner and outer membranes of the bacterial envelope and secretes translocon and effector proteins. Translocon proteins allow access of effector proteins to the eukaryotic cells by forming pores in the host cell membrane and forming a connecting channel-like complex between the bacterium and the eukaryotic membrane [4]. *S. enterica* encodes two distinct virulence-associated T3SS with roles in different phases of pathogenesis. Important virulence characters of *S. enterica* are encoded by genes within *Salmonella* Pathogenicity Islands (SPI), such as the interaction with enterocytes resulting in diarrhea, the invasion of non-phagocytic cells, and the ability to survive phagocytosis and to proliferate within eukaryotic host cells. Two major SPI, SPI1 and SPI2, encode T3SS that translocates bacterial effectors [5]. The function of SPI2 is essential for the

second hallmark of *Salmonella* pathogenesis, the ability to cause systemic infections and to proliferate within host organs.

Salmonella translocates T3SS effector proteins into the host cell cytoplasm mediated by either the SPI1-T3SS from the extracellular stage and from the SCV at an early stage after entry or the SPI2-T3SS from the SCV at later stages during intracellular life [1, 3, 6]. Efficient immune response mainly requires T cells induction which depends on the strength of antigen presentation. The antigen presentation strength by its role depends on antigen access to the respective processing compartment, the antigen-processing efficacy, and antigen abundance [7]. *Salmonella* T3SS-mediated translocation can be used for efficient delivery of heterologous antigen fusions to SPI1 or SPI2 effector proteins to the cytosol of APC, leading to prominent CD8 T cell priming in orally immunized mice [8].

Live attenuated strains of *Salmonella* that synthesize and secrete foreign antigens are being developed as vaccines for a number of infectious bacterial, viral and parasitic diseases and cancer. Recent advances in the understanding the genetics of *Salmonella* virulence has led to the development of attenuated *Salmonella* strains with single or multiple defined mutations in known virulence genes. Live attenuated *Salmonella* vaccines are potentially superior because of their ability to induce cell-mediated immunity in addition to antibody responses, oral delivery, single-dose administration, low cost and easy storage, induction of immune responses at multiple mucosal sites and, interestingly, their possible use as carriers for the delivery of recombinant antigens to the immune system.

Balance between the attenuation and virulence should be considered while designing efficient live attenuated *Salmonella* vaccine. Attenuation must produce an organism with reduced ability to grow within the host. Ideally, the growth rate of the live vaccine should not be significantly increased in immune compromised hosts. On the other hand, excessive attenuation can compromise the ability of the vaccine to persist in the tissues and to induce protective immunity. Recent progress in the knowledge of the genetics of *Salmonella* virulence and modern recombinant DNA technology offers the possibility to introduce multiple, defined, attenuating and irreversible mutations into the bacterial genome.

A number of genes involved in the survival of *Salmonella* in the host have already been identified and targeted for the construction of live attenuated *Salmonella* vaccines. These include both 'house-keeping genes' such as those involved in the biosynthesis of bacterial structural components (e.g. LPS, outer membrane proteins) or in the synthesis of essential metabolites (purines, pyrimidines, histidine, methionine, and aromatic amino acids, or second messenger cAMP), and 'true virulence genes', specifically involved in bacterial resistance to host-defence mechanisms. For example, *phoP*-regulated genes confer resistance to defensins; *htrA*, and *sod*C are involved in resistance to oxidative stress and genes within the SPI2 region are essential for bacterial growth in the tissues, and mediate bacterial resistance to NADPH-oxidase-mediated anti-microbial mechanisms within phagocytes [9-13]. *S.* Tphimurium mutant carrier provides a mouse model with systemic infections that resemble typhoid fever in humans (Miller and Pegues 2000).Gene *aro* function is biosynthesis of aromatic amino acids and related compounds. *aroA* mutant strains were proven to be safe and widely used as carrier

for vaccination. SL5000 and SL7207 *aroA* mutant carrier strains were widely used to deliver heterlogous antigens and induce humoral and cellular responses [14]. *S.*Typhimurium *pur* double mutation with other genes was also used to induce both humoral and cellular immune responses. The function of genes *pur* is biosynthesis of adenine and *htrA* is protection of bacteria against heat stress and oxidative stress.The use of *htrA*/*purD* double mutant strain to deliver tumor associated antigen survivin induced protective CD4 and CD8 responses [15]. LH1 160(*purB/phoP/phoQ*) and Vnp20009 (*purI/xyl/msbB*) mutants induced humoral responses by oral and i.v. routes, respectively [16-17].

galE mutants are incapable of synthesising uridine diphosphate-galactose (UDP-Gal) from uridine diphosphate-glucose (UDP-Glu). *S.* Typhimurium *galE* mutant strains were attenuated and effective as live oral vaccines in the mouse model but showed variable success in vaccination in veterinary. Moreover, it was found that *S.* Typhi *galE* mutants lacking of the Vi antigen, was found to be virulent in humans (reviewed in [12]. Other trails were done to utilize different mutations to increase the immunogenicity of heterologous antigens and at the same time achieve required attenuation. RfaH is a transcriptional antiterminator that reduces the polarity of long operons encoding secreted and surface-associated cell components of *Salmonella*, including O antigen and lipopolysaccharide core oligosaccharides [18]. *rfaH* mutant *S.*Typhimurium vaccine enhanced immunogenicity of heterologous antigens and was sufficiently attenuated in mice [19].

Trails for attenuation of virulence genes also showed variable success in vaccination. Double mutation in *ssaV*, a SPI2-encoded protein, and *aroC* showed variable humoral

responses when used in humans [20]. SPI2 effector SifA is required for the formation of lysosomal glycoprotein-containing structures, called SIFs, and to maintain the integrity of the phagosomal membrane of the SCV during intracellular proliferation [6]. Recently, new systems were developed in *S.* Typhimurium to enhance the safety and immunogenicity including regulated delay in *in vivo* attenuation [21], antigen synthesis [22] and cell lysis [23].

In this study we are discussing the efficacy of *S.*Typhimurium *sifA* mutant strain in comparison to other proved *S.*Typhimurium carrier strains in particular MvP728 (Δ*htrA*/*purD*), MvP 468 (Δ*aroA*) and MvP 760 (Δ*rfaH*). We transferred plasmid pWSK29 *PsseJ sseJ*::OVA::HA or Pwsk29 *PsseJ sseJ*::*lisA*$_{51\text{-}363}$::HA to tested strains. In vitro studies showed that s*ifA* mutants stimulated specific CD8 T-cells proliferation more than *aroA* and *rfaH* mutants when used to deliver OVA model antigen to B3Z T-cells that have *lacZ*reporter. In the same experiment *S.*Typhimurium *sifA* mutant stimulated T-cell proliferation non significantly more than *htrA/purD* mutant. We suppose the efficient use of *S.*Typhimurium *sifA* mutant as carrier for vaccination.

4.2. Materials and Methods

4.2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. *Salmonella enterica* serovar Typhimurium strain NCTC 12023 (*S.*Typhimurium) was used as wild-type strain. Bacteria were routinely cultured in LB broth and on LB agar plates. If required for the selection of recombinant strains or to maintain plasmids, carbenicillin (50 µg/ml) and/or

kanamycin (50 µg/ml) were added. Synthetic minimal media with limiting (PCN-P media) or non-limiting (PCN media) amounts of phosphate were used for the analyses of promoters under control of the SsrAB regulatory system *in vitro* and have been described before [9]. Synthetic media were supplemented with 1 mM adenine for growth of auxotrophic *purD* strains. Attenuated carrier strains were previously constructed by our group and are specified in Table1.

4.2.2. Construction of plasmids

For generation of expression cassette consisting of gene fusion *sseJ*, hSurvivin and HA tag under control of P_{sseJ} promoter. The gene encoding hSurvivin was amplified by PCR using hSurv-For-EcoRV, hSurvivin-HA-Rev-XbaI and p3342 as a template, which later digested with EcoRV and XbaI. The digested hSurvivin was ligated to EcoRV and XbaI digested low copy plasmid pWSK29. The resulting plasmid pWSK29::*hsurvivin* was digested with KpnI and EcoRV. The gene *sseJ* was PCR amplified using SseJ-Pro-For-KpnI and SseJ-Rev-EcoRV. The resulting amplified *sseJ* was digested with KpnI and EcoRV and ligated to digested pWSK29::*hsurvivin* resulting in plasmids Pwsk29 P_{sseJ} *sseJ*::hSurvivin::HA (p3550).

For generation of expression cassette consisting of gene fusion *sseJ*, OVA and HA tag under control of SPI2 promoter P_{sseJ} . The gene encoding OVA including the CD4 and CD8 epitopes was amplified by PCR using Ova-For-NaeI, OVA-HA-Rev-XbaI and pOMP as a template, which later digested with NaeI and XbaI. P_{sseJ} *sseJ*::hSurvivin (p3550) was digested with EcoRV and XbaI. Digested OVA fragment was ligated to large fragment of digested plasmids to obtain plasmids P_{sseJ} *sseJ*::OVA::HA (p3554).

For generation of expression cassette consisting of gene fusion *sseJ*, LisA and HA tag under control of SPI2 promoters *PsseJ.* *PsseJ*::SseJ was amplified using SseJ-Pro-For-KpnI and SseJ-Rev-EcoRV, which were digested with KpnI and EcoRV. Plasmid p2810 harboring P_{sseA} *sscB*::sseF::LisA::HA was digested with KpnI and EcoRV, the large fragment was ligated to amplified digested *PsseJ*::SseJ resulting in P_{sseJ} sseJ::lisA::HA (p3557). The obtained plasmids were confirmed by colony PCR, diagnostic digestion and sequenced using T7-Seq and T3-Seq primers.

Table 1. Strains and plasmids used in this study

***S. enterica* serovar Typhimurium strain or plasmid**	**Relevant characteristics[a]**	**Source or reference**
Strains		
NCTC 12023	Wild type	Lab Stock
P2D6	Δ*ssaV*; Kan^r	Lab Stock
MvP503	Δ*sifA*	Lab Stock
MvP468	Δ*aroA*	Lab Stock
MvP481	Δ*purD* Kan^r	Lab Stock
MvP679	Δ*galE*	Lab Stock
MvP728	Δ*purD* Δ*htrA*	Lab Stock
MvP729	Δ*galE* Δ*htrA*	Lab Stock
MvP740	Δ*purD* Δ*galE*	Lab Stock
MvP760	Δ*rfaH*	Lab Stock
Plasmids		
pWSK29	Low copy number; Amp^r	Lab Stock
pOMP-OVA	*lacZ*::OVA	Lab stock
p2810	pWSK29 P_{sseA} *sscB sseF*::$lisA_{51\text{-}363}$::HA	Lab stock
P3342	pwsk29 P_{sseA} *sscB sseF*::hSurvivin::HA	Lab stock
p3550	pwsk29 P_{sseJ} *sseJ*::hSurvivin	This study
p3554	pWSK29 P_{sseJ} *sseJ*:: OVA::HA	This study
P3557	pWSK29 P_{sseJ} *sseJ*:: $lisA_{51\text{-}363}$::HA	This study

[a] Kan^r, kanamycin resistance; Amp^r, ampicillin resistance.

Table2. Oligonucleotides used in this study

Designation	Sequence
SseJ-Rev-EcoRV	ACGGATATCTTCAGTGGAATAATGATGAGC
SseJ-Pro-For-KpnI	TACGGTACCTCACATAAAACACTAGCAC
Ova-For-NaeI	ATAGCCGGCGCAATGCCTTTCAGAGTGAC
OVA-HA-Rev-XbaI	AGATCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTAAGGG GAAACACATCTGCCAAAG
LisA-51-For-EcoRV	CTAGATATCACGCCAATCGAAAAGAAAC
LisA-363-HA-Rev-XbaI	GAGTCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTAGAGG TTGCCGTCGATGATTTG
hSurvivin-HA-Rev-XbaI	ATTTCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTAATCCA TAGCAGCCAGCTGCTC
hSurvivin-For-EcoRV	TACGATATCGGTGCCCCGACGTTGCCCCC
T3-Seq	AATTAACCCTCACTAAAGG
T7-Seq	TAATACGACTCACTATAGGG

4.2.3. Western blotting

Plasmids p3554 and p3557 were transferred to tested mutants. In order to quantify the amounts of recombinant protein produced by the various strains, Western blots were analyzed using the Odyssey detection system and signal for model antigens were normalized by the levels of constitutively synthesized control protein DnaK.

4.2.4. In vitro stability of plasmid in recombinant *Salmonella* strains

The in vitro stability of the plasmid encoding the model antigen (Llo) was determined by growing the resulting clones without antibiotic pressure. Bacteria were subcultured for 7

days, and the percentage of cells retaining the plasmid was determined by plating in the presence or absence of antibiotic.

4.2.5. SPI2-T3SS-dependent translocation of fusion proteins by intracellular *Salmonella*.

HeLa cells or raw macrophage cells were infected with various *Salmonella* mutant strains harboring plasmids for the expression of *SseJ*::OVA::HA under the control of *PsseJ* promoter. At 16 h after infection, the cells were fixed and processed for immuno-staining of *Salmonella* LPS (rabbit anti-*Salmonella* O1,4,5, Difco, BD) and the HA epitope tag (Roche) The cells were analyzed by microscopy using a Leica laser-scanning confocal microscope.

4.2.6. Analyses of invasion of epithelial cells

HeLa cells were seeded in 24-well plates at a density of 5 x 10^5 cells per well one day before infection. For infection under SPI1- inducing conditions, bacteria were sub-cultured 1:30 from an overnight culture in LB with appropriate antibiotics and incubated 3.5 h at 37°C. A master-mix of the inoculum of approximately 1 x 10^5 bacteria per well multiplicity of infection (MOI 1) for HeLa cell infection was prepared in DMEM and 300 µl were added to each well. The plates were centrifuged onto the cells at 500 rpm for 5 min. After infection for 25min, the HeLa cells were washed three times with PBS and incubated for 1 h in cell culture medium containing 100µg/ml gentamicin (Sigma). The cells were lysed with 500 µl per well 0.1 % Triton X-100 10 min at RT. Serial dilutions of the inoculum and the lysates were plated onto Mueller Hinton (MH) plates to determine intracellular bacteria. The percentage of invaded bacteria (1 h versus inoculum x 100) was calculated.

4.2.7. Phagocytosis and intracellular replication in macrophages

In order to assess the relative uptake and intracellular replication, 2 x 10^5 macrophages were seeded and an MOI of 1 as well as bacterial over night cultures were used for infection. At 2 h and 16 h post infection, the infected cells were washed twice with PBS and lysed with 500 µl of 0.1 % Triton-X-100 10 min at RT. The lysates were adjusted to 1 ml with PBS and serial dilutions of the inoculum and the lysates were plated onto MH plates to enumerate the intracellular colony forming units (cfu). The percentage of phagocytosis/relative uptake (2 h versus inoculum x 100) and x-fold intracellular replication (16 h versus 2 h) were calculated.

4.2.8. Quantification of T cell stimulation

The SL-H2-K^b-specific, murine $CD8^+$ T cell hybridoma B3Z express the *lacZ* reporter gene under the control of the NFAT enhancer. Briefly, 10^5 BM-DC from BALC/c mice per well of 96-well plates were infected with bacterial strains grown to stationary phase. Infection was performed for 1 h at MOI of 25 for WT or mutant strains. The plate was centrifuged for 5 min at 500 x *g* to synchronize the infection. After the infection period, non-internalized bacteria were removed by two washes with phosphate-buffered saline (PBS). To kill remaining extracellular bacteria, infected cells were incubated for 1 h in medium containing 100 µg x ml^{-1} of gentamicin. After a washing step, medium containing 25 µg x ml^{-1} gentamicin was added. B3Z T-cells were added to plate and co-cultured with a DC: T-cell ratio ranging from 1:8 to 1:0.125 in a total volume of 200 µl per well for 24 h. Cells were centrifuged at maximal speed and lysed by addition of 100 µl substrate solution (0.15 mM chlorophenyl red β-galactopyranoside, 0.5% (v/v)

Nonidet P-40 in PBS). After incubation for 6 - 8 h at 37°C, the absorbance was determined at 595 nm.

4.3. Results

4.3.1. Generation and evaluation of expression cassettes

To test of efficacy of various live attenuated carrier strains to deliver SPI2–effector antigen fusions in vaccination approaches, expression cassettes containing the promoter of *sseJ* and a genes encoding a hybrid protein consisting of SPI2-T3SS translocated effector proteins SseJ, and model antigens hSurvivin, ovalbumin (OVA) or listeriolysin (Llo) were generated. For these model antigens, a large panel of tools is available for the characterization of immune responses. The expression cassettes were located on low copy number plasmids that have previously was shown to be compatible with SsrAB-regulated in vivo expression. The modular design of the various expression cassettes is presented in **Fig. 4.1**.

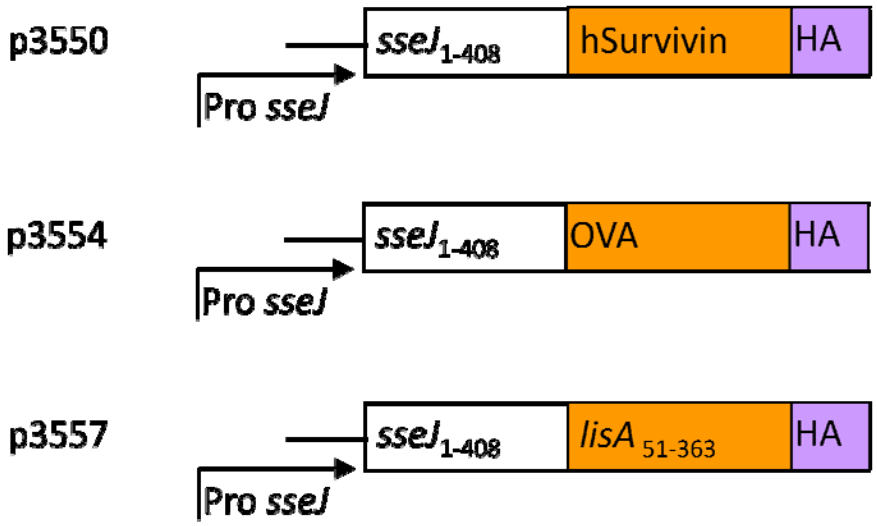


Fig.4.1. Generation of expression cassettes for the expression of heterologous vaccine antigens. Expression cassettes consist of hybrid genes for the expression of fusion proteins consisting of SPI2 effector protein SseJ model antigens hSurvivin, Llo or OVA and a C-terminal epitope tag HA for the standardized detection of the amounts of fusion protein. The expression is controlled by *in vivo*-activated promoter P_{sseJ} of the SsrAB regulon that control the expression of genes within SPI2. All plasmids were generated on the basis of low copy number vector pWSK29, and plasmid designations are indicated by p3550, etc.

Strains harboring the expression cassettes were tested for the levels of recombinant fusion proteins. We first used in vitro culture conditions known to induce the expression of genes of the SsrAB regulon and the synthesis of SPI2 effector proteins. The synthesis of SPI2 SseJ-OVA-HA fusion proteins was observed in all tested mutant strains analyzed here (**Fig. 4.2**). In order to quantify the amounts of recombinant protein produced by the various strains, Western blots were analyzed using the Odyssey detection system and signal for model antigens were normalized by the levels of constitutively synthesized control protein DnaK. The levels of expression of *sseJ*::OVA::HA in different tested mutant strains were not significantly affected except in Δ*ssaV* mutant strain which showed the lowest expression level.

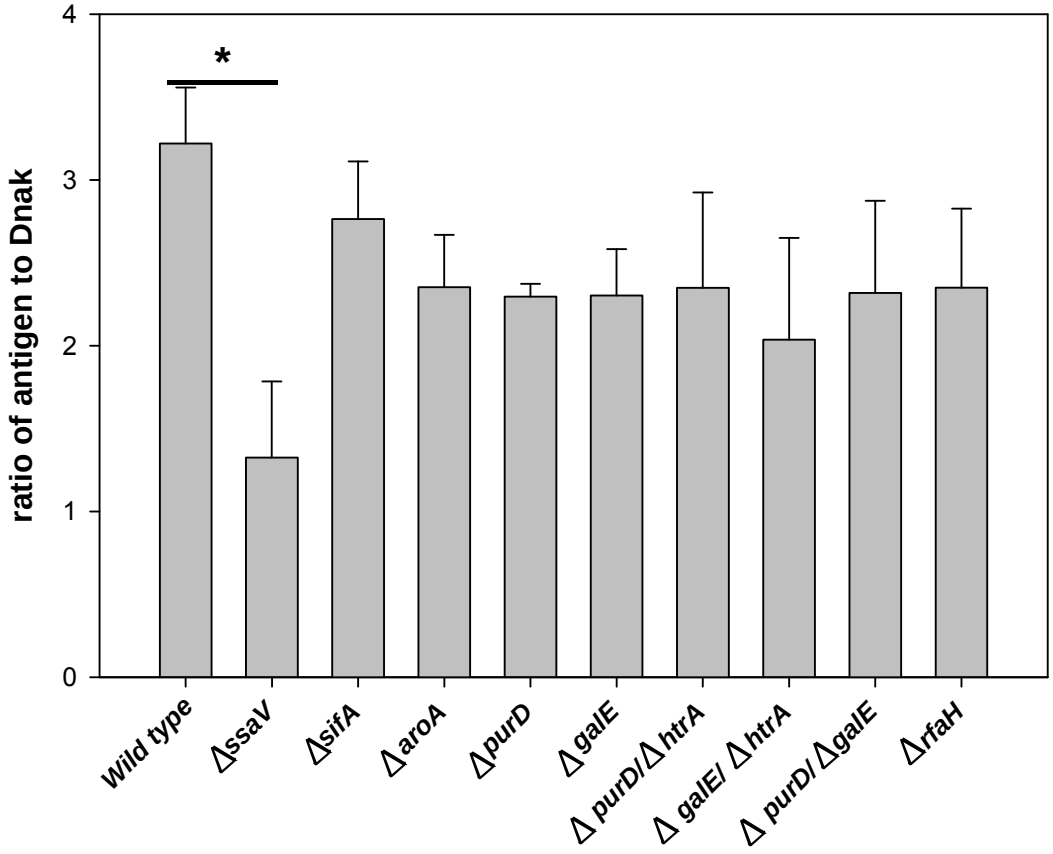
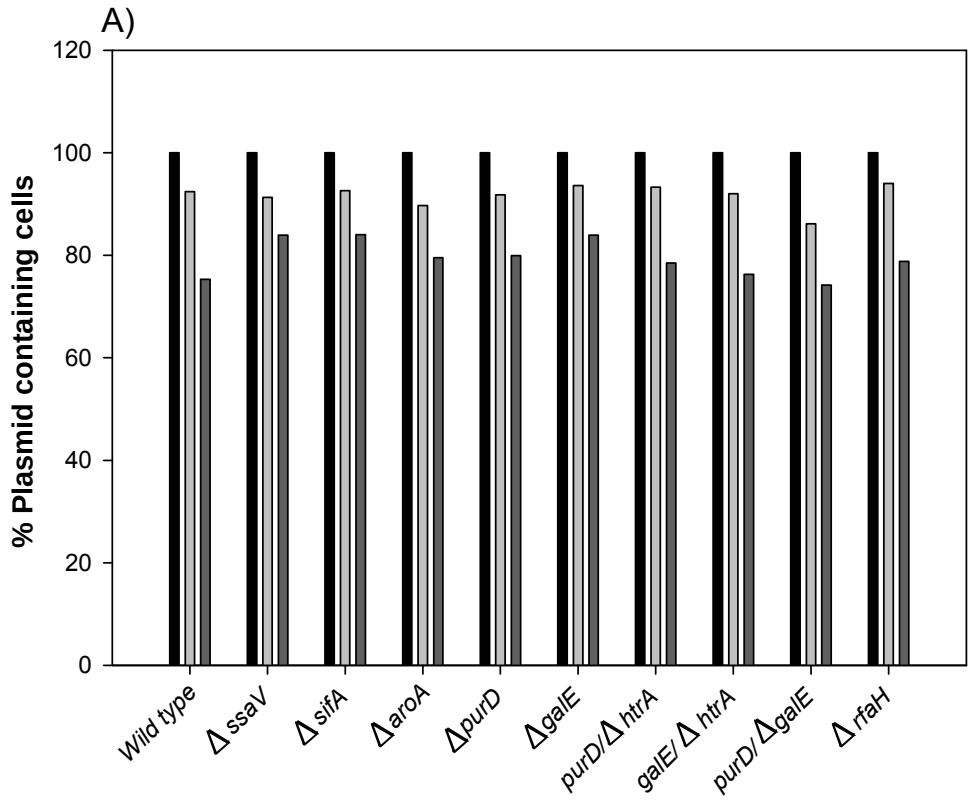


Fig.4.2. Synthesis of fusion proteins with OVA model antigen. The *S. enterica* serovar Typhimurium wild-type or various mutant strains harboring plasmids of the of fusion proteins consisting of SPI2 effector protein SseJ, model antigen OVA and a C-terminal epitope tag HA under control of P_{sseJ} promoter were grown in SPI2-inducing minimal media (PCN-P, pH 5.8). Synthetic media were supplemented with 1mM adenine for growth of auxotrophic *purD* strains. Bacteria were harvested after 6 h of culture under inducing conditions and equal amounts of bacteria as adjusted by OD_{600} were lysed and subjected to SDS-PAGE and Western blot analyses for the detection of the HA epitope tag was performed. Blots were probed with fluorescently labeled secondary antibodies and signal intensities were quantified using the Odyssey system (Li-Cor). As loading controls, the cytosolic heat shock protein DnaK was detected on the same blot and signals were quantified. The ratios of the HA to DnaK signals were

calculated and means and standard deviations for three samples were shown. The experiment was performed at least three times and means and standard deviations are shown. Means and standard deviations of triplicate assays are shown. Statistical significances were determined by Student's t-test and are indicated as: n.s. not significant; *P< 0.050.

The in vitro stability of the plasmid encoding the model antigen (Llo) was tested. Bacteria were subcultured for 7 days, and the percentage of cells retaining the plasmid was determined by plating in the presence or absence of antibiotic. The obtained results showed that pWSK29 P_{sseJ} *sseJ*::*lisA*$_{51\text{-}363}$::HA was stable in *S*. Typhimurium wild type and various mutant strains after growth in LB in the presence or absence of antibiotic (**Fig. 4.3**).



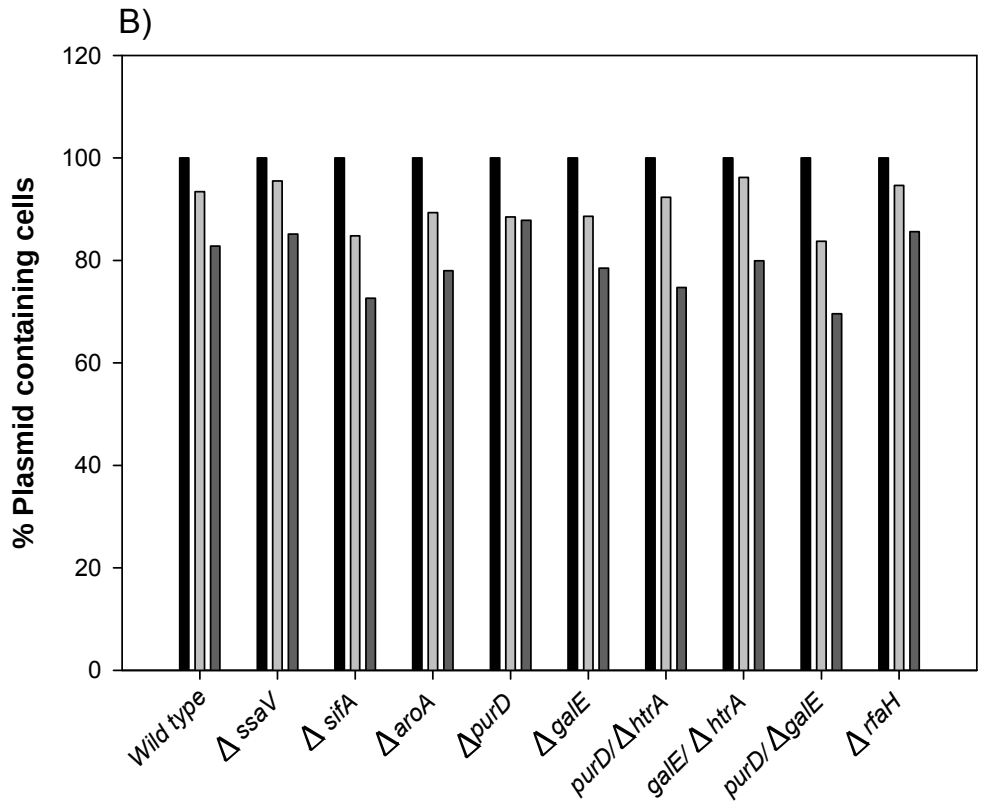


Fig. 4.3. In vitro stability of low copy number plasmids harboring expression cassette in recombinant *Salmonella.* *S*. Typhimurium wild type or various mutant strains harbor an expression cassette consisting of *sseJ*::OVA::HA under control of P_{sseJ}. The bacteria were cultured in LB without selective pressure and subcultures were prepared each day over a period of 7 d. Serial dilutions of overnight cultures were plated 1(black bars) or 3(grey bars) or 7 days (dark grey bars) onto either an(A) LB agar or (B) LB agar supplemented with carbenicillin for determination of the CFU/ml.

4.3.2. In vitro Intracellular behavior of *S.* Typhimurium mutants

In order to assess the relative invasiveness and intracellular replication of tested mutants harbouring the expression cassette encoding SseJ-antigen fusion under control of P_{sseJ}, HeLa cells and macrophages were infected for invasion assay and intracellular

replication assay, respectively. For invasion assays, the percentage of invaded bacteria (1 h post infected HeLa cells versus inoculum x 100) was calculated (**Fig 4.4**). But in intracellular replication assay, the percentage of phagocytosis/relative uptake (2 h post infected macrophage versus inoculum x 100) and x-fold intracellular replication (16 h post infected macrophage cells versus 2 h) were calculated (**Fig 4.5**). The invasion assay showed no significant difference between different mutant strains in their capability to invade HeLa cells in comparison to wild type strain, except Δ*aroA* mutant which was significantly less invasive than wild type strain. The secretion system apparatus Δ*ssaV* mutant showed the lowest replication inside macrophages. However, Δ*rfaH* mutants were significantly more intercellular replicated inside macrophage than other mutant strains in comparison to wild type. Δ*galE*, Δ*galE*/Δ*htrA*, Δ*galE*/Δ*purD* and Δ*htrA*/Δ*purD* and Δ*aroA* replication levels were significantly low in comparison to wild type. Interestingly, Δ*sifA* strain replicated significantly in macrophages more than Δ*htrA*/Δ*purD* strain.

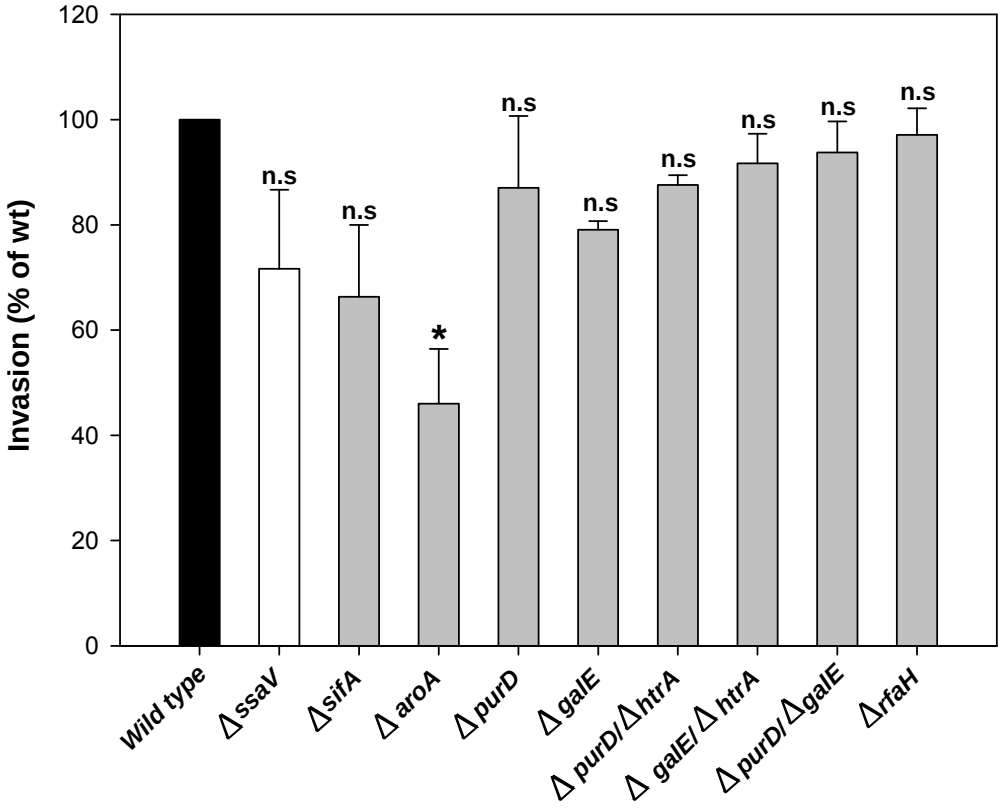


Fig.4.4. Invasion assay of wild type or various mutant strains harboring low copy plasmid encoding SseJ::OVA::HA under control of P_{sseJ}. Epithelial cells were infected with *S.* Typhimurium WT or various mutant strains. For determination of invasion, bacteria were added to the cells at an MOI of 1for HeLa cells. After incubation for 25 min at 37°C non-internalised bacteria were removed by washing and the remaining extracellular bacteria were killed by addition of medium containing 100 µg ml-1 gentamicin for 1 h. Subsequently, the cells were washed and internalised bacteria were released by cell lysis. Serial dilutions of the bacterial suspensions were plated onto agar plates for the quantification of intracellular bacteria. Invaded bacteria are expressed as percentage of the inoculum. Means and standard deviations shown are deduced from one representative experiment out of a series of three independent experiments performed in triplicates. Means and standard deviations of triplicate assays are shown.

Statistical significance were determined by Student's t-test and are indicated as: n.s. not significant; *P< 0.05.

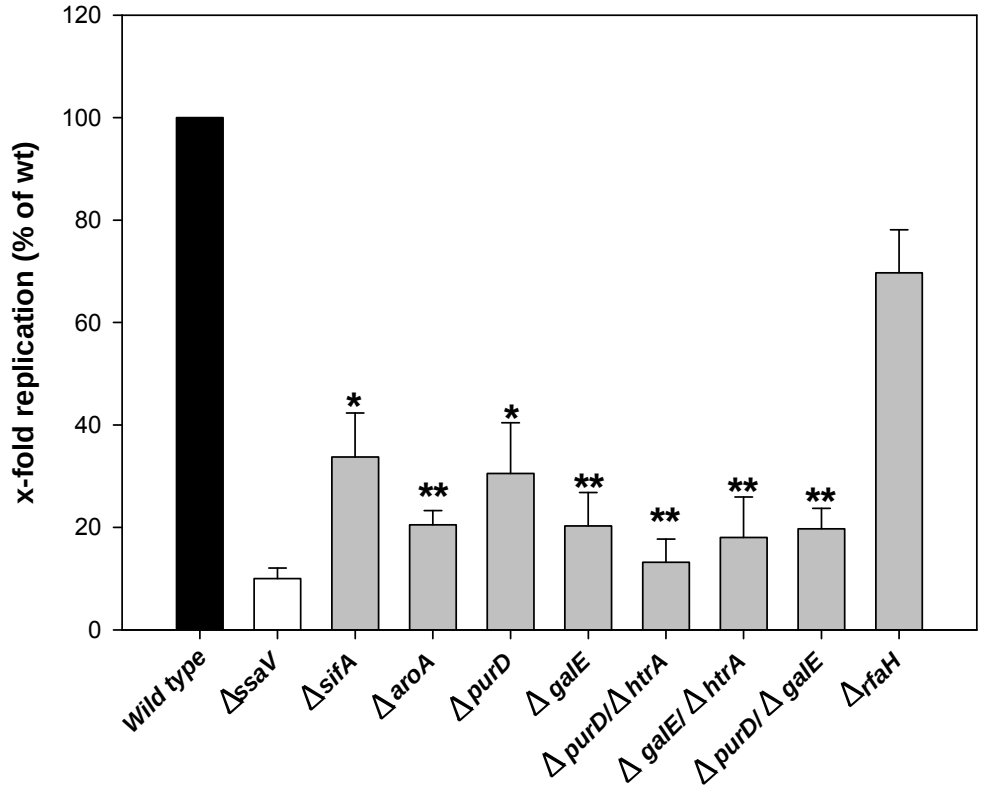


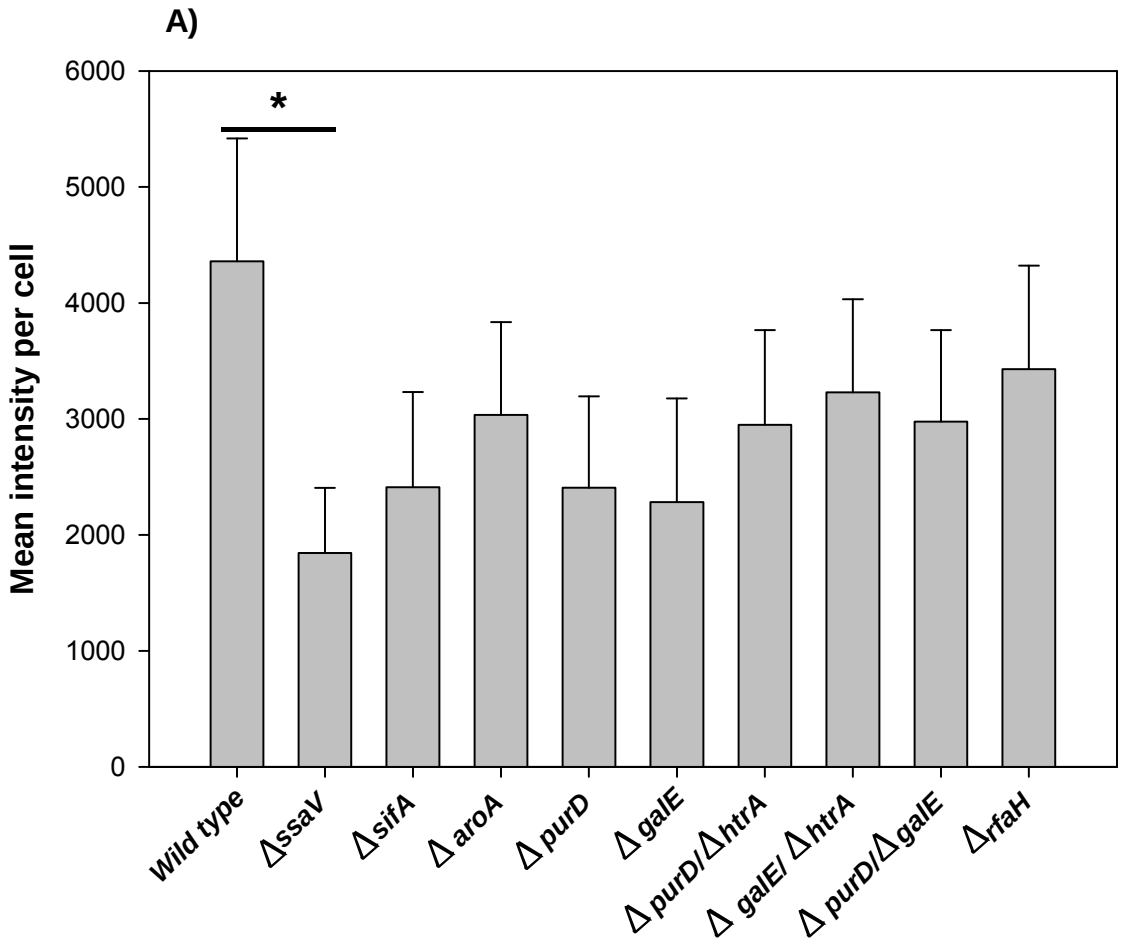
Fig.4.5. Intracellular replication of wild type or various mutant strains in macrophages harboring low copy plasmid encoding SseJ-OVA-HA under control of P_{sseJ}. RAW264.7 macrophages were infected at an MOI of 1 with *S.* Typhimurium WT, a strain deficient in the SPI2-encoded T3SS (*ssaV*) or various mutant strains. For the quantification of intracellular replication, infected cells were lysed 2 h and 16 h after infection and the cfu of intracellular bacteria was quantified. The x-fold intracellular replication is the ratio of cfu recovered at 16 h versus 2 h after infection. Means and standard deviations of triplicate assays are shown. Statisical significance were determined by Student's t-test and are indicated as: *P< 0.050; **P< 0.010.

4.3.3. Evaluation of translocation efficiency and Quantification of T-cell responses to antigens presented by intracellular *Salmonella* mutants

The translocation of the OVA model antigen by intracellular Salmonella in HeLa cells and macrophages was analyzed. We investigated the translocation of SseJ-OVA-HA fusion protein by mutant strains harboring expression cassettes with PsseJ promoters. The intensities of the immuno-fluorescence staining were variable, indicating different amounts of translocated protein. For quantification, infected HeLa cells or macrophages harboring similar numbers of intracellular *Salmonella* were selected and the signal intensities for the fluorescence channel for SseJ-OVA-HA staining were quantified (**Fig 4. 6**). The comparison of expression cassette with SseJ effector did not indicate a significant variation in translocation by different mutants except the Δ*ssaV* mutant.

We next compared the antigen-dependent simulation of T-cells after uptake of recombinant *Salmonella* strains with expression of recombinant antigens under control of P_{sseJ} promoter. BM-DC were infected with *S. enterica* serovar Typhimurium attenuated carrier strains harboring plasmids for the expression of SseJ::OVA::HA under the control of P_{sseJ}. Subsequently, infected BM-DCs were incubated with the B3Z T-cell line. B3Z is a T-cell hybridoma that recognizes the OVA-derived SIINFEKL epitope in the context of H2Kb and express *lacZ* reporter gene under control of the NFAT enhancer. The β-galactosidase activity thus is a measure of the antigen-dependent stimulation [24]. Very low stimulation was observed with the vector controls without expression cassettes, while addition of the SIINFEKL peptide was used as positive control resulting in maximal stimulation. Highest stimulation was obtained with *htrA*/*purD* mutant strain as well as *sifA* mutant (**Fig 4.7**). *sifA* mutants stimulated specific

T-cells proliferation non-significantly compared to *htrA*/*purD* mutants. Δ*sifA* strain stimulated significantly T-cells proliferation more than Δ*rfaH*, Δ*aroA* and wild type strains.



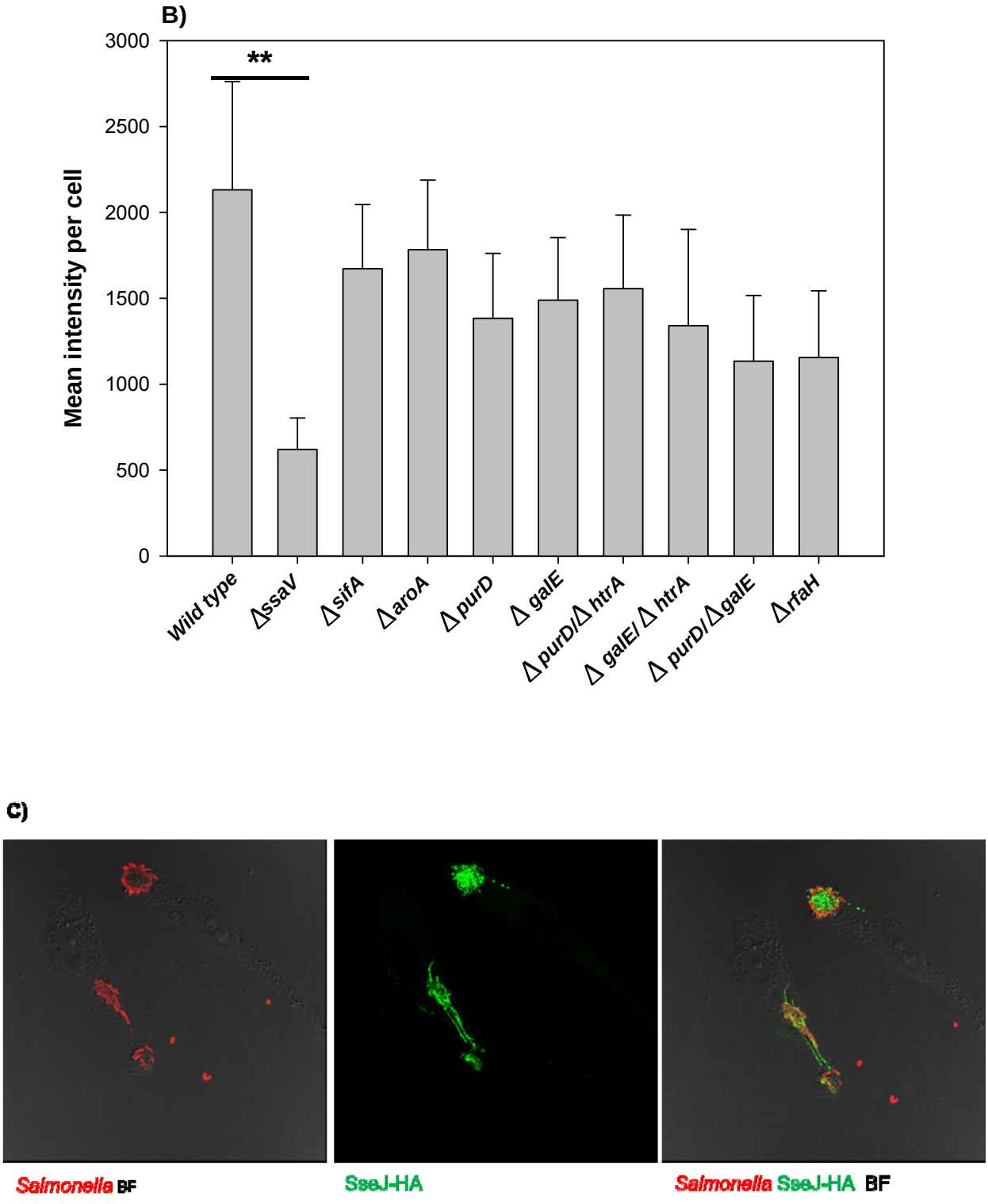


Fig 4.6. Translocation of fusion proteins by intracellular *Salmonella*. The wild type or various mutant strains harboring plasmid with cassette for the expression of fusion proteins consisting of SPI2 effector

proteins SseJ, model antigen OVA and a C-terminal epitope tag HA under control of P_{sseJ} promoter were used to infect (A) HeLa cells or (B) RAW macrophages at MOI of 100. The cells were fixed 16 h after infection and processed for immune-staining. Infected cells with similar amounts of intracellular *Salmonella* were selected for the various conditions and the signal intensities of the Cy3 channel for the anti-HA strains were measured with identical exposure times. The mean signal intensities and standard deviations for at least 25 infected cells per strains were shown. n.s. not significant; *P< 0.050; *P< 0.010.(C) HeLa cells were fixed 16 h after infection and processed for immune-staining for intracellular *Salmonella* (red) and translocated fusion protein effector-OVA-HA (green) was performed.

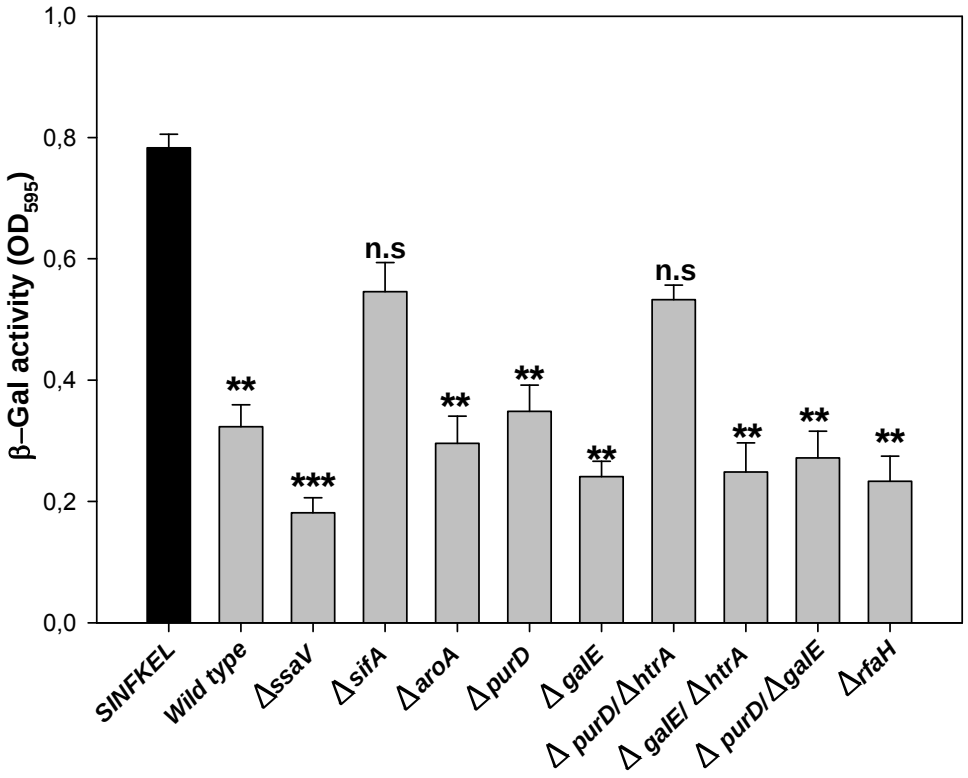


Fig. 4.7. The effect of various mutant carrier strains on the stimulation of T-cell. Murine BM-DC were infected at an MOI of 25 with wild type or various carrier strains. Strains harbored plasmid with

expression of fusion proteins consisting of cassettes for SPI2 effector proteins SseJ model antigens OVA and a C-terminal epitope tag HA. As positive control, BM-DC were infected with WT *Salmonella* and stimulated with the SIINFEKL peptide. The infected BM-DC were incubated with B3Z reporter cell line and after co-culture for 24 h, the β-galactosidase substrate chlorophenyl red β-galactopyranoside was added. After additional incubation for 6 h, the reaction was stopped and the β-galactosidase product was quantified photometrically by measurement of the absorbance at 595 nm. T-cell stimulation was analyzed at ratio of infected BM-DC to T-cells 1:4. The means and standard deviations of triplicate samples are shown and the data sets are representative for 4 independent experiments. The experiments were performed in triplicates and repeated at least three times and means and standard deviations are shown. Statistical significance was determined by Student's t-test and are indicated as: n.s. not significant; *P< 0.050; **P< 0.010; ***P< 0.001.

4.4. Discussion:

The selection of suitable mutant carrier is crucial for designing live recombinant *Salmonella* vaccines. A sufficient attenuation in virulence is required to prevent undesired side effects like bacteremia, diarrhea or fever. However, the balance between attenuation and over-attenuation must be considered as over-attenuation can lead to poor immunogenicity of the vaccine. In vivo studies indicate that various independent genetic defects can yield adequately attenuated *Salmonella* strains. The proper mutant carrier strains should be mutated to keep the balance between attenuation and virulence, to carry at least two independent attenuating mutations to minimize the hypothetical risk of reversion to virulence and to be genetically stable. For specific applications, it might be necessary to establish systems to minimize the possibility of

horizontal gene transfer from the vaccine strain to members of the mucosal flora or environmental microorganisms.

Mutants deficient in the biosynthesis of aromatic amino acids or purines, adenylate cyclase or cAMP receptor protein, carrying mutations affecting the global regulatory system *phoP phoQ* or lacking the DNA adenine methylase have been most widely characterized as carriers, reviewed in [14, 25].

In this study we investigated the intracellular behavior of different *S*. Typhimurium mutant strains in vitro, the invasion of HeLa cells, intracellular replication in RAW macrophages, and most important the expression and translocation of SPI2 effector SseJ antigen fusions. SsaV is a component of the SPI2-T3SS, required for secretion of most T3SS effectors [26]. The expression level of fusion protein is dependent on the promoter P_{sseJ} stength and efficacy of translocation due to efficiently translocated SseJ effector proteins which we described in previous chapters of this work. It was not a surprise that we did not observe significant difference in the expression and translocation in HeLa cells or RAW macrophage of SseJ protein fusions in all mutants except in the SPI2 deficient *ssaV* mutant strain. Also, the *ssaV* mutant strain showed significantly low intracellular replication in RAW macrophage, in contrast Δ*rfaH* replicated significantly more than other mutants in RAW macrophages.

Our results showed that Δ*aroA* mutants are significantly less virulent in vitro as well as less replication intracellulary than wild type strain. This finding is consistent with other independent results. *Salmonella* mutants auxotrophic for aromatic amino acids have reduced virulence for animals [27]. Moreover, *aro* mutants were so attenuated that these mutants are avirulent even for a sensitive model such as gnotobiotic pigs [28].

The extreme attenuation was expected as a reason why in at least some cases the *aro* mutants were not immunogenic and did not efficiently protect animals from subsequent infection, especially when highly virulent *Salmonella* strains were used for the challenge [29]. The reduced virulence of *aro* mutants has been explained by their inability to produce aromatic metabolites, mainly aromatic amino acids which are not freely available inside a host that is why *aro* mutants were expected to be incapable of intracellular replication. This has been supported by in vitro experiments in minimal media in which *aro* mutants did not grow as long as the aromatic amino acids or their precursors were added. Also *aro* mutants are defective in cytoplasmic or outer membrane or periplasmic space function, which could make them more sensitive to some components of the innate immune response [30]. Previously, *htrA, aroA and ssaV* mutations were used in construction of several *Salmonella* carrier strains to deliver heterologous antigens (reviewed in [14]). Recently it was found that, the regulated delayed expression of *rfaH* in an attenuated *Salmonella* vaccine enhances immunogenicity of outer membrane proteins and a heterologous antigen. As consequence *rfaH* mutations can be combined with other attenuating mutations to produce an attenuated live *Salmonella* carrier capable of delivering a protective antigen to induce protective immunity [19].

We are interested to evaluate the efficacy of these mutant carriers in stimulation of specific T cells. In this direction we transferred plasmid pWSK29 *PsseJ sseJ*::OVA::HA to tested strains. In vitro studies showed that s*ifA* mutants stimulated specific CD8 T-cells proliferation more than *aroA* and *rfaH* mutants when used to deliver OVA model

antigen to B3Z T-cells that have *lacZ* reporter. In the same experiment *S.*Typhimurium *sifA* mutant stimulated T-cell proliferation non significantly more than *htrA/purD* mutant. We suppose the efficient use of *S.* Typhimurium *sifA* mutant as carrier for vaccination, this finding consistent with previous results of our group[24]. In recent report, an independent group examined whether the muted CD8 T cell priming during infection with *Salmonella* is due to its poor intracellular proliferation or to its active inhibition of antigen presentation. They used various mutants and various in vitro and in vivo models of antigen presentation. They found that that *Salmonella* displays selectively poor intracellular proliferation, generates a poor intracellular antigen levels and MHC-peptide complexes and hence results in poor CD8T cell priming [31]. This finding may indicate that *sifA* mutant may show in vivo efficient CD8 T-cell stimulation. A systematic in vivo comparison between different mutants in stimulating immune responses is currently in process.

The translation of appraoches that work with *S.* Typhimurium for mice into *a S.* Typhi-based vaccine for humans is facing several obstacles. The pathogen *S.* Typhimurium provides a mouse model with systemic infections that resemble typhoid fever in humans, in contrast *S*. Typhi which cause systematic infection strictly in human not mices. Moreover, the mixed results obtained when translating the *S*.Typhimurium mutations in *S*.Typhi. For example, Δ*galE*, Δ*aroC*/Δ*aroD*, Δ*cya*/Δ*crp* and Δ*phoPQ* mutants of *S.* Typhimurium were completely attenuated in mice and induced protective immunity to challenge with wild-type *S.* Typhimurium. In contrast, *S.* Typhi Δ*galE*, Δ*aroC*/Δ*aroD* and Δ*cya*/Δ*crp* mutants were not sufficiently attenuated and caused significant reactogenicity in humans [14, 32].

AS a summary of this work, s*ifA* and *htrA*/*purD* mutant strains stimulated significantly specific CD8 T cells, taken in consideration that s*ifA* mutant strains replicated intracellulary more than *htrA*/*purD* which may be reflect on its in vivo immune stimulation behavior. *aroA* mutant strain is well known as save vaccine and as carrier for delivery of heterologous antigens but several reports showed its variable success in stimulation of effective immunity. Other tested mutants listed in this work showed less specific CD8 T cells. An in vivo analysis of the efficacy of different mutants in stimulating immune responses is currently in process.

4.5. References

1. Hansen-Wester, I. and M. Hensel, *Salmonella pathogenicity islands encoding type III secretion systems.* Microbes Infect, 2001. **3**(7): p. 549-59.
2. Hensel, M., *Salmonella pathogenicity island 2.* Mol Microbiol, 2000. **36**(5): p. 1015-23.
3. Hensel, M., *Evolution of pathogenicity islands of Salmonella enterica.* Int J Med Microbiol, 2004. **294**(2-3): p. 95-102.
4. Cornelis, G.R., *The type III secretion injectisome.* Nat Rev Microbiol, 2006. **4**(11): p. 811-25.
5. Gerlach, R.G. and M. Hensel, *Salmonella pathogenicity islands in host specificity, host pathogen-interactions and antibiotics resistance of Salmonella enterica.* Berl Munch Tierarztl Wochenschr, 2007. **120**(7-8): p. 317-27.
6. Kuhle, V. and M. Hensel, *Cellular microbiology of intracellular Salmonella enterica: functions of the type III secretion system encoded by Salmonella pathogenicity island 2.* Cell Mol Life Sci, 2004. **61**(22): p. 2812-26.
7. Yewdell, J.W. and J.R. Bennink, *Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses.* Annu Rev Immunol, 1999. **17**: p. 51-88.
8. Rüssmann, H., *Bacterial type III translocation: a unique mechanism for cytosolic display of heterologous antigens by attenuated Salmonella.* Int J Med Microbiol, 2003. **293**(1): p. 107-112.
9. Deiwick, J., et al., *Environmental regulation of Salmonella pathogenicity island 2 gene expression.* Mol Microbiol, 1999. **31**(6): p. 1759-73.
10. Spreng, S., G. Dietrich, and G. Weidinger, *Rational design of Salmonella-based vaccination strategies.* Methods, 2006. **38**(2): p. 133-43.
11. Garmory, H.S., K.A. Brown, and R.W. Titball, *Salmonella vaccines for use in humans: present and future perspectives.* FEMS Microbiol Rev, 2002. **26**(4): p. 339-53.
12. Mastroeni, P., et al., *Salmonella: immune responses and vaccines.* Vet J, 2001. **161**(2): p. 132-64.
13. Pasetti, M.F., M.M. Levine, and M.B. Sztein, *Animal models paving the way for clinical trials of attenuated Salmonella enterica serovar Typhi live oral vaccines and live vectors.* Vaccine, 2003. **21**(5-6): p. 401-18.
14. Becker, P.D., M. Noerder, and C.A. Guzman, *Genetic immunization: bacteria as DNA vaccine delivery vehicles.* Hum Vaccin, 2008. **4**(3): p. 189-202.
15. Xiong, G., et al., *Novel cancer vaccine based on genes of Salmonella pathogenicity island 2.* Int J Cancer, 2010. **126**(11): p. 2622-34.
16. Angelakopoulos, H. and E.L. Hohmann, *Pilot study of phoP/phoQ-deleted Salmonella enterica serovar typhimurium expressing Helicobacter pylori urease in adult volunteers.* Infect Immun, 2000. **68**(4): p. 2135-41.
17. Toso, J.F., et al., *Phase I study of the intravenous administration of attenuated Salmonella typhimurium to patients with metastatic melanoma.* J Clin Oncol, 2002. **20**(1): p. 142-52.

18. Carter, J.A., et al., *O-antigen modal chain length in Shigella flexneri 2a is growth-regulated through RfaH-mediated transcriptional control of the wzy gene.* Microbiology, 2007. **153**(Pt 10): p. 3499-507.
19. Kong, Q., et al., *Regulated delayed expression of rfaH in an attenuated Salmonella enterica serovar typhimurium vaccine enhances immunogenicity of outer membrane proteins and a heterologous antigen.* Infect Immun, 2009. **77**(12): p. 5572-82.
20. Hindle, Z., et al., *Characterization of Salmonella enterica derivatives harboring defined aroC and Salmonella pathogenicity island 2 type III secretion system (ssaV) mutations by immunization of healthy volunteers.* Infect Immun, 2002. **70**(7): p. 3457-67.
21. Curtiss, R., 3rd, et al., *Salmonella enterica serovar typhimurium strains with regulated delayed attenuation in vivo.* Infect Immun, 2009. **77**(3): p. 1071-82.
22. Xin, W., et al., *Analysis of type II secretion of recombinant pneumococcal PspA and PspC in a Salmonella enterica serovar Typhimurium vaccine with regulated delayed antigen synthesis.* Infect Immun, 2008. **76**(7): p. 3241-54.
23. Kong, W., et al., *Regulated programmed lysis of recombinant Salmonella in host tissues to release protective antigens and confer biological containment.* Proc Natl Acad Sci U S A, 2008. **105**(27): p. 9361-6.
24. Xu, X., et al., *Efficacy of intracellular activated promoters for generation of Salmonella-based vaccines.* Infect Immun, 2010. **78**(11): p. 4828-38.
25. Shi, H., et al., *Live recombinant Salmonella Typhi vaccines constructed to investigate the role of rpoS in eliciting immunity to a heterologous antigen.* PLoS One, 2010. **5**(6): p. e11142.
26. Browne, S.H., et al., *Identification of Salmonella SPI-2 secretion system components required for SpvB-mediated cytotoxicity in macrophages and virulence in mice.* FEMS Immunol Med Microbiol, 2008. **52**(2): p. 194-201.
27. Hoiseth, S.K. and B.A. Stocker, *Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines.* Nature, 1981. **291**(5812): p. 238-9.
28. Trebichavsky, I., et al., *Attenuated aroA Salmonella enterica serovar Typhimurium does not induce inflammatory response and early protection of gnotobiotic pigs against parental virulent LT2 strain.* Vaccine, 2006. **24**(20): p. 4285-9.
29. Nnalue, N.A., *Mice vaccinated with a non-virulent, aromatic-dependent mutant of Salmonella choleraesuis die from challenge with its virulent parent but survive challenge with Salmonella typhimurium.* J Med Microbiol, 1990. **31**(4): p. 225-33.
30. Sebkova, A., et al., *aro mutations in Salmonella enterica cause defects in cell wall and outer membrane integrity.* J Bacteriol, 2008. **190**(9): p. 3155-60.
31. Albaghdadi, H., et al., *Selectively reduced intracellular proliferation of Salmonella enterica serovar typhimurium within APCs limits antigen presentation and development of a rapid CD8 T cell response.* J Immunol, 2009. **183**(6): p. 3778-87.
32. Bumann, D., et al., *Recombinant live Salmonella spp. for human vaccination against heterologous pathogens.* FEMS Immunol Med Microbiol, 2000. **27**(4): p. 357-64.

Chapter 5

Discussion

5. Discussion

5.1 The immune response against intracellular bacteria

Live attenuated strains of *Salmonella* that synthesize and secrete foreign antigens are being developed as vaccines against a number of infectious bacterial, viral and parasitic diseases and for treatment of cancer. Moreover, the use of live attenuated *Salmonella* to present recombinant antigens to the immune system is an attractive strategy for the construction of multivalent vaccines [1-3]

The CD4 T cells are specific for peptides presented by MHC class II molecules which translocate antigens from the phagosomal compartment to the cell surface. Therefore, intracellular bacteria remaining in the phagosome such as *Salmonella* are a preferred antigenic target for CD4 T cells. The CD4 T cells, also termed T helper cells (Th cells), produce various cytokines that have a major influence in ensuing the immune response. The so-called Th2 cells activate eosinophils and basophils and are critical for B cell maturation into antibody producing plasma cells. Accordingly, Th2 cells are responsible for control of helminthic infections, bacterial infection and for toxin neutralization. The so-called Th1 cells produce cytokines such as interferon-γ (IFN-γ) and interleukin 2 (IL-2), which activates cytolytic T lymphocytes (CTL) and macrophages. In contrast, CD8 T cells recognize antigenic peptides presented by MHC class I molecules which transport antigens from the cytoplasm to the cell surface. Accordingly, CD8 T cells are responsible for the response against viral pathogens and also for those intracellular bacteria which egress into the cytoplasm. However, *L. monocytogenes* which release to cytoplasm of APC also can stimulate CD4 T cells, and most microbes remaining in the phagosome as *Salmonella* can stimulate CD8 T cells [1].

Dendritic cells are the most professional APC and are the key link between innate and adaptive immunity. Immature DC can internalize and process *Salmonella* for peptide presentation on MHC-II as well as MHC-I, initiating an immune response. However, *Salmonella* remain confined in vacuolar compartments, they can be processed for MHC-I presentation of bacterial antigens to CD8+ T cells [4]. Due to the cytotoxicity to infected cells, DC can either present directly or indirectly the *Salmonella* antigens. Direct presentation of antigens to T cells upon phagocytic processing of *Salmonella* that does not induce their death and later indirectly present bacterial antigens to T cells as by engulfing antigenic material from neighboring cells that have undergone *Salmonella*-induced apoptotic death [5].

5.2 *Salmonella* as carrier for delivering heterologous antigens

Live attenuated strains of *Salmonella* that synthesize and secrete foreign antigens are being developed as vaccines for a number of infectious diseases by bacterial, viral and parasitic pathogens and against cancer. The use of live attenuated *Salmonellae* to deliver recombinant antigens to the immune system is an attractive strategy for the construction of multivalent vaccines. *Salmonella*-based vaccines provide a number of advantages over other antigen delivery strategies, the most important that *Salmonella* vaccine delivering heterologous antigens stimulate innate immunity and also activate both the arms of the adaptive immune system by which they exert efficient immune response.

The use of intracellular bacteria that have access to the host cell cytosol may allow a more specific targeting of DNA vaccine vectors to professional APC. Due to its invasive properties and their preference for macrophages and DC, *Salmonella* are ideally suited for a more direct targeting of DNA vaccines to professional APC [6]. Recombinant *Salmonella* should be metabolically attenuated in order to die and lyse in these cells, consequently the plasmid molecules are liberated to transfect host cells [38]. The plasmid DNA is released into the phagosome or subsequently enter the nucleus and plasmid-encoded antigens can be expressed [2]. At the same time, the invaded cells are activated by bacterial endotoxins and other bacterial components which stimulate innate immune responses. This might lead to direct and indirect presentation of antigen in the context of MHC I and MHC II molecules to CD8 and CD4 T cells, thereby eliciting cellular and promoting humoral immune responses.

The main difference between “protein delivery” and “DNA delivery” strategies is that in the first one, antigens are delivered in form of protein synthesized by the bacterial carrier, whereas in the latter bacterial carrier delivers plasmid DNA into the eukaryotic target cell, where it is translated and also post-translationally modified. The strategy using live attenuated *Salmonella* to deliver plasmid-encoded antigens under the transcriptional control of eukaryotic promoters has been used successfully in vaccination against bacteria, viruses, fungi, parasites and even against cancer as we described in details in previous sections of this work, rewiwed in [7]. Recently, a novel bacterial delivery approach has been described “RNA delivery” and tried in *Listeria*. They used *L. monocytogenes* strains that produce and deliver plasmid-encoded mRNA of a candidate protein under the control of a T7 promoter variant. They showed that

mRNA encoding the model antigen OVA delivered by *L. monocytogenes* carriers leads to presentation of OVA in the context of major histocompatibility complex (MHC) class I molecules in vivo and to induction of an specific CD8 T-cell response [8-9]. This approach is tested in *Listeria* but also can be translated to *Salmonella* carrier strains as a new strategy.

5.3. *Salmonella* TTSS-mediated heterologous antigen delivery

As a consequence of intracellular location of *Salmonella*, proteins delivered are trapped in the phagolysosome and presented to the immune system preferentially in the context of MHC class II molecules. There have been several approaches in the literature for bioengineering *Salmonella* strains which could deliver antigens directly to the intracellular compartment instead of the phagosome. The use of intracellular bacteria that have access to the host cell cytosol may allow a more specific targeting of DNA vaccine vectors to professional APC. Due to its invasive properties and their preference for macrophages and DC, *Salmonella* are ideally suited for a more direct targeting of DNA vaccines to professional APC [1, 6]. The strategy of using live attenuated *Salmonella* to deliver plasmid-encoded antigens under the transcriptional control of eukaryotic promoters has been used successfully in vaccination [7]. *Salmonella* carrier DNA vaccines have been employed in prophylactic immunization against bacteria, viruses, fungi, parasites and even against cancer [2, 10-11].

Localization within the SCV prevents delivery of expressed foreign proteins to the MHC class I-restricted antigen presentation pathway and hinders the use of *Salmonella* as vaccine carrier to induce specific CD8 T-cells which is crucial for protection against

viruses, intracellular bacteria, and tumors. Heterologous antigens can be expressed in *Salmonella* as fusions with recombinant or native proteins. This approach has been used mainly to direct the expression of the desired antigen to a particular location of the bacterial cell and increase the immunogenicity of foreign antigens by fusing them to proteins that could exert a carrier effect [12]. Towards this end *Salmonella* type III secretion system (T3SS)-mediated translocation can be used for efficient delivery of heterologous antigens to the cytosol of antigen-presenting cells leading to prominent CD8 T-cell responses [13-15].

Salmonella translocates type III effector proteins into the host cell cytoplasm mediated either by the SPI1-encoded T3SS from the extracellular location and from the SCV at an early stage of invasion, or by the SPI2-encoded T3SS from within the SCV at a significantly later stage during intracellular life [16-18]. *Salmonella* T3SS-mediated translocation can be used for efficient delivery of heterologous SPI1-antigen or SPI2-antigen fusions to the cytosol of APC, leading to prominent CD8 T-cell priming in orally immunized mice [13].

SPI2 effector proteins, especially those which are expressed only when the *Salmonella* are inside the host cell DCs and macrophage [19-20] have been considered as promising carrier candidates for antigen delivery to MHC-I pathway. Vaccination of mice with *Salmonella* strains expressing *sifA*/*iap* led to the induction of P60-specific CD8 T cells [21]. Previously, our group focused on testing and comparing several SPI2-antigen fusions and used different antigen models that include P60, Ova albumin, Listerolysin O (LLO) and tumor-associated antigen survivin.

5.4. Strategy and Optimization

In *Salmonella*-mediated DNA transfer strategy, recombinant *Salmonella* should be metabolically attenuated to lyse and die in these cells, consequently the plasmid molecules are liberated to transfect host cell [22]. We hypothesize that the use of a strategy of T3SS-mediated heterologous antigen delivery has advantage over *Salmonella*-mediated DNA transfer strategy. By using the strategy of T3SS-mediated heterologous antigen delivery, the immune response will be induced due to the translocation of SPI2 effector proteins fusions. Moreover the carrier strains can transport the SPI2 fusion proteins via T3SS regardless the lysis of carrier strain which may be helpful to use transfer more lysis resistant immunogenic carrier strains (**Fig 5.1**).

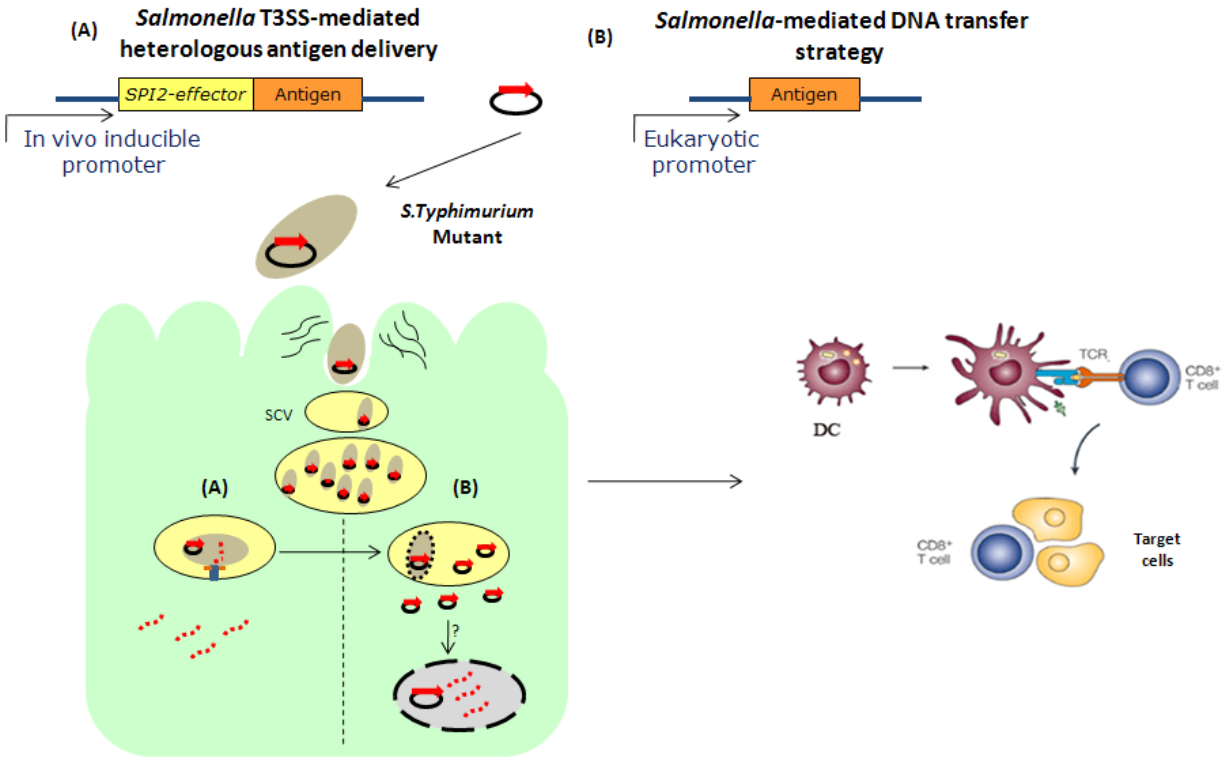


Fig 5.1. *Salmonella* T3SS-mediated heterologous antigen delivery strategy versus vaccination mediated by DNA transfer strategy. (A) In the *Salmonella* T3SS-mediated heterologous antigen delivery strategy the antigen fusion protein will be delivered by using suitable carrier strain to cytosol of the infected host cell either via T3SS or lysis of carrier strains. (B) In the *Salmonella* mediated DNA transfer strategy, the antigen fusion will be delivered to cytosol after lysis of carrier strain and release of DNA which later will transfect the host DNA and synthesis the antigen proteins.

The use of T3SS-mediated heterologous antigen delivery as a strategy for vaccination may result in abundant antigen proteins in cytosol which in consequence lead to efficient presentation and efficient specific T-cells stimulation in particular CD8 T-cells.

For optimization of *Salmonella*-based vaccines, we hypothesized that the efficient immune response mainly requires T cell induction which depends on the strength of antigen presentation. The antigen presentation strength by its role depends on antigen access to the respective processing compartment, the antigen-processing efficacy, and antigen abundance. In other words, the efficacy of *Salmonella*-based vaccines is mainly depending on four factors at least; (i) the strength of fusion protein expression, (ii) the efficacy of translocation of SPI2-effector fusion protein, (iii) the use of suitable carrier and, finally (iv), the stability of construct itself.

By using TTSS-mediated heterologous antigen delivery as strategy, attenuated strains of *S.* Typhimurium expressing chimeric proteins engaged by SseF, another SPI2 effector, under control of an interacellular activated promoters efficiently induced both CD4 and CD8 T cells [15, 23-24]. The efficacy of this strategy was tested by other

groups, as vaccination of mice with *Salmonella* strains expressing SifA/P60 and SspH2/p60 led to the induction of P60-specific CD8 T cells [14, 21].

5.5. The expression level

Several factors such as the cellular location of the expressed antigen may affect the magnitude and type of immune response induced against an expressed antigen. This influence may be due to degradation of the antigen in certain cellular compartments or modification of the antigen presentation to the immune system. While efficient antigen display is an indispensable requirement for induction of T cells, the strength of antigen presentation might depend on antigen abundance. Thus it is hypothesized that more the antigen expression, more is the immunogenicity of antigens. Surprisingly, the highest levels of expression are not necessarily required for an elevated immune response to heterologous antigens [25].This finding by our group was consistent with other independent studies by other groups [26]. As with any foreign antigen expression system in bacteria, certain proteins may be toxic to carrier bacteria especially when they are expressed at high levels.

From previous results of our group, the expression levels of genes encoding SPI2 effector proteins are highly divergent and also amount of translocated effector protein. Using in vitro conditions for induction of the SsrAB virulon, as well as analyses of intracellular bacteria, pronounced differences was observed in the expression levels of genes encoding the SPI2-T3SS effector and P_{sifA} and P_{sseJ} both showed the highest expression levels [27]. But by comparison of various promoters of the SsrAB regulon for

expression of heterologous antigen fusions to SSPI2-T3SS effector protein SseF, P_{sifB} was identified as a promoter with superior stimulation of immune responses to vaccination [25].

Chromsomal constructs are more stable and have lower expression levels than plasmid constructs. Our and other's findings proved that high expression levels are not mandatory for strong immune response. In this direction, there are some trails to construct chromosomal foreign antigen fusion prior for using them in vaccination. An efficient and robust methods that may be used to construct recombinant antigen-expressing *S*. Typhimurium [28] or *S*. Typhi [29] strains were developed. For example, three different influenza antigen expression cassettes as well as a green fluorescent protein gene reporter into four different loci were inserted on the Ty21a chromosome. that strong inducible expression of all four heterologous genes was confirmed by fluorescent microscopy and Western blotting analysis [29].

5.6. The efficacy of translocation

A further parameter for rational design will be the selection of optimal T3SS proteins as vehicles for delivery of the heterologous antigen. In the present as well as in previous approaches [30-31], we used SPI2-T3SS effector SseF as fusion partner for translocation of vaccine antigens. SseF, as well as fusion proteins with SseF, are targeted to endosomal membranes and remain membrane-associated for extended periods of time [32-33]. In this study, we made a systematic comparison of the efficacy of various SPI2-T3SS effectors in translocating heterologous vaccine antigens and in stimulating immune responses.

To quantify the translocation of fusion proteins, the signal intensities for the fluorescence channel for SPI2-effector-antigen staining were quantified. We next compared the antigen-dependent simulation of T-cells after uptake of recombinant Salmonella strains with expression of recombinant antigens. BM-DC were infected with S. enterica serovar Typhimurium attenuated carrier strain MvP728 deficient in *purD* and *htrA* **[15]** harboring plasmids for the expression of SPI2-effector::OVA::HA under the control of P_{sseA}. Subsequently, infected BM-DC were either incubated with the T cell hybridoma that recognizes the OVA-derived SIINFEKL epitope B3Z T-cell line or OVA-specific T-cells isolated from OT-I or OT-II transgenic mice as described in previous chapters (**Fig 5.2**).

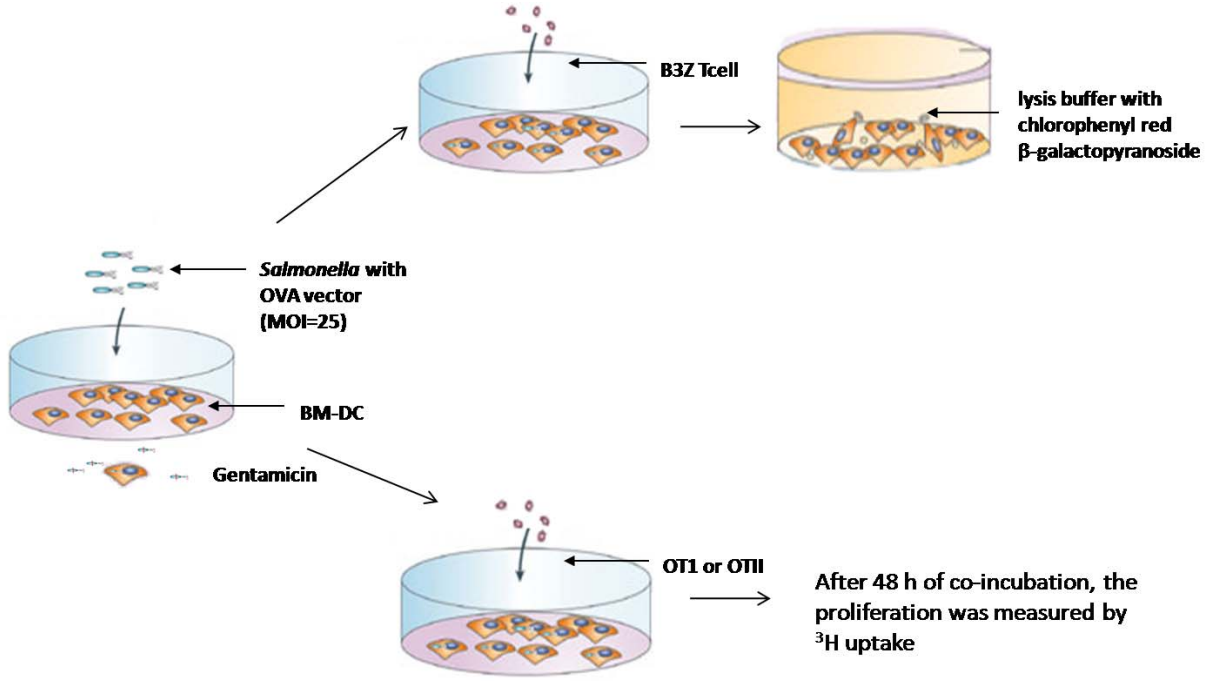


Fig 5.2. Antigen presentation assay.

SPI2-effectors SseF, SifA, SseJ, SteC and SseL are localized inside host cells on the membrane of SCV and SIFs [17]. These SPI2 effector proteins which are expressed only when the *Salmonella* is inside host cells such as DCs and macrophage were considered as promising carrier candidates for antigen delivery to MHC-I pathway. Our results showed that the antigen proteins significantly processed and presented for MHC class I complex more than MHCII complex. It is consistent with the finding of other independent group, that a subgroup of SPI2-T3SS effector proteins interfere with MHC-II presentation in DCs. [34].

Purified SseJ has deacylase and acyltransferase activity in vitro, and SseJ catalytic-triad mutants that reduce deacylase activity are attenuated for virulence in mice, indicating that SseJ enzymatic activity contributes to intracellular replication in host tissues [35]. SseJ antigen fusion protein stimulated significantly not only in vitro T cell proliferation but also in vivo specific CTL. From our current results, we would suggest SseJ as the optimal SPI2 effector for the generation of expression cassettes for recombinant antigens in *Salmonella.*

SPI-2-dependent F-actin meshwork formation depends on the kinase activity of SteC, which resembles more closely eukaryotic than prokaryotic kinases [36]. SteC antigen fusion protein showed a significant efficiency in vitro. In contrast to in vitro results, SteC fusion proteins showed a very weak in vivo T cell response. The high expression level of SteC proteins under control of P_{sseA} promoter should be taken in consideration as it might be toxic to cells and in consequence reducing the immune response.

SifA is required for the formation of *Salmonella*-induced filaments (Sif) and maintenance of the vacuolar membrane that surrounds replicating bacteria and is essential for virulence in the mouse model of systemic *Salmonella* infection [37]. SseL functions as a deubiquitinase that modulates host inflammatory responses. *sseL* mutant strains is attenuated for virulence in mice[38].The low level expression of SseL and SifA effectors under control of P_{sseA} clearly negatively affect the efficacy of translocation in vitro. SseL effector protein showed weaker stimulation of specific T cells in vivo and in vitro. While SifA fusion protein in contrast to in vitro stimulated significant specific CTLs in vivo. The synthesis level of the effector proteins under intracellular conditions may vary considerably between in vitro and in vivo conditions and the reasons for the different levels of immune stimulation are not clear.

5.7. Cytosolic translocated SPI2 effectors

Focusing on the cytosolic translocated SPI2 effectors might be beneficial in inducing an efficient immune response. Interestingly, *Yersinia* outer protein E (YopE) for heterologous antigen delivery by the *Salmonella* TTSS was analyzed. YopE was fused to the immuno-dominant T cell antigens listeriolysin O (LLO) and p60 from Listeria monocytogenes. *Salmonella* secretes and translocates these chimeric proteins into the cytosolic compartments of host cells but not into endosomal compartments. This led to efficient MHC class I-restricted antigen presentation of listerial nonamer peptides. Mice orally vaccinated with attenuated *S.* Typhimurium expressing translocated hybrid YopE proteins revealed efficient and specific CD8 T-cell response, protecting mice against a challenge with *L. monocytogenes*. These findings suggest that YopE is a versatile

carrier molecule for T3SS-mediated foreign antigen delivery by *Salmonella* vaccine strains [39-40].

SspH2 co-localizes with the polymerizing actin cytoskeleton in the host cell and interacts with filamin and profiling, and localized in cytoplasm of the host cell [17]. SspH2/p60 hybrid proteins led to concomitant p60-specific CD4 and CD8 T-cell priming, indicating that the SPI2 effector protein SspH2 might be an attractive carrier molecule for antigen delivery when T-cell immune responses against complex microbes or tumors are needed [14, 21]. In this work we focused on the membranr-associated SPI2 effctors, we are planning to make a systematic comparison of cytosolic and membrane-associated SPI2 effectors as a vehicle for foreign antigen delivery.

5.8. Fusions of intracellular activated promoters and SPI2 effectors for optimization of *Salmonella*-based vaccines

For optimization of the *Salmonella* based vaccines we tested the different SPI2 membrane-associated effectors under control of various in vivo-induced promoters. P_{sifB} has been suggested as the optimal promoter for the generation of expression cassettes for recombinant antigens in *Salmonella* [25]. From our current results we suggest SseJ as the most efficient SPI2 effector. A systematic comparison between different cassettes described previously in chapter 3 was done. Expression cassettes of *sseJ*, *sseL*, *steC*, *sifA* or *sseF* fusion antigens under control of P_{sifB}, P_{sifA}, P_{sseA} or P_{sseJ} were analyzed in vitro. P_{sifB}::*sseJ* showed the highest specific T-cell stimulation. This observation is in agreement with our hypothesis. Moreover P_{sifB}::*sseJ* also showed a significant in vivo specific CTL stimulation in comparison to P_{sifB}::*sseF* or P_{sseA}::s*seJ*

(data not published). A detailed systematic analysis of the efficacy of P_{sifB}::*sseJ* using different antigen models is currently in process.

5.9. Role of the carrier strain

A mandatory requirement for the use of live recombinant *Salmonella* for vaccination is a sufficient attenuation in virulence to prevent undesired side effects like bacteremia, diarrhea or fever. However, a critical balance between attenuation and over-attenuation must be considered as over-attenuation can lead to poor immunogenicity of the vaccine. In vivo studies indicate that various independent genetic defects can yield adequately attenuated *Salmonella* strains. The choice of the attenuating mutations should (i) keep the balance between attenuation and virulence, (ii) carry at least two independent attenuating mutations to minimize the hypothetical risk of reversion to virulence and (iii) be genetically stable. For specific applications, it might be necessary to establish systems to minimize the possibility of horizontal gene transfer from the vaccine strain to members of the mucosal flora or environmental microorganisms. Mutants deficient in the biosynthesis of aromatic amino acids or purines, adenylate cyclase or cAMP receptor protein, carrying mutations affecting the global regulatory system *phoP phoQ* or lacking the DNA adenine methylase have been most widely characterized as carriers. Attenuated strains of *Salmonella* have been approved for use in human and in veterinary medicine [41-42].

The pathogen *S.* Typhimurium provides a mouse model with systemic infections that resemble typhoid fever in humans and this model is also useful for the preclinical test of candidate carrier strains. The *aroA* mutant strains were proven to be safe and are

widely used as carrier for vaccination [41]. Other trails were done to utilize different mutations to increase the immunogenicity of heterologous antigens and at the same time achieve required attenuation. *rfaH* mutant *S.* Typhimurium vaccine enhanced immunogenicity of heterologous antigens and was sufficiently attenuated in mice [43]. *htrA/purD* double mutant carrier strain showed an efficient immune response [15]. Mutations in specific *Salmonella* virulence genes, such as SPI2 genes, were investigated in order to develop efficient carriers [41, 44-45].

Our In vitro studies showed that *sifA* mutants as well as *htrA/purD* mutants stimulated specific CD8 T-cell proliferation more efficiently than *aroA* mutant when used to deliver OVA antigen model to B3Z T-cells. The *sifA* mutants replicate significantly more inside RAW macrophage than *htrA/purD* mutants which may reflected on its immunogenicity in vivo. *sifA* mutant strains showed less in vivo invasion and intracellular replication in comparison to wild type strain. The in vivo attenuation of virulence of the *sifA* mutant is not directly linked to the mislocalization of the SCV but due to the release of bacteria into the macrophage cell cytoplasm, where is toxic to *Salmonella* [46].The *rfaH* mutant of *S.* Typhimurium did not efficiently stimulate T cell proliferation. *rfaH* mutant strains showed the capability to replicate inside macrophage more than *sifA* or *htrA/purD* strains. There is no significant difference observed in expression level and translocation of SPI2 effector SseJ under control of P_{sseJ} promoter in all mutant strains used in this study except for the *ssaV* mutant strain. A systematic in vivo comparison between immune response triggered by the various carrier strains has to be performed by future studies.

5.10. *S.* Typhi mutant carrier

Translating the analyses of *S.* Typhimurium in murine models mice to *S.* Typhi-based vaccines for humans has led to mixed results. For example, Δ*galE*, Δ*aroC*/Δ*aroD*, Δ*cya*/Δ*crp* and Δ*phoPQ* mutants of *S.* Typhimurium were completely attenuated in mice and induced protective immunity to challenge with wild-type *S.* Typhimurium. In contrast, *S.* Typhi Δ*galE*, Δ*aroC*/Δ*aroD* and Δ*cya*/Δ*crp* mutants were not sufficiently attenuated and caused significant reactogenicity in humans [41, 47]. Taking into consideration the higher virulence behavior of *S.* Typhi than *S.* Typhimurium, it is needed to devise new strategies to sufficiently attenuate *S. Thypi* and overcome the reactogenicity. In this direction *S.*Typhi triple mutations Δcya/Δcrp/Δcdt and ΔaroC/ΔaroD/Δhtra were reported to be safe and immunogenic in humans [48-49]. It was not a surprising to find that mutations which highly attenuated *S.*Typhimurium were safe and immunogenic when transferred to *S.*Typhi. Further, Δ*aroC*/Δ*ssaV S.* Typhi Ty2 mutant was reported to be safe and immunogenic in humans, while *S.*Typhimurium mutant was strongly attenuated and showed variable humoral response [50]. Another example includes a *phoP* mutant of *S.* Typhimurium, which is completely attenuated in mice, led to development of a safe and immunogenic Δ*phoPQ S.* Typhi (Ty800) vaccine strain [51-52].

Nonreactogenic *S.*Typhi strains are often hyperattenuated and induce poor immune responses, even to *Salmonella* antigens themselves [52]. Using live *S.* Typhi vectors in humans still face major difficulty in balancing between immunogenicity to vectored antigen and reactogenicity. It is believed that the recombinant *S.* Typhi strains do not colonize lymphoid tissues to a sufficient level to stimulate a strong immune response

against the foreign antigen. The host immune system may also be stimulated to the *Salmonella* carrier, inducing a strong response against *Salmonella* antigens instead of the vectored antigen[42]. Another obstacle, the lack of an animal model due to *S.* typhi's strict human host specificity hinders significantly the understanding of its pathogenesis and its using as a carrier for vaccination.

A series of vaccine candidates based on attenuation of *S* Typhi by deletions in the aroC, aroD, and htrA genes designated "CVD 908," "CVD 908-htrA," and "CVD 909," have been in the last decade developed and tested in volunteers with variable success [48]. It might be useful to invistigate those clinically efficient mutant strains as carriers to deliver foreign antigens. For example, mutant strain CVD 908-*htrA* was genetically engineered for stable plasmid-based for vaccination against anthrax [53].

To our knowledge, there is no studies answered the question, are effector proteins in *S*.Typhi are equally effective as they are in *S*.Typhimurium and are promters working identically. Galen et al, suggested that antibody responses to antigens delivered by *S*.Typhi live carriers are inversely related to the metabolic burden imposed by expression of the foreign antigen and the immune responses can be improved when antigens are expressed from low-copy-number plasmids and exported out of the cytoplasm of less attenuated live vectors [54]. This finding is compatible with other findings in *S*.Typhimurium, as the higher immune response to forign antigens were not accompanied with higher expression levels [25-26].

5.11. Concluding remarks and Outlook

In this work we aimed to optimize *Salmonella* as a carrier for vaccination. The efficacy of *Salmonella*-based vaccines depends on several factors. First, we hypothesized that the use of T3SS-mediated antigen delivery strategy is more advantageous than using the *Salmonella* DNA carrier strategy. This hypothesis needs a realistic and systematic analysis to compare experimentally between two strategies. The antigen abundance is a crucial factor; meaning not only expression levels but also translocation efficacy. While efficient antigen display is an indispensable requirement for induction of T cells, the strength of antigen presentation might depend on antigen abundance. Thus, it is hypothesized that the higher the antigen expression, the higher is the immunogenicity of antigens. Surprisingly, our group's current results showed that the highest levels of expression are not necessarily required for an elevated immune response to heterologous antigens. P_{sifB}, moderate strength promoter, is the optimal promoter for the generation of expression cassettes for recombinant antigens in *Salmonella*. As a consequence the stable chromosomal fusions with their low expression levels may induce an effective immune responses and should be tested in comparison to low copy plasmids.

The selection of optimal T3SS proteins as vehicles for delivery of the heterologous antigen is an important additional parameter. Subcellular localization of the translocated protein in APC and the route of processing for presentation is an important parameter that will affect the efficacy of translocated recombinant antigens. The proteins of the subgroup of SPI2-T3SS effectors investigated in this study are all characterized by their association with endosomal membranes after translocation into host cells and the rather

long half-life in host cells. The antigen abundance mainly depends on transloacation efficacy of SPI2 effectors to cytosol which in turn will be processed and presented for MHC class I complex more than MHC II complex..

SseJ fusion proteins were efficiently translocated and stimulated specific CD8 T-cells in vitro and in mice. SifA and SseL fusion proteins were lower efficiently translocated and also showed lower efficiently specific CTL stimulation epically SseL effector proteins.SteC fusion proteins were efficiently translocated and stimulated specific CD8 T cells in vitro. In contrast to in vivo results this showed a great decrease in its efficacy. The higher expression levels of SteC fusion proteins may be toxic to carrier strains which adversely affect its efficacy in vivo. As a conclusion the efficacy of translocation of SPI2 effector proteins is a conditionally required for an elevated immune response to heterologeous antigens. SseJ is the optimal SPI2 effector as a vehicle for recombinant antigens for generation of *Salmonella*-based vaccines. Cytosolic translocated SPI2 effectors might be beneficial in inducing an efficient immune response. It is needed to establish a systematic comparison between membrane-associated SPI2 effectors and cytosolic translocted SPI2 effectors as a vehicle for recombinant antigens delivery.

P_{sifB}::*sseJ* showed an efficient immune response in vitro and in vivo. This result meets with our hypothesis by using the most efficient SPI2 effector SseJ and most efficient promoter P_{sifB}. *sifA* mutant strain is a promising carrier strain. The systematic in vitro comparison between *SifA* mutants and *htrA/purD* mutants showed that s*sifA* mutants stimulated specific CD8 T cells similarly to *htrA/purD* mutants. Moreover *sifA* mutants were significantly replicate inside raw macrophage more than *htrA/purD* which may

reflect on its in vivo activity. The in vivo testing is planned to show the efficacy of *sifA* mutant as a carrier strain.

All these optimizations may be helpful to construct of multivalent *Salmonella* based vaccines, especially if it is possible to use attenuated *S.* Typhi mutants. This work can introduce efficient vaccines against cancer, virus, parasites and intracellular bacteria which consequently reflect on the human health and the economy especially in developing countries.

5.12. References

1. Mollenkopf, H., G. Dietrich, and S.H. Kaufmann, *Intracellular bacteria as targets and carriers for vaccination.* Biol Chem, 2001. **382**(4): p. 521-32.
2. Loessner, H. and S. Weiss, *Bacteria-mediated DNA transfer in gene therapy and vaccination.* Expert Opin Biol Ther, 2004. **4**(2): p. 157-68.
3. Medina, E. and C.A. Guzman, *Use of live bacterial vaccine vectors for antigen delivery: potential and limitations.* Vaccine, 2001. **19**(13-14): p. 1573-80.
4. Yrlid, U. and M.J. Wick, *Antigen presentation capacity and cytokine production by murine splenic dendritic cell subsets upon Salmonella encounter.* J Immunol, 2002. **169**(1): p. 108-16.
5. Sundquist, M., A. Rydstrom, and M.J. Wick, *Immunity to Salmonella from a dendritic point of view.* Cell Microbiol, 2004. **6**(1): p. 1-11.
6. Loessner, H., et al., *Improving live attenuated bacterial carriers for vaccination and therapy.* Int J Med Microbiol, 2008. **298**(1-2): p. 21-6.
7. Schoen, C., et al., *Bacteria as DNA vaccine carriers for genetic immunization.* Int J Med Microbiol, 2004. **294**(5): p. 319-35.
8. Schoen, C., et al., *Bacterial delivery of functional messenger RNA to mammalian cells.* Cell Microbiol, 2005. **7**(5): p. 709-24.
9. Loeffler, D.I., et al., *Comparison of different live vaccine strategies in vivo for delivery of protein antigen or antigen-encoding DNA and mRNA by virulence-attenuated Listeria monocytogenes.* Infect Immun, 2006. **74**(7): p. 3946-57.
10. Vassaux, G., et al., *Bacterial gene therapy strategies.* J Pathol, 2006. **208**(2): p. 290-8.
11. Shahabi, V., et al., *Live, attenuated strains of Listeria and Salmonella as vaccine vectors in cancer treatment.* Bioeng Bugs, 2010. **1**(4): p. 235-9.
12. Mastroeni, P., et al., *Salmonella: immune responses and vaccines.* Vet J, 2001. **161**(2): p. 132-64.
13. Russmann, H., *Bacterial type III translocation: a unique mechanism for cytosolic display of heterologous antigens by attenuated Salmonella.* Int J Med Microbiol, 2003. **293**(1): p. 107-12.
14. Panthel, K., et al., *Salmonella type III-mediated heterologous antigen delivery: a versatile oral vaccination strategy to induce cellular immunity against infectious agents and tumors.* Int J Med Microbiol, 2008. **298**(1-2): p. 99-103.
15. Xiong, G., et al., *Novel cancer vaccine based on genes of Salmonella pathogenicity island 2.* Int J Cancer, 2010. **126**(11): p. 2622-34.
16. Hansen-Wester, I. and M. Hensel, *Salmonella pathogenicity islands encoding type III secretion systems.* Microbes Infect, 2001. **3**(7): p. 549-59.
17. Kuhle, V. and M. Hensel, *Cellular microbiology of intracellular Salmonella enterica: functions of the type III secretion system encoded by Salmonella pathogenicity island 2.* Cell Mol Life Sci, 2004. **61**(22): p. 2812-26.
18. Hensel, M., *Evolution of pathogenicity islands of Salmonella enterica.* Int J Med Microbiol, 2004. **294**(2-3): p. 95-102.
19. Hensel, M., *Salmonella pathogenicity island 2.* Mol Microbiol, 2000. **36**(5): p. 1015-23.

20. Abrahams, G.L. and M. Hensel, *Manipulating cellular transport and immune responses: dynamic interactions between intracellular Salmonella enterica and its host cells.* Cell Microbiol, 2006. **8**(5): p. 728-37.
21. Panthel, K., et al., *Salmonella pathogenicity island 2-mediated overexpression of chimeric SspH2 proteins for simultaneous induction of antigen-specific CD4 and CD8 T cells.* Infect Immun, 2005. **73**(1): p. 334-41.
22. Darji, A., et al., *Oral delivery of DNA vaccines using attenuated Salmonella typhimurium as carrier.* FEMS Immunol Med Microbiol, 2000. **27**(4): p. 341-9.
23. Husseiny, M.I. and M. Hensel, *Evaluation of an intracellular-activated promoter for the generation of live Salmonella recombinant vaccines.* Vaccine, 2005. **23**(20): p. 2580-90.
24. Husseiny, M.I. and M. Hensel, *Evaluation of Salmonella live vaccines with chromosomal expression cassettes for translocated fusion proteins.* Vaccine, 2009. **27**(28): p. 3780-7.
25. Xu, X., et al., *Efficacy of intracellular activated promoters for generation of Salmonella-based vaccines.* Infect Immun, 2010. **78**(11): p. 4828-38.
26. Pathangey, L., et al., *Effect of expression level on immune responses to recombinant oral Salmonella enterica serovar Typhimurium vaccines.* Vaccine, 2009. **27**(20): p. 2707-11.
27. Xu, X. and M. Hensel, *Systematic analysis of the SsrAB virulon of Salmonella enterica.* Infect Immun, 2010. **78**(1): p. 49-58.
28. Husseiny, M.I. and M. Hensel, *Rapid method for the construction of Salmonella enterica Serovar Typhimurium vaccine carrier strains.* Infect Immun, 2005. **73**(3): p. 1598-605.
29. Yu, B., et al., *A method to generate recombinant Salmonella typhi Ty21a strains expressing multiple heterologous genes using an improved recombineering strategy.* Appl Microbiol Biotechnol, 2011. **91**(1): p. 177-88.
30. Husseiny, M.I., F. Wartha, and M. Hensel, *Recombinant vaccines based on translocated effector proteins of Salmonella Pathogenicity Island 2.* Vaccine, 2007. **25**(1): p. 185-93.
31. Xiong, G., et al., *Novel cancer vaccine based on genes of Salmonella pathogenicity Island 2.* Int J Cancer, 2009: p. in press.
32. Kuhle, V., D. Jäckel, and M. Hensel, *Effector proteins encoded by Salmonella pathogenicity island 2 interfere with the microtubule cytoskeleton after translocation into host cells.* Traffic, 2004. **5**(5): p. 356-370.
33. Kuhle, V. and M. Hensel, *SseF and SseG are translocated effectors of the type III secretion system of Salmonella pathogenicity island 2 that modulate aggregation of endosomal compartments.* Cell. Microbiol., 2002. **4**(12): p. 813-824.
34. Halici, S., et al., *Functional analysis of the Salmonella Pathogenicity Island 2-mediated inhibition of antigen presentation in dendritic cells.* Infect. Immun., 2008. **76**: p. 4924-4933.
35. Nawabi, P., D.M. Catron, and K. Haldar, *Esterification of cholesterol by a type III secretion effector during intracellular Salmonella infection.* Mol Microbiol, 2008. **68**(1): p. 173-85.

36. Poh, J., et al., *SteC is a Salmonella kinase required for SPI-2-dependent F-actin remodelling.* Cell Microbiol, 2008. **10**(1): p. 20-30.
37. Deiwick, J., et al., *The translocated Salmonella effector proteins SseF and SseG interact and are required to establish an intracellular replication niche.* Infect Immun, 2006. **74**(12): p. 6965-72.
38. Gal-Mor, O., et al., *The Salmonella enterica PhoP directly activates the horizontally acquired SPI-2 gene sseL and is functionally different from a S. bongori ortholog.* PLoS One, 2011. **6**(5): p. e20024.
39. Russmann, H., et al., *Protection against murine listeriosis by oral vaccination with recombinant Salmonella expressing hybrid Yersinia type III proteins.* J Immunol, 2001. **167**(1): p. 357-65.
40. Igwe, E.I., G. Geginat, and H. Russmann, *Concomitant cytosolic delivery of two immunodominant listerial antigens by Salmonella enterica serovar typhimurium confers superior protection against murine listeriosis.* Infect Immun, 2002. **70**(12): p. 7114-9.
41. Becker, P.D., M. Noerder, and C.A. Guzman, *Genetic immunization: bacteria as DNA vaccine delivery vehicles.* Hum Vaccin, 2008. **4**(3): p. 189-202.
42. Shi, H., et al., *Live recombinant Salmonella Typhi vaccines constructed to investigate the role of rpoS in eliciting immunity to a heterologous antigen.* PLoS One, 2010. **5**(6): p. e11142.
43. Kong, Q., et al., *Regulated delayed expression of rfaH in an attenuated Salmonella enterica serovar typhimurium vaccine enhances immunogenicity of outer membrane proteins and a heterologous antigen.* Infect Immun, 2009. **77**(12): p. 5572-82.
44. Link, C., et al., *An SopB-mediated immune escape mechanism of Salmonella enterica can be subverted to optimize the performance of live attenuated vaccine carrier strains.* Microbes Infect, 2006. **8**(8): p. 2262-9.
45. Nagarajan, A.G., et al., *SopB of Salmonella enterica serovar Typhimurium is a potential DNA vaccine candidate in conjugation with live attenuated bacteria.* Vaccine, 2009. **27**(21): p. 2804-11.
46. Beuzon, C.R., S.P. Salcedo, and D.W. Holden, *Growth and killing of a Salmonella enterica serovar Typhimurium sifA mutant strain in the cytosol of different host cell lines.* Microbiology, 2002. **148**(Pt 9): p. 2705-15.
47. Bumann, D., et al., *Recombinant live Salmonella spp. for human vaccination against heterologous pathogens.* FEMS Immunol Med Microbiol, 2000. **27**(4): p. 357-64.
48. Tacket, C.O. and M.M. Levine, *CVD 908, CVD 908-htrA, and CVD 909 live oral typhoid vaccines: a logical progression.* Clin Infect Dis, 2007. **45 Suppl 1**: p. S20-3.
49. Tacket, C.O., et al., *Phase 2 clinical trial of attenuated Salmonella enterica serovar typhi oral live vector vaccine CVD 908-htrA in U.S. volunteers.* Infect Immun, 2000. **68**(3): p. 1196-201.
50. Hindle, Z., et al., *Characterization of Salmonella enterica derivatives harboring defined aroC and Salmonella pathogenicity island 2 type III secretion system (ssaV) mutations by immunization of healthy volunteers.* Infect Immun, 2002. **70**(7): p. 3457-67.

51. Galan, J.E. and R. Curtiss, 3rd, *Virulence and vaccine potential of phoP mutants of Salmonella typhimurium.* Microb Pathog, 1989. **6**(6): p. 433-43.
52. Hohmann, E.L., C.A. Oletta, and S.I. Miller, *Evaluation of a phoP/phoQ-deleted, aroA-deleted live oral Salmonella typhi vaccine strain in human volunteers.* Vaccine, 1996. **14**(1): p. 19-24.
53. Galen, J.E., et al., *Mucosal immunization with attenuated Salmonella enterica serovar Typhi expressing protective antigen of anthrax toxin (PA83) primes monkeys for accelerated serum antibody responses to parenteral PA83 vaccine.* J Infect Dis, 2009. **199**(3): p. 326-35.
54. Galen, J.E., et al., *A new generation of stable, nonantibiotic, low-copy-number plasmids improves immune responses to foreign antigens in Salmonella enterica serovar Typhi live vectors.* Infect Immun, 2010. **78**(1): p. 337-47.

Summary

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen which can enter host cells and replicates within a specialized membrane compartment. *Salmonella* is also an interesting vehicle for the display of recombinant antigens to professional antigen presenting cells (APC). Heterologous antigens can be expressed in *Salmonella* as fusions with recombinant or native proteins. This approach has been used mainly to direct the expression of the desired antigen to a particular location of the bacterial cell and to increase the immunogenicity of foreign antigens by fusing them to proteins that could exert a carrier effect. The type III secretion system (T3SS)-mediated translocation by *Salmonella* can be used for efficient delivery of heterologous antigens into the cytosol of APC, leading to stimulation of both CD4 and CD8 T-cells. In this work, we investigated the use of a subset of effector proteins of the SPI2-encoded T3SS as SseJ, SifA, SseL and SteC. These effectors proteins are associated with endosomal membranes after translocation. Our In-vitro and in-vivo experiments for vaccination show that effector SseJ is the most suitable fusion partner. In previous work it was shown that s*ifB* promoter was the most efficient in-vivo inducible promoter. Here we show that SseJ antigen fusion protein under control of the s*ifB* promoter is most efficient in comparison with effector fusions under control of other in-vivo inducible promoters. By comparison of various attenuated carrier strains, we observed that the *htrA*/*purD* double mutant strain can be used efficiently as attenuated carrier for vaccination and observed the efficient stimulation in-vitro T-cell proliferation by the delta *sifA* mutant strain as carrier.

List of Abbreviations

APCs	Antigen Presenting Cells
PAI	Pathogenicity Islands
SPI	*Salmonella* Pathogenicity Islands
SCV	*Salmonella* Containing Vacuole
PMNs	Polymorphonuclear Leukocytes
T3SS	Type III Secretion Systems
IS	Insertion Sequence
STM	Signature Tagged Mutagenesis
INOS	Inducible Nitric Oxide Synthase
CTLs	Cytotoxic T-lymphocytes
Th	T-helper cells
IFN-γ	Interferon-γ
IL	Interleukin
TAA	Tumor-Associated Antigens
DAP	Diaminopimelic acid
TLR	Toll Like Receptors
NKT	Natural Killer T cells
DCs	Dendritic Cells

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4. Xu X., **Hegazy W.A.E.**, Hensel M., Matilista L. (2011), Fusions of intercellular activated promoters and SPI2 effectors for optimization of *Salmonella*-based vaccines. (Manuscript under preparation to be submitted to *Int. J. Cancer*).
5. **Hegazy W.A.E.** and AbdelSalam H. (2005) Genotypic and phenotypic characterization of clinical and environmental *Burkholderia cepacia* isolates. *N. Egypt. J. Microbiol*., 12: 265-286.

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